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Chlorella modulates insulin signaling pathway and prevents high-fat diet-induced insulin resistance in mice

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ABSTRACT

Aims: The search for natural agents that minimize obesity-associated disorders is receiving special attention. In this regard, the present study aimed to evaluate the prophylactic effect of *Chlorella vulgaris* (CV) on body weight, lipid profile, blood glucose and insulin signaling in liver, skeletal muscle and adipose tissue of diet-induced obese mice.

Main methods: Balb/C mice were fed either with standard rodent chow diet or high-fat diet (HFD) and received concomitant treatment with CV for 12 consecutive weeks. Triglyceride, free fatty acid, total cholesterol and fractions of cholesterol were measured using commercial assay. Insulin and leptin levels were determined by enzyme-linked immunosorbent assay (ELISA). Insulin and glucose tolerance tests were performed. The expression and phosphorylation of IRβ, IRS-1 and Akt were determined by Western blot analyses.

Key findings: Herein we demonstrate for the first time in the literature that prevention by CV of high-fat diet-induced insulin resistance in obese mice, as shown by increased glucose and insulin tolerance, is in part due to the improvement in the insulin signaling pathway at its main target tissues, by increasing the phosphorylation levels of proteins such as IR, IRS-1 and Akt. In parallel, the lower phosphorylation levels of IRS-1^{ser307} were observed in obese mice. We also found that CV administration prevents high-fat diet-induced dyslipidemia by reducing triglyceride, cholesterol and free fatty acid levels.

Significance: We propose that the modulatory effect of CV treatment preventing the deleterious effects induced by high-fat diet is a good indicator for its use as a prophylactic–therapeutic agent against obesity-related complications.

Introduction

Obesity is a worldwide epidemic that results in enormous costs to health-care systems (Stumvoll et al., 2005). Data from the World Health Organization (WHO) have shown that the incidence of obesity worldwide has doubled since the 1980s (WHO, 2012). Considered as a chronic inflammatory process (Donath and Shoelson, 2011; Gregor and Hotamisligil, 2011), obesity is strongly associated with type 2 diabetes (T2D) and other complications such as cardiovascular disease, cancer, osteoarthritis, work disability, sleep apnea and depression (Seidell, 2000; Visscher and Seidell, 2001).

Insulin resistance is an important pathophysiological mechanism that predicts the progression to T2D (Stumvoll et al., 2005; Donath and Shoelson, 2011; Gregor and Hotamisligil, 2011). In many cases, insulin resistance is associated with a complex network of signaling pathways, including reduced insulin-stimulated tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate (IRS) as well as Akt serine phosphorylation in the main target tissues of insulin, including the liver, skeletal muscle and adipose tissue (Hotamisligil et al., 1996; Pessin and Saltiel, 2000; Saltiel and Kahn, 2001; Boura-Halfon and Zick, 2009). The mechanism responsible for the above mentioned alterations is the chronic activation of several serine kinases, such as c-Jun N-terminal kinase (JNK) and inhibitor of kappa β kinase (IKKβ), which gives rise to obesity-induced insulin resistance, mainly by promoting serine phosphorylation of IRS-1 (Aguirre et al., 2002; Hirosumi et al., 2002; Shoelson et al., 2006; Solinas and Karin, 2010). Thus, several studies have recommended that the phosphorylation levels of IRS-1 on serine residue 307 (IRS-1^{ser307}) in rodents or serine residue 312 (IRS-1^{ser312}) in humans could be used as an insulin resistance marker (Aguirre et al., 2002; Shoelson et al., 2006; Donath and Shoelson, 2011).

Despite some efficacy in producing weight loss, improving metabolic parameters and reducing the burden of complications,

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the pharmacological treatment of obesity and T2D has several side effects, which, in many cases, may worsen health conditions (Narayan et al., 2000). Therefore, the search for natural agents that minimize these undesirable effects, and are still effective in regulating the disturbances observed in obesity, has been receiving increasing attention from the scientific community (Lee et al., 2012; Hidaka et al., 2004; Chang et al., 2013). In this sense, *Chlorella vulgaris* (CV), a microscopic single-celled freshwater alga that is rich in nutrients and is commonly used as a food supplement, has emerged as an alternative prophylactic treatment for obesity-related complications.

CV has been considered a biological response modifier (Noda et al., 1998), as demonstrated by its protective activities against viral and bacterial infections in normal and immunosuppressed mice (Tanaka et al., 1986; Dantas and Queiroz, 1999; Hasegawa et al., 2000; Queiroz et al., 2003; Souza-Queiroz et al., 2008), as well as against tumors (Konishi et al., 1985; Tanaka et al., 1998; Ramos et al., 2010). In this regard, studies from our laboratory have shown that CV up modulates myelossupression in the bone marrow and stromal cells by inducing a significant recovery in the reduced number of myeloid progenitor cells (CFU-GM) found in tumor-bearing, stressed, infected and lead-exposed mice (Queiroz et al., 2003, 2008, 2011; Souza-Queiroz et al., 2008, 2013, submitted for publication; Ramos et al, 2010).

CV has also been shown to have the capacity to induce apoptosis in HepG2 cells (Saad et al., 2006) and to reduce heavy metals in blood and tissues, such as bone, kidney and liver (Singh et al., 2004; Queiroz et al., 2008). CV is considered to be a complete food, containing all the ingredients necessary to promote human health. Its well-balanced nutrients include carbohydrates, proteins, nucleic acids, essential amino acids, fatty acids (ω-3 and ω-6), vitamins, dietary fiber, growth factors and antioxidants (lutein, α- and β-carotene, ascorbic acid and tocopherol) (Vijayavel et al., 2007; Rodriguez-Garcia and Guil-Guerrero, 2008; Panahi et al., 2012).

Clinical and experimental studies in the literature have shown that different species of *Chlorella*, i.e., *Chlorella pyrenoidosa* (CP) and CV, may have a series of biochemical and physiological effects, such as decreasing serum cholesterol fractions, triglycerides and glucose levels in addition to reducing body weight (Jong-Yuh and Mei-Fen, 2005; Cherng and Shih, 2006; Lee and Kim, 2009; Lee et al., 2008; Mizoguchi et al., 2008). Based on these findings and on those we obtained previously with the alga, we designed the present study aiming to evaluate the prophylactic effect of CV on body weight, lipid metabolism, blood glucose and insulin signaling in liver, skeletal muscle and adipose tissue of diet-induced obese mice, a question still open in the literature.

Material and methods

Mice

Six-week-old male Balb/C mice were maintained under specific pathogen-free (SPF) conditions in a regimen of 12 h dark/light cycles and a controlled environment (room temperature: 22 ± 3 °C, humidity: 55 ± 5%). The animals were randomly divided into four groups (n = 6 mice/group) and treated for 12 consecutive weeks, as follows: standard rodent chow and vehicle (control—CTL), standard rodent chow and *C. vulgaris* (CTL + CV), high-fat diet and vehicle (DIO) and high-fat diet + *C. vulgaris* (DIO + CV). The high-fat diet (HFD) consisted of 55% calories from fat, 29% from carbohydrate and 16% from protein, as described previously (Caricilli et al., 2011; Oliveira et al., 2011; Araujo et al., 2012). The animals received water and their respective diets ad libitum for the whole 12-week period. Body weight and fasting blood glucose were measured weekly. At the end of the experiment, insulin and glucose tolerance tests were performed as previously described (Oliveira et al., 2011). All animal studies were approved by the Animal Care and Use Committee at the State University of Campinas (process: 1987-1) and are in accordance with the guidelines for the Care and Use of Laboratory Animals. The CV treatment was administered as described below.

CV and treatment

The dried alga *C. vulgaris*, a strain of unicellular green alga, was kindly provided by Research Laboratories, *Chlorella* Industry Co., Ltd., Fukuoka, Japan. The nutritional and fatty acid compositions of the alga are shown in Tables 1 and 2. CV was prepared in distilled water and doses of 50 mg/kg/day were given orally once daily by gavage of 0.2 ml volume/mouse in a prophylactic/therapeutic manner, starting 5 days before HFD and lasting for the 12 weeks of study. CTL and DIO groups received vehicle (distilled water) only. In all groups, the experiments were performed during the morning, 24 h after the last administration of CV. The selection of CV dose was based on the preliminary dose–response studies performed in our laboratory (Dantas and Queiroz, 1999; Justo et al., 2001). The treatment schedule used here was standardized thenceforth to be used in all works produced with the alga by our group (Dantas and Queiroz, 1999; Justo et al., 2001; Queiroz et al., 2002, 2003, 2008, 2011; Souza-Queiroz et al., 2004, 2008, 2013; Ramos et al., 2010). Prophylactic–therapeutic administration was also used in all our studies, since our aim is to investigate the modulating effects of CV as a functionally whole food able to protect the host, acting as a biological response modifier, and what also justifies the use of the oral route for the administration of the alga.

Antibodies, chemicals, and buffers

Reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA, USA). All antibodies were from Santa Cruz Technology (Santa Cruz, CA), except anti-Akt, anti-phospo-Akt, and anti-phospho-IRS-1^{Ser307}, which were obtained from Cell Signaling Technology (Beverly, MA). Sodium thiopental and human recombinant insulin (humulin R) were from Eli Lilly and Co. (Indianapolis, IN, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified elsewhere.

Serum analysis

After an overnight fasting (12 h), a blood sample was collected from the vena cava. Serum was separated by centrifugation (2500 ×g) for 15 min at 4 °C and stored at −80 °C until assay. Triglyceride, total cholesterol and fractions of cholesterol were measured using colorimetric assay kits (Labtest Diagnostica, Minas Gerais, Brazil). Serum free fatty acid (FFA) levels were analyzed using a NEFA-kit-U (Wako Chemical, Neuss, Germany) with oleic acid as a standard. The insulin and leptin levels were determined by enzyme-linked immunosorbent assay (ELISA) (Linco, St. Charles, MO) according to the manufacturer's instructions.

Insulin and glucose tolerance test

For measurements of insulin and glucose tolerance, intraperitoneal (i.p.) challenge was performed at the end of the 12 h fasting. Glucose

Table 1
Nutritional composition of CV according to the physico-chemical methods for food analysis of the Adolfo Lutz Institute.

Composition	Quantity (/100 g)
Moisture and volatiles	5.2 g
Ash	7.0 g
Total lipids	8.6 g
Proteins	57.4 g
Total carbohydrates	21.8 g
Calories	394 kcal
Iron	95 mg

Table 2
Fatty acid composition of CV according to the physico-chemical methods for food analysis of the Adolfo Lutz Institute.

Fatty acids	Quantity (g/100 g)
Saturated	1.67
Monounsaturated	1.60
Polyunsaturated	4.09
Omega-3	1.49
Omega-6	2.00
Total trans-isomers	<0.01
Not identified	0.88

tolerance tests were conducted with 25% D-glucose in 0.9% saline, so that the final dose was 11.1 mmol/kg body weight in all animals. Insulin tolerance tests were conducted with 100 U/ml human insulin in 0.9% saline, so that the final dose was 1.5 U/kg. Glucose levels (mg/dl) were measured from tail blood using a handheld glucometer (Optium Xceed, Abbot, Berkshire, England) before (0 min) and at 15, 30, 60 and 120 min after injection. Glucose tolerance was assessed by area under the curve analysis, and insulin tolerance by glucose clearance over the initial 60 min of the insulin challenge (Matthews et al., 1985).

Tissue extraction and protein analysis by immunoblotting

After an overnight fasting (12 h), mice were anesthetized, and after 10–15 min, when anesthesia was assured by the loss of pedal and corneal reflexes, the abdominal cavity was opened, the portal vein exposed, and 0.2 ml of normal saline was injected with or without insulin (10^{-6} mol/l). At 30 and 90 s after insulin injection, the liver, gastrocnemius and adipose tissue were removed, minced coarsely, and homogenized immediately in extraction buffer at 4 °C (1% Triton X-100, 100 mM Tris–HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride, and 0.1 mg of aprotinin/ml) with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments). Insoluble material was removed by centrifugation for 30 min at 9000 ×*g* in a 70 Ti rotor (Beckman, Fullerton, CA, USA) at 4 °C. The protein concentrations of the supernatants were determined by Bradford dye-binding method (Caricilli et al., 2011; Oliveira et al., 2011; Araujo et al., 2012). In direct immunoblot experiments, protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-IR, anti-phospho-IR^{tyr}, anti-IRS-1, anti-phospho-IRS-1^{tyr}, anti-Akt, anti-phospho-Akt^{ser473} and anti-IRS-1^{ser307}. The homogeneity of gel loading was evaluated by blotting the membranes with antibodies against IR, IRS-1 and Akt as appropriate.

Statistical analysis

Data are expressed as means SEM and the number of independent experiments is indicated. The results of blots are presented as direct comparisons of bands or spots on autoradiographs and were quantified by optical densitometry (Scion Image, Scion Corporation, Frederick, MD). For statistical analysis, the groups were compared using a one-way ANOVA followed by Bonferroni's posthoc test. The level of significance adopted was $P < 0.05$.

Results

Administration of C. vulgaris improves insulin resistance in mice fed on high-fat diet

Fig. 1 shows comparative data between insulin resistance of mice fed on standard rodent chow, treated (CTL + CV) or non-treated (CTL) with CV, and mice fed on high-fat diet, treated (DIO + CV) or non-treated (DIO) with CV. Our findings corroborate previous studies (Jeong et al., 2009) showing that treatment with CV produces no changes in body weight (Fig. 1A). In addition, no changes were produced by the alga on gonadal fat pad (Fig. 1B), or food intake (data not shown). Glucose concentrations in fasting serum were similar among all animals at baseline, i.e., before the administration of HFD or CV (data not shown). At the end of the 12 weeks of treatment higher glucose levels were observed in all groups receiving HFD (DIO and DIO + CV), compared to CTL group. However, significantly lower levels were observed in DIO + CV groups, compared to DIO groups (Fig. 1C). Similarly, HFD also led to less effective glucose clearance after glucose injection during the i.p. GTT, which was significantly reversed by the administration of CV (Fig. 1D). The DIO group showed higher fasting insulin levels in comparison with CTL, however, unlike its effect on fasting glucose, the administration of CV showed a tendency to reduce insulin levels in obese mice, which was not statistically significant (Fig. 1E). In contrast, the i.p. ITT clearly demonstrated that CV administration prevented the development of HFD-induced insulin intolerance (Fig. 1F) Additionally, as expected, DIO mice exhibited higher circulating levels of leptin than the control animals, and this effect was not prevented by the treatment with CV (Fig. 1G). Of importance, CV treatment produced no changes in CTL mice in all parameters evaluated in the present study.

C. vulgaris prevents high-fat diet-induced dyslipidemia in mice

To better characterize the modulating effects of CV on obese mice, we carried out a complete investigation of the lipid profile in all groups. Elevated triglyceride levels, observed in the DIO group, were restored to control levels by the treatment with CV (Fig. 2A). Similarly, the alga also restored to control values the higher levels of total cholesterol (Fig. 2B), and of cholesterol fractions (with the exception of high-density lipoprotein (HDL)) (Fig. 2C–E). In addition, CV brought the elevated levels of FFA produced by HFD to values under physiological levels (Fig. 2F). Together, these results show the ability of CV to maintain the balance in lipid profile of obese mice.

Effects of C. vulgaris administration on insulin signaling in liver, muscle and adipose tissue of mice fed on high-fat diet

The results of CV administration on insulin signaling pathway in its main target tissues demonstrated that CV treatment produced no effects on the levels of insulin-stimulated phosphorylation of IRβ^{tyr} and IRS-1^{tyr} and Akt^{ser} phosphorylation, in all tissues studied, in normal (CTL) mice (Fig. 3A–I). As expected, feeding a high-fat diet for 12 weeks resulted in a remarkable reduction in insulin-stimulated phosphorylation levels of IRβ^{tyr} and IRS-1^{tyr} and Akt^{ser} phosphorylation in liver, skeletal muscle and adipose tissue, when compared to the CTL group (Fig. 3A–I). On the other hand, DIO + CV mice exhibited higher phosphorylation of these proteins in all studied tissues when compared to the DIO group (Fig. 3A–I). No changes in basal phosphorylation or expression levels were observed among groups (Fig. 3A–I). Taken together, these data indicate that prophylactic–therapeutic administration of CV is able to prevent the deleterious effects induced by a high-fat diet on insulin signaling pathways.

The effect of administration of C. vulgaris on serine phosphorylation levels of IRS-1 in mice

Since the phosphorylation levels of IRS-1^{ser307} can be used as a marker of insulin resistance in obesity, we investigated this parameter in liver, muscle and adipose tissue of all studied groups. As expected, the DIO group exhibited higher phosphorylation levels of IRS-1^{ser307} in all three tissues, compared to the CTL group (Fig. 4A–C). CV administration, as before, had no effects on normal (CTL) animals, except for the induction of lower phosphorylation levels of IRS-1^{ser307} in the muscle, what seems to indicate a positive modulating effect of the alga (data not shown). In addition, the DIO + CV group presented lower

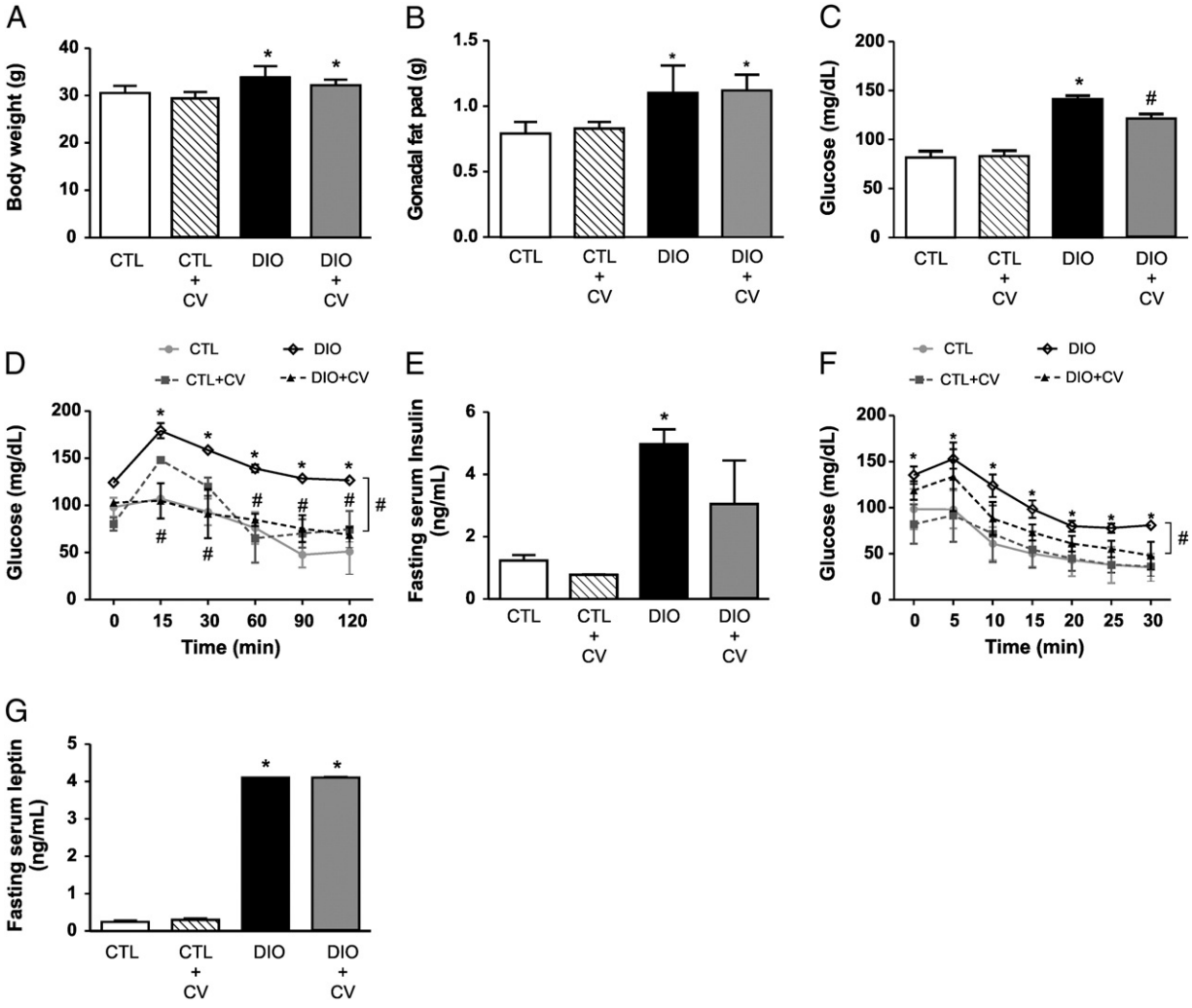

Fig. 1. Physiological, metabolic and insulin tolerance parameters in mice fed a high-fat diet (HFD) and treated in a prophylactic–therapeutic manner with daily oral doses of 50 mg/kg *Chlorella vulgaris* (CV). Treatment started 5 days prior to the onset of the administration of HFD and was extended for 12 weeks concomitantly with HFD. In all groups, the experiments were performed during the morning, 24 h after the last administration of CV. Control mice (CTL and DIO) received vehicle only. Results represent means ± SD of 6 mice/group. A: Body weight. B: Gonadal fat pad. C: Fasting serum glucose. D: Glucose response curve during the glucose tolerance test. E: Fasting serum insulin. F: Glucose response curve during the insulin tolerance test. G: Fasting serum leptin. Data are presented as means ± SEM of six mice per group. *P < 0.05 vs. control, #P < 0.05 vs. DIO.

phosphorylation levels of IRS-1^{ser307} in all studied tissues, compared to non-treated obese mice (Fig. 4A–C). Overall, these results indicate that the administration of CV reduces the increased levels of IRS-1^{ser307} normally observed during the development of obesity.

Discussion

Obesity can currently be treated by a limited number of medicines, and although they demonstrate some efficacy by reducing body weight and improving metabolic parameters, they produce a good number of undesirable side-effects (Narayan et al., 2000; Hidaka et al., 2004; Lee et al., 2012). Therefore, the search for alternative therapies, particularly natural products, is attracting increasing attention from the scientific community (Hidaka et al., 2004; Chou et al., 2012; Lee et al., 2012; Chang et al., 2013). In this context, CV has emerged as an alternative treatment and prophylactic agent against obesity-related complications.

Herein we have demonstrated for the first time in the literature that prevention by CV of high-fat diet-induced insulin resistance in obese mice, as shown by increased glucose and insulin tolerance, is in part due to the improvement in the insulin signaling pathway in liver, skeletal muscle and adipose tissue, by increasing phosphorylation levels of proteins such as IR, IRS-1 and Akt. In parallel, lower phosphorylation levels of IRS-1^{ser307} were observed in obese mice. We also found that CV administration prevents high-fat diet-induced dyslipidemia by reducing triglyceride, cholesterol and free fatty acid levels. Additional mechanisms reported in the literature for the antihyperinsulinemic effects of CV are due to the modulation of adipose tissue hypertrophy and adipocytokine secretion (Noguchi et al., 2013). An important aspect of our results, which corroborates the adaptogenic aspects attributed to the alga, consists in its ability to modulate the defective response in the body, without altering the functions in the normal host. Taken together, these data show that the prophylactic–therapeutic administration of CV was able to prevent the deleterious effects induced by high-fat diet induced obesity in mice.

It is well established that insulin resistance is the intersection between obesity and T2D, and all additional complications (Shoelson et al., 2006; Donath and Shoelson, 2011; Gregor and Hotamisligil, 2011). Insulin is essential in the metabolic process, mainly by increasing glucose uptake in both muscle and adipose tissue and inhibiting hepatic glucose output (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002). Moreover, under physiological conditions, insulin stimulates lipogenesis, and glycogen and protein synthesis, and inhibits lipolysis, glycogenolysis and protein breakdown (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002). Therefore, insulin resistance promotes profound dysregulation in these processes, which in turn results in higher circulating levels of glucose and lipids (Saltiel and Kahn, 2001). In this respect, our results

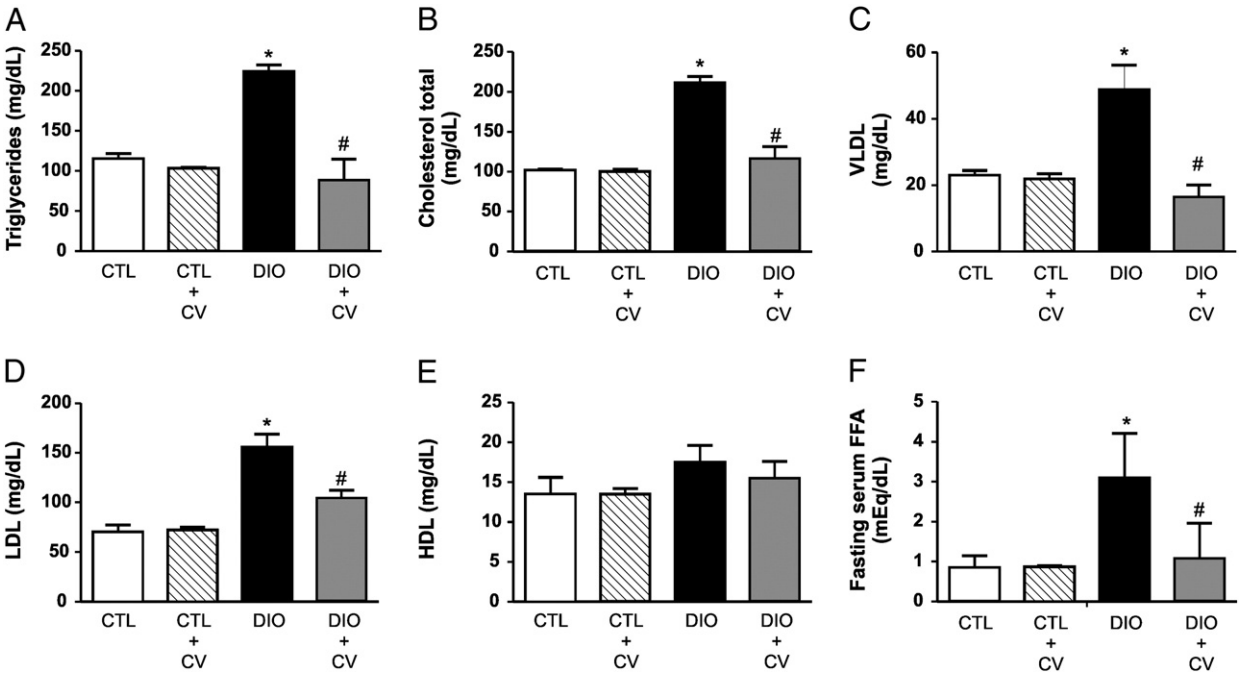

Fig. 2. Physiological, metabolic and insulin tolerance parameters in mice fed a high-fat diet (HFD) and treated in a prophylactic–therapeutic manner with daily oral doses of 50 mg/kg *Chlorella vulgaris* (CV). Treatment started 5 days prior to the onset of the administration of HFD and was extended for 12 weeks concomitantly with HFD. In all groups, the experiments were performed during the morning, 24 h after the last administration of CV. Control mice (CTL and DIO) received vehicle only. A: Fasting serum triglycerides. B: Fasting serum total cholesterol. C: Fasting serum VLDL. D: Fasting serum LDL. E: Fasting serum HDL. F: Fasting serum FFA. Data are presented as means ± SEM of six mice per group. *P < 0.05 vs. control, #P < 0.05 vs. DIO.

show that CV was able to prevent the hyperglycemia developed in obese mice. Previous studies in the literature corroborate our findings by reporting hypoglycemic effects for several species of the *Chlorella* genus, such as, *C. vulgaris* (Noguchi et al., 2013), *C. pyrenoidosa* (Rodriguez-Lopez and Lopez-Quijada, 1971; Jong-Yuh and Mei-Fen, 2005; Jeong et al., 2009; Senthilkumar et al., 2012) and *Chlorella sorokiniana* (Chou et al., 2008). In this latter work, the authors reported that this alga was able to provoke dual activation of peroxisome proliferator activated receptors α/γ, which are known to improve glucose and insulin levels and to reduce hyperlipidemia (Chou et al., 2008). In addition, increased expression of glucose transporter type-4 (GLUT-4) has been reported for CV, which allows for an influx of glucose in the liver and muscle (Lee and Kim, 2009). Taken together, these studies indicate that CV has the ability to down-modulate the hyperglycemic response.

Relevant to the understanding of the mechanisms involved in the modulation produced by CV in the obese mice is the fact that, in spite of its efficacy in reducing glucose levels, the alga was not able to reduce the remarkable increase in insulin levels observed in HFD fed mice, which is corroborated by previous findings with *C. pyrenoidosa* and CV (Rodriguez-Lopez and Lopez-Quijada, 1971; Hirosumi et al., 2002; Cherng and Shih, 2006; Jeong et al., 2009; Mehran et al., 2012). In support of this view, a previous study compared *C. pyrenoidosa* with glibenclamide (a hypoglycemic sulfonylurea drug that is known to stimulate insulin secretion) and observed that the algae did not cause any alterations in insulin levels, reinforcing the hypothesis that it reduces glycemia via a mechanism other than that used by glibenclamide (Jong-Yuh and Mei-Fen, 2005).

Importantly, the present results demonstrating the ability of CV to modulate insulin signaling pathway are pioneer in the literature. It is well known that insulin resistance is associated with reduced phosphorylation of IR, IRS-1 and Akt in its target tissues (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002; Boura-Halfon and Zick, 2009). CV treatment increased the phosphorylation levels of IR, IRS-1 and Akt proteins in the liver, skeletal muscle and adipose tissue, and also lowered phosphorylation levels of IRS-1^{ser307}. In addition, our findings help to explain the increased levels of GLUT-4 induced by CV in obese mice, found by Lee and Kim (2009), since the activation of insulin signaling pathways may be responsible for increasing GLUT-4 translocation and consequently the influx of glucose into the liver and muscle. Taken together, these data suggest that CV acts by increasing the insulin sensitivity of the main target tissues and consequently prevents the deleterious effect on insulin signaling induced by a high-fat diet.

Insulin resistance is frequently correlated with dyslipidemia (Saltiel and Pessin, 2002), which is characterized by increased triglycerides and cholesterol levels (Howard et al., 2003). In this respect, another important modulation produced by CV was the ability to restore to normal values the increased levels of triglycerides, total cholesterol, cholesterol fractions, as well as FFA concentration, observed in the obese mice. This is consistent with previous studies showing that CV administration decreased fat absorption, promoted its intestinal excretion, and reduced FFA synthesis (Okudo et al., 1975; Sano et al., 1988; Shibata et al., 2001; Chovanèíková and Simek, 2001; Cherng and Shih, 2006; Lee et al., 2008); this is in agreement with the water-soluble fiber content presented by the alga (Kay, 1991). Considering that lipid deposition in non-adipose tissue leads to insulin resistance, one possible mechanism behind the restoration of insulin signaling and improved glucose tolerance, observed in our study, might be related to the reduced lipid absorption, along with its increased excretion rate, which potentially lowers lipid in muscle and liver. The potential decrease of lipid in liver and muscle is supported by the decrease in phosphorylation of IRS-1^{ser307}. In addition, CV contains polyunsaturated fatty acids, such as omega-3 (ω-3), which have been demonstrated to exert anti-inflammatory effects and improve insulin sensitivity, up-regulating genes implicated in glucose transport and insulin signaling in adipose tissue and liver (Gonzalez-Periz et al., 2009). Moreover, CV contains the ideal ratio of polyunsaturated fatty acids, i.e., the ratio of ω-6 and ω-3 is 1.34:1, important for reducing the risk of chronic diseases (Simopoulos, 2002).

Another proposed mechanism involved in insulin resistance is the phenomenon known as leptin resistance. This mechanism is known to be disrupted in many obese individuals, and even though their leptin

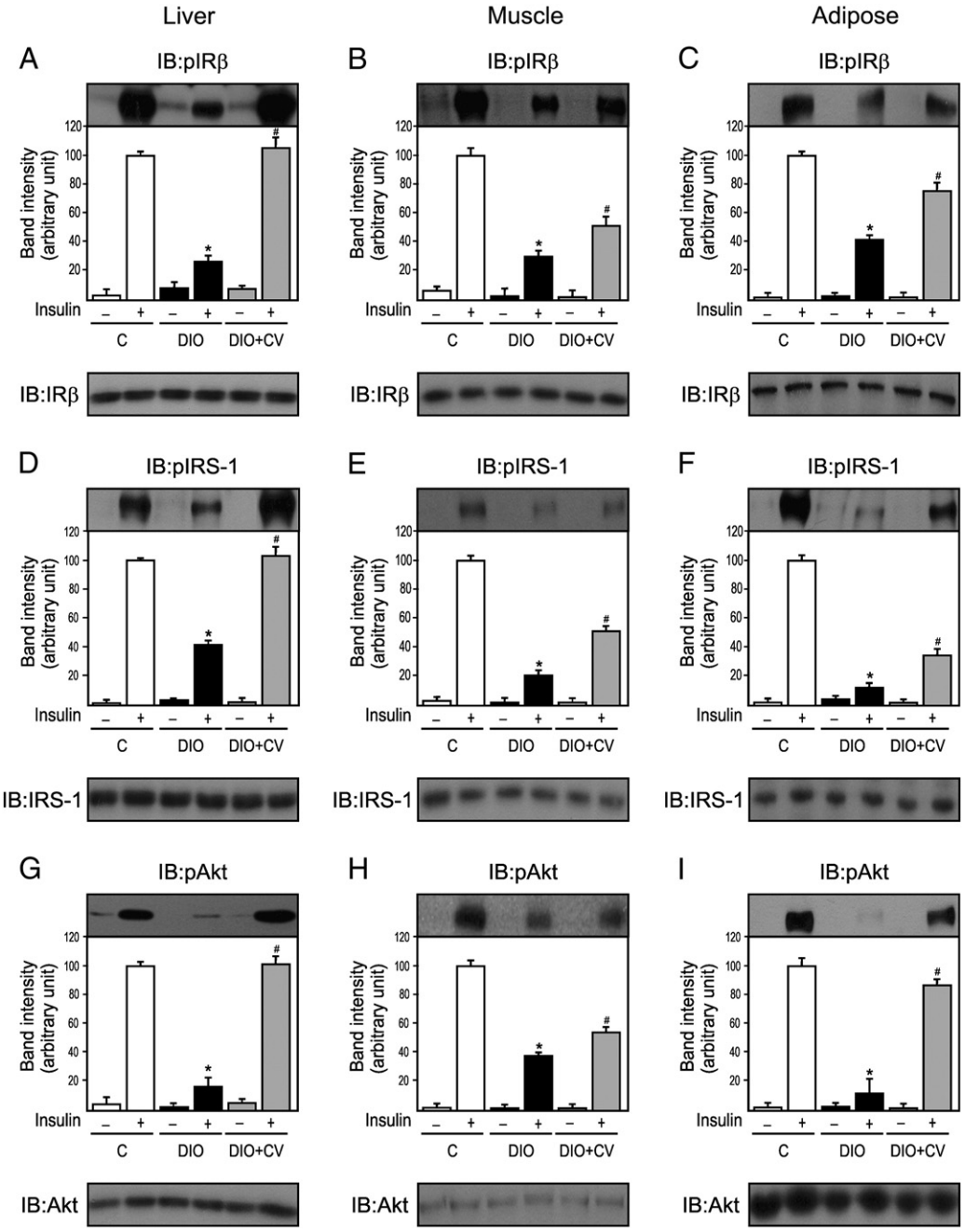

Fig. 3. Physiological, metabolic and insulin tolerance parameters in mice fed a high-fat diet (HFD) and treated in a prophylactic–therapeutic manner with daily oral doses of 50 mg/kg *Chlorella vulgaris* (CV). Treatment started 5 days prior to the onset of the administration of HFD and was extended for 12 weeks concomitantly with HFD. In all groups, the experiments were performed during the morning, 24 h after the last administration of CV. Control mice (CTL and DIO) received vehicle only. Representative blots show tyrosine phosphorylation of IRβ in liver (A), muscle (B) and adipose tissue (C) of control, DIO, DIO + CV mice (top). Total protein expression of IRβ (A–C, bottom). Tyrosine phosphorylation of IRS-1 in liver (D), muscle (E) and adipose tissue (F) of control, DIO, DIO + CV mice (top). Total protein expression of IRS-1 (D–F, bottom). Serine phosphorylation of AKT in liver (G), muscle (H) and adipose tissue (I) of control, DIO, DIO + CV mice (top). Total protein expression of AKT (G–I, bottom). Data are presented as means ± SEM from six mice per group, *P < 0.05 vs. control, #P < 0.05 vs. DIO. IB, immunoblot.

levels are commonly elevated, this does not result in reduction of appetite and caloric intake. Leptin resistance can be triggered in rats by ad libitum consumption of energy-dense, highly palatable foods over a period of several days (Wang et al., 2001). Surprisingly, we observed no protective effect of CV treatment on the high levels of leptin observed in mice fed with HFD. A possible explanation is that leptin is a hormone that regulates long-term energy balance in many mammals, producing long-term inhibition of appetite in response to formation of body fat. Studies in the literature corroborate this hypothesis by showing that leptin enters the brain by a saturable transport system, and the capacity of leptin transport is lower in obese individuals, providing a mechanism for leptin resistance (Caro et al., 1996). Additional studies showed that postprandial rise in serum insulin is not associated with any change in serum leptin concentrations, also suggesting the possibility that long-term changes in insulin secretion are necessary to alter serum leptin concentrations (Considine et al., 1996).

A potential explanation for the modulatory effect of CV preventing the development of insulin resistance might be related to its ability to inhibit the elevation of glucocorticoid hormones induced by stressing factors (Hasegawa et al., 2000). It is well known that these hormones

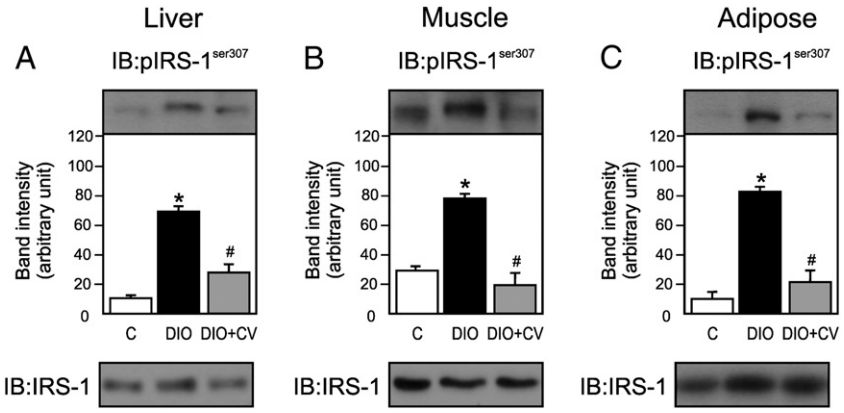

Fig. 4. Physiological, metabolic and insulin tolerance parameters in mice fed a high-fat diet (HFD) and treated in a prophylactic–therapeutic manner with daily oral doses of 50 mg/kg *Chlorella vulgaris* (CV). Treatment started 5 days prior to the onset of the administration of HFD and was extended for 12 weeks concomitantly with HFD. In all groups, the experiments were performed during the morning, 24 h after the last administration of CV. Control mice (CTL and DIO) received vehicle only. Representative blots show serine phosphorylation of IRS-1 in liver (A), muscle (B) and adipose tissue (C) of control, DIO, DIO + CV mice (top). Total protein expression of IRS-1 (A–C, bottom). Data are presented as means ± SEM from six mice per group, *P < 0.05 vs. control, #P < 0.05 vs. DIO. IB, immunoblot.

counteract insulin, contribute to hyperglycemia-causing hepatic gluconeogenesis and inhibit the peripheral utilization of glucose which eventually leads to insulin resistance, what is produced by decreasing the translocation of glucose transporters (especially GLUT4) to the cell membrane (Piroli et al., 2007). Corroborating this assumption is the ability of CV to increase the expression of glucose transporter type-4 (GLUT-4) (Lee and Kim, 2009).

The results presented here corroborate our previous findings related to the ability of CV to act as a biological response modifier by increasing non-specific host resistance, thus promoting a more appropriate response against different types of environmental or psychogenic stresses. In this context, we studied the modulating effects of CV in the following experimental conditions: single acute psychogenical and physical stress (Souza-Queiroz et al., 2004, 2008), chronic stress (Souza-Queiroz et al., 2013), exposure to heavy metals (Queiroz et al., 2003, 2008, 2011), infection (Dantas and Queiroz, 1999; Dantas et al., 1999; Queiroz et al., 2002) and tumor (Justo et al., 2001; Ramos et al., 2010, submitted for publication). Altogether, our findings suggest that adjuvant colony-stimulating factors produced by CV treatment, which act synergistically for highly enriched numbers of CFU-GM in combinations of modulatory cytokines, may inhibit the suppressive effects of different types of stress on critical pools of hematopoietic progenitor cells and mitigate the inhibition of Th1 function induced by uncontrollable stress, thus potentiating immune surveillance. Collectively, our findings suggest that stress-related immune-hematopoietic dysregulation might be one core mechanism behind a diverse set of health risks.

Irrespective of the mechanisms involved, the modulatory effect of CV may have an important role in the prophylactic activity suggested to the algae in protecting hosts from immune and non-immune stressful situations, leading to an increased ability of the immune system to respond to challenge. Although the ultimate mechanisms of this action need additional studies, these findings can provide a venue for promoting the translation of this understanding into effective prevention strategies and new treatment regimens to improve human health.

Authors' contributions

J.F.V.: researched the data, contributed to the discussion and wrote/reviewed/edited the manuscript; A.G.O.: researched the data and wrote/reviewed/edited the manuscript; T.G.A.: contributed to the discussion and reviewed/edited the manuscript; S.R.B.: researched the data; C.O.T.: contributed to the discussion, M.J.A.S.: designed the study, contributed to the discussion and reviewed/edited manuscript; M.L.S.Q.: designed the study, contributed to the discussion and reviewed/edited manuscript. M.L.S.Q. and M.J.A.S. jointly designed the study through the intersection of their lines of research.

Conflict of interest

No potential conflicts of interest relevant to this work were reported.

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