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ARTICLE

Vitamin E supplementation and caloric restriction promotes regulation of insulin secretion and glycemic homeostasis by different mechanisms in rats

Paula R. Venturini, Bruna Fontana Thomazini, Camila Andréa Oliveira, Armindo A. Alves, Thaís Furtado Camargo, Caio E.C. Domingues, Helena C.L. Barbosa-Sampaio, and Maria Esméria C. do Amaral

Abstract: Vitamin E and caloric restriction have antioxidant effects in mammals. The aim of this study was to evaluate effects of vitamin E supplementation and caloric restriction upon insulin secretion and glucose homeostasis in rats. Male Wistar rats were distributed among the following groups: C, control group fed ad libitum; R, food quantity reduction of 40%; CV, control group supplemented with vitamin E [30 mg·kg^{-1}·day^{-1}]; and RV, food-restricted group supplemented with vitamin E. The experiments ran for 21 days. Glucose tolerance and insulin sensitivity was higher in the CV, R, and RV groups. Insulin secretion stimulated with different glucose concentrations was lower in the R and RV groups, compared with C and CV. In the presence of glucose and secretagogues, insulin secretion was higher in the CV group and was lower in the R and RV groups. An increase in insulin receptor occurred in the fat pad and muscle tissue of groups CV, R, and RV. Levels of hepatic insulin receptor and phospho-Akt protein were higher in groups R and RV, compared with C and CV, while muscle phospho-Akt was increased in the CV group. There was a reduction in hepatic RNA levels of the hepatocyte growth factor gene and insulin degrading enzyme in the R group, and increased levels of insulin degrading enzyme in the CV and RV groups. Thus, vitamin E supplementation and caloric restriction modulate insulin secretion by different mechanisms to maintain glucose homeostasis.

Key words: muscle, islets, fat pad, liver, large intestine.

Résumé : La vitamine E et la restriction calorique exercent des effets antioxydants chez les mammifères. Le but de cette étude était d'évaluer les effets d'une supplémentation en vitamine E et d'une restriction calorique sur la sécrétion d'insuline et l'homéostasie du glucose chez le rat. Les rats ont été répartis en 4 groupes : groupe contrôle alimenté *ad libitum* (C), groupe avec restriction alimentaire de 40 % (R), groupe C supplémenté à la vitamine [30 mg·(kg de masse corporelle)$^{-1}$·jour^{-1}] (CV), groupe R supplémenté à la vitamine E (RV) pendant 21 jours. La tolérance au glucose et la sensibilité à l'insuline étaient plus élevées chez les groupes CV, R et RV. La sécrétion d'insuline stimulée par différentes concentrations de glucose était plus faible chez les groupes R et RV comparativement aux groupes C et CV. En présence de glucose et de sécrétagogues, la sécrétion d'insuline était plus élevée dans le groupe CV et plus faible chez les animaux des groupes R et RV. Le récepteur d'insuline était plus abondant dans le coussinet adipeux et le tissu musculaire des groupes CV, R et RV. Le récepteur d'insuline et la phospho-Akt hépatiques étaient plus abondants chez les groupes R et RV comparativement aux groupes C et CV, alors que la phospho-Akt du muscle était plus abondante chez le groupe CV. Les niveaux d'ARN du facteur de croissance hépatique et de l'enzyme de dégradation de l'insuline dans le foie étaient réduits chez le groupe R, alors que l'enzyme de dégradation de l'insuline était accrue chez les groupes CV et RV. Tant la supplémentation en vitamine E que la restriction calorique modulent la sécrétion d'insuline par différents mécanismes afin de maintenir l'homéostasie du glucose. [Traduit par la Rédaction]

Mots-clés : muscle, ilots, coussinet adipeux, foie, gros intestin.

Introduction

The impact of diet on human nutrition and its role in the quality of life are of interest in various areas of scientific research. Caloric restriction (CR) can have beneficial effects on metabolism by reducing levels of blood glucose and insulin, potentially increasing longevity. CR has been studied as an effective treatment to increase the life expectancy of many species, ensuring healthy ageing and protecting against metabolic diseases (Masoro 2009; Xiang and He 2011; Redman et al. 2018). CR is beneficial in metabolic control because of its antioxidant effect (He et al. 2012). The classical mechanism used to explain the effects of reduced calorie intake involves insulin signaling, decreased body fat, and reductions in reactive oxygen species (ROS) (Guarente 2005; Mercken et al. 2013). These reductions in ROS production and cellular oxidative injury are considered the main positive effects of CR (Armeni et al. 2003). The benefits have been attributed to regulatory proteins that reduce insulin secretion and simultaneously increase peripheral insulin sensitivity (Whiteman et al. 2002; Boucher et al. 2014). One of the main molecules activated by insu-

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P.R. Venturini, B.F. Thomazini, C.A. Oliveira, A.A. Alves, T.F. Camargo, and M.E.C. do Amaral. Graduate Program in Biomedical Sciences, Centro Universitário Hermínio Ometto, UNIARARAS, Araras, São Paulo, Brazil.
C.E.C. Domingues. School of Biology, Centro Universitário Hermínio Ometto, UNIARARAS, Araras, São Paulo, Brazil.
H.C.L. Barbosa-Sampaio. Department of Structural and Functional Biology, Institute of Biology, State University of Campinas, São Paulo, Brazil.
Corresponding author: Maria Esméria Corezola do Amaral (email: esmeria@fho.edu.br).

lin through the insulin receptor (IR) and insulin receptor substrates (IRS) is PI3K, which exerts a variety of important metabolic functions by the activation (phosphorylation) of Akt (Saltiel and Kahn 2001). In addition, insulin degrading enzyme (IDE) is an important enzyme for the clearance of insulin, and deficiency of this enzyme has been associated with type 2 diabetes in humans (Groves et al. 2003; Rudovich et al. 2009). IDE knockout mice showed hyperinsulinemia associated with peripheral insulin resistance and decreased insulin receptor expression (Farris et al. 2003; Abdul-Hay et al. 2011). These data suggest that IDE might be important in strategies to treat diseases associated with insulin resistance.

Besides CR, another option that has been investigated in glucose homeostasis is vitamin E supplementation. Vitamin E is a classical lipophilic antioxidant whose most important function is to protect molecules and tissues against the deleterious effects of free radicals (Rimbach et al. 2002; Minamiyama et al. 2008). This vitamin aids in the stability of cell membranes and can inhibit inflammatory responses, exerting an action similar to that of steroids (Birringer et al. 2007; Costacou et al. 2008; Patel et al. 2011). In addition, dietary vitamin E supplementation has been associated with a reduction of body mass index (BMI) (Zillikens et al. 2010), suggesting that vitamin E could modulate SIRT1, depending on the dose. These findings indicate that vitamin E could be of interest for the treatment of metabolic diseases. Supplementation with α-tocopherol has been found to prevent cardiovascular risk by means of Akt activation in the insulin signaling pathway (Minamiyama et al. 2008) vitamins E and C have been shown to improve insulin performance and to reduce the incidence of type 2 diabetes. A relationship between vitamin E supplementation and insulin sensitivity was observed in healthy users (Facchini et al. 1996), and low plasma vitamin E concentrations were associated with increased risk of type 2 diabetes (Öhrvall et al. 1993).

Both CR (Swindell 2012) and vitamin E supplementation (Mayer-Davis et al. 2002) are classical strategies known to have beneficial metabolic effects by exerting anti-inflammatory (Pae et al. 2012) and antioxidant (Greń 2013) activities in animals and humans. However, there have been few studies concerning their impacts in metabolic signaling pathways. Here, we used these two nutritional strategies (CR and vitamin E supplementation) in an investigation of glucose homeostasis, with the aim of improving the activity of insulin as well as nutrient-stimulated insulin secretion from the isolated islets of rats.

Materials and methods

Ethical approval and animals

All surgical and experimental procedures were approved by the Ethics Committee of the Hermínio Ometto University Center (UNIARARAS) (protocol No. 073/2011) and were conducted according to the guidelines of COBEA (Brazilian College of Animal Experimentation). Male Wistar rats, 2 months old and weighing 200–300 g, were provided by the animal facility of Centro Universitário Hermínio Ometto, UNIARARAS. The rats were maintained in individual cages at a temperature of 22 ± 1 °C, under a 12-h light/dark cycle, for one week before the beginning of CR and vitamin E supplementation. After this period, the animals were distributed among 4 groups: (*i*) C, control group; (*ii*) CV, control group supplemented with vitamin E [by gavage, 30 mg·kg^{-1}·day^{-1}]; (*iii*) R, rats subjected to CR; and (*iv*) RV, rats subjected to CR and supplemented with vitamin E [by gavage, 30 mg·kg^{-1}·day^{-1}]. A dietary intake of 18 mg vitamin E·kg^{-1}·day^{-1} is recommended for normal growth in rats (National Research Council 1995). In our study, the vitamin E concentration used was similar to a supplement dose. This dose can be considered low because higher doses of vitamin E (around 500 mg) have been used elsewhere (Chiang et al. 2011). The control rats were fed a commercial isocaloric diet, ad libitum (=100%), and the rats in R group received 60% of the food consumed by C group, corresponding to food restriction of 40% of the control intake. All groups had free access to water for the 21 days of the experiment. After 21 days, the animals were euthanized by deep anesthesia (3× the dose required for general anesthesia) using a combination of xylazine anesthetic [10 mg·(kg body mass)$^{-1}$] and ketamine [90 mg·(kg body mass)$^{-1}$], by intraperitoneal injection (i.p.).

Chemicals and antibodies

Vitamin E (Pharmanostra), bovine serum albumin (BSA, fraction V), collagenase type V, tris-(hydroxymethyl)aminomethane (Tris), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Triton X-100, Tween 20, aprotinin, and glycerol were purchased from Sigma (St. Louis, Missouri, USA). The reagents and apparatus for sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting were from Bio-Rad (Richmond, California, USA). Nitrocellulose membranes (Hybond ECL, 0.45 μm) and the SuperSignal West Pico Chemiluminescent Substrate Western blotting analysis system were from ThermoFisher Scientific Inc. (Rockford, Illinois, USA). Glucose was purchased from Fluka Biochemika (Buchs, Switzerland).

Hormonal and biochemical analyses and evaluation of body mass

After euthanization, blood samples from the different groups of rats were collected by cardiac puncture, and the serum was stored frozen at –20 °C for subsequent analysis. Serum levels of protein, cholesterol, and triglycerides were measured using commercial kits, according to the manufacturer's instructions (Laborlab, Guarulhos, São Paulo, Brazil). Insulin was measured using ELISA (Cayman Chemical, Ann Arbor, Michigan, USA). The hepatic and muscle glycogen content of the rats were determined as described by Lo et al. (1970).

Intraperitoneal glucose tolerance test (Ip.GTT) and intraperitoneal insulin tolerance test (Ip.ITT)

Ip.GTT

After fasting for 8 h, blood was collected from the tails of the rats (time zero). The animals then received 2 g glucose·(kg body mass)$^{-1}$, i.p., and blood samples were collected at 30, 60, 90, and 120 min to measure the glucose concentration. The glucose response during the Ip.GTT was calculated by estimating the total area under the curve using the trapezoidal method (Le Floch et al. 1990).

Ip.ITT

Insulin [1.5 U·(kg body mass)$^{-1}$] was administered (i.p.) and blood samples were collected after 0, 5, 10, 15, 20, 25, and 30 min to measure the serum levels of glucose. The constant rate for glucose disappearance (Kitt) was calculated using the formula $0.693/t_{1/2}$. Glucose $t_{1/2}$ was calculated from the slope of the least-squares analysis of the plasma glucose concentrations during the linear decay phase (Bonora et al. 1987).

Colonic mucosal histology analyses

We chose a morphometric tool to analyze colon histology (Thomazini and Dolder 2017). After fixation, the colon tissues were paraffin embedded and sections 5 μm thick taken at 40 μm intervals were stained with hematoxylin and eosin. Data were collected using ImagePro Plus (version 4.5.0.29; Media Cybernetics). The thickness of the colon wall was measured in 10 different regions; crypt height was measured in 15 crypts; and the height of the absorptive epithelium was determined from 20 absorptive cells.

Insulin secretion by isolated islets

Islets were isolated manually after collagenase digestion of the pancreas, as described by Boschero et al. (1990). Groups of 5 islets (n = 12 wells/group) were first incubated for 30 min at 37 °C in Krebs-bicarbonate solution containing 5.6 mmol·L^{-1} glucose and

Table 1. Serum biochemical parameters, hepatic and muscle glycogen, and colonic histology data for the different rat groups: control (C), control + vitamin E [30 mg·kg^{-1}·day^{-1}] (CV), caloric restriction (R), and caloric restriction + vitamin E (RV).

Parameter	C	CV	R	RV
Insulin (ng·mL^{-1})	0.98±0.17	0.69±0.10	0.35±0.02*	0.45±0.05*
Glucose (mg·dL^{-1})	89.0±1.3	75.0±7.1	61.7±7.5*	60.80±7.3*
Protein (g·L^{-1})	2.5±0.33	3.3±0.30	3.4±0.44	2.8±0.50
Cholesterol (mg·dL^{-1})	43.4±8.3	59.3±2.1*	29.0±3.8*	35.0±15.1
Triglycerides (mg·dL^{-1})	93.0±3.8	120.0±5.2*	57.0±11.4*	72.6±11.2
MDA (mmol·L^{-1})	0.55±0.080	0.29±0.022*	0.6±0.080	0.25±0.030*
Hepatic glycogen [g·(100 g tissue)$^{-1}$]	0.42±0.07	0.31±0.04	0.35±0.04	0.30±0.03
Muscle glycogen [g·(100 g tissue)$^{-1}$]	0.21±0.02	0.26±0.02	0.28±0.01	0.21±0.04
Colonic histology data				
Colonic mucosal height (μm)	282.9±31.80	358.7±57.50*	318.5±24.82	366.0±38.10*
Crypt height (μm)	142.6±8.80	162.3±58.60*	164.62±6.48*	153.2±3.01
Absorptive epithelium height (μm)	16.53±0.34	18.0±41.40	17.33±0.83	18.45±1.48

Note: MDA, malondialdehyde; data shown are the mean ± SEM (n = 8 rats); *, $p < 0.05$ compared with the control group.

equilibrated with 95% O_2–5% CO_2, at pH 7.4. The solution was then replaced with fresh Krebs-bicarbonate buffer and the islets were incubated for a further hour in the presence of increasing concentrations of glucose (2.8 to 27.7 mmol·L^{-1}) or 2.8 mmol·L^{-1} glucose plus 10 mmol·L^{-1} arginine, 10 mmol·L^{-1} leucine, 10 mmol·L^{-1} KIC (alpha-ketoisocaproate), and 5 mmol·L^{-1} glutamine. The incubation medium contained (in mmol·L^{-1}): 115 NaCl, 5 KCl, 10 $NaHCO_3$, 2.56 $CaCl_2$, 1 $MgCl_2$, and 15 HEPES, as well as 0.3% (*w*/*v*) BSA. The glucose concentration producing a response that was 50% of the maximum (EC_{50}) was calculated as the mean negative logarithm (pD2). The insulin content of each sample was measured as described above, using an ELISA kit.

Western blotting

For protein extraction, the liver, fat pad (epididymal), and gastrocnemius muscle samples were chopped and homogenized in buffer containing 10 mmol·L^{-1} EDTA, 100 mmol·L^{-1} Trizma base, 10 mmol·L^{-1} sodium pyrophosphate, 100 mmol·L^{-1} sodium fluoride, 100 mmol·L^{-1} sodium orthovanadate, 2 mmol·L^{-1} PMSF, 0.1 mg·mL^{-1} aprotinin (Sigma), and deionized water. The supernatant was collected to measure the protein concentration by the biuret method. Aliquots of the supernatant were treated with Laemmli buffer containing 100 mmol·L^{-1} DTT (Sigma). Samples containing 70 μg protein were boiled for 5 min and loaded onto 10% SDS–PAGE. The gels were run using a Mini-Protean apparatus (Bio-Rad) and transferred to nitrocellulose membranes (Hybond ECL, 0.45 μm). The membranes were washed in basal solution (1 mol·L^{-1} Trizma base, 5 mol·L^{-1} NaCl, 0.005% Tween 20, and deionized water) and incubated in blocking solution (basal solution plus 5% Molico skim milk). Membranes were incubated with a polyclonal antibody against phospho-Akt ser473 (1:1000; cat. No. 9271S; Cell Signaling Technology, Danvers, Massachusetts, USA), IR (1:200; cat. No. sc-711; Santa Cruz Biotechnology), and β-actin (1:1000; cat. No. 49705; Cell Signaling Technology). Specific protein bands were visualized by incubating the membranes with appropriate secondary antibodies, followed by exposure to the Super Signal West Pico Chemiluminescent Substrate kit, and were developed with the Syngene G:BOX documentation system. The band intensities were quantified by optical densitometry using the free Scion Image for Windows software (Scion Corporation, Frederick, Maryland, USA). The densitometric values of phospho-Akt ser473 and IR were expressed relative to the protein densitometric value of β-actin (Ni et al. 2016).

Detection of HGF and IDE mRNA by reverse-transcription PCR

Total RNA was isolated from approximately 100 mg of rat liver using TRIzol reagent (Invitrogen, Carlsbad, Calif.), including the digestion of contaminating DNA with amplification grade DNase I (Invitrogen), following the manufacturer's instructions. RNA purity and concentration were determined spectrophotometrically. Synthesis of cDNA employed 2 μg of RNA in the presence of DTT, dNTP, random primers, RNAseOUT, and SuperScript II Reverse Transcriptase (Invitrogen), in a final volume of 20 μL. The mRNA levels of the hepatocyte growth factor (HGF) and insulin degrading enzyme (IDE) genes were investigated by semiquantitative RT–PCR. Primer sequences used in the PCR reactions were chosen based on the sequences available in GenBank. HGF was amplified using gene-specific forward (5′-TTCCCAGCTAGTCTATGGAC-3′) and reverse (5′-GGTGCTGACTGCATTTCTC-3′) primers with an expected amplicon of 237 bp [melting temperature (T_m) = 59 °C], while the primers used for IDE (forward 5′-AGGAAATGTTGGCTGTGGACGCA-3′ and reverse 5′-CCTGGCAAGAACGTGGACGGATA-3′) amplified a predicted amplicon of 62 bp (T_m = 57 °C).

The amplified products were separated on a 2.0% agarose gel stained with ethidium bromide, and the gel was photographed using LPix-Touch (Loccus Biotecnologia). The signal intensities of the bands were measured densitometrically using Scion Image software (Scion Corporation). Each value was determined as the mean of 3 densitometry readings. The results are expressed as the average ratios for the relative expression of transcripts normalized with *β-actin* as the control housekeeping gene.

Statistical analysis

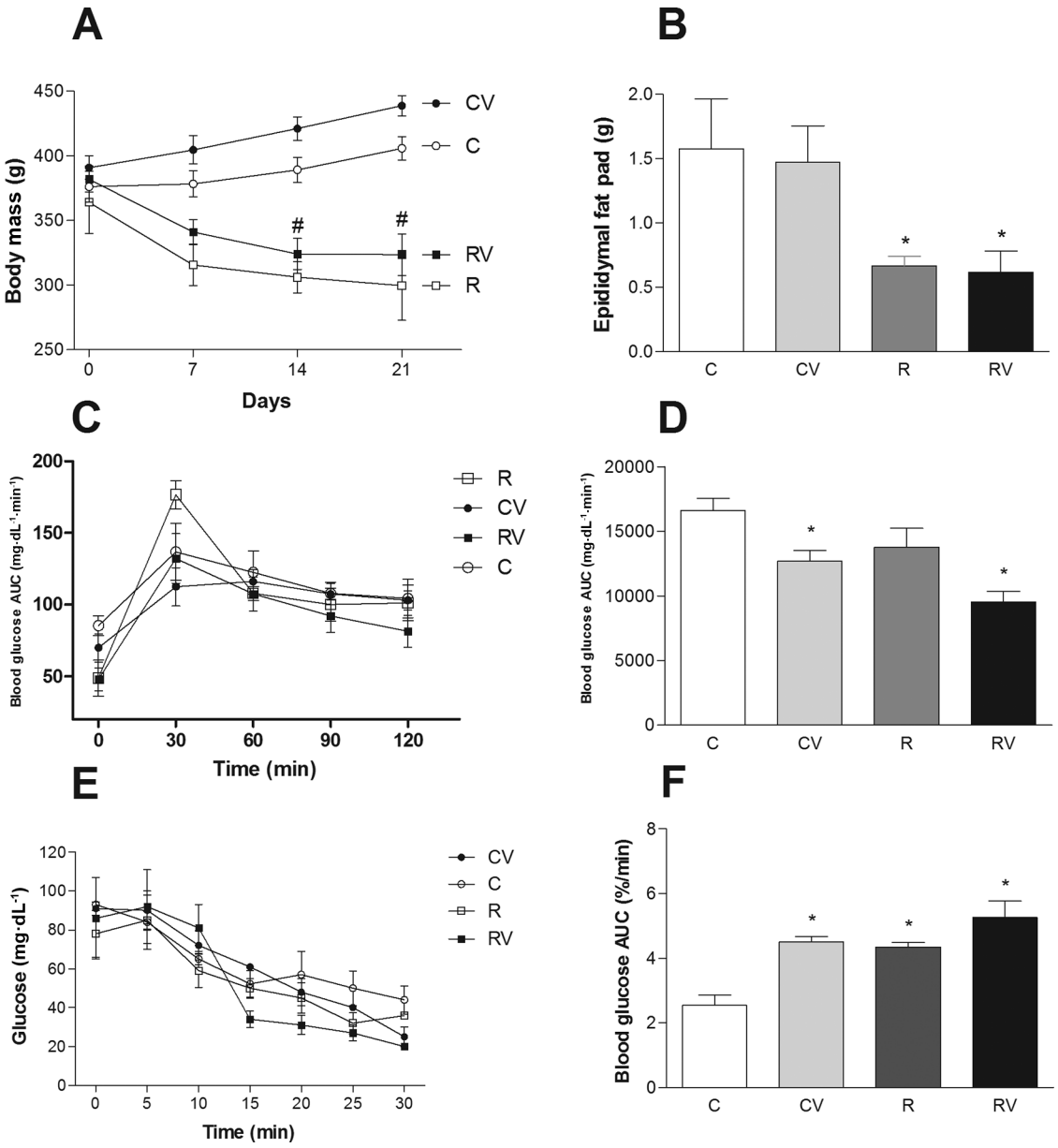
The results obtained for the four groups were compared using one-way analysis of variance (ANOVA) and the Tukey post-hoc test. Comparison of groups C and CV against groups R and RV employed unpaired t-tests. These statistical procedures were performed with GraphPad Prism 5 software. The results reported are the mean ± SEM, and values for $p < 0.05$ were considered statistically significant.

Results

Characteristics of the animals

Table 1 shows the biochemical parameters for the serum and Fig. 1 shows the body mass and glucose parameters for the different groups. Reductions in body mass, mass of the epididymal fat pad (Figs. 1A and 1B), glucose levels, and circulating insulin (Table 1) were observed in the R and RV groups, compared with the C and CV groups. Protein levels and hepatic and muscle glycogen contents were similar for all 4 groups. Serum levels of malondialdehyde (MDA) were significantly lower in the CV and RV groups compared with the C and R groups. The levels of cholesterol and triglyceride were significantly lower in the R group compared with the other groups, and were increased in the CV group com-

Fig. 1. Body mass (A), epididymal fat pad mass (B), area under the curve (AUC) for the glucose tolerance test (C), AUC for the intraperitoneal glucose tolerance test (D), and the constant for glucose decay (Kitt) during the test were calculated (E) and glucose disappearance rates during the insulin tolerance test (F), for the control group (C), control group treated with vitamin E [30 mg·kg^{-1}·day^{-1}] (CV), group subjected to caloric restriction (R), and the group subjected to caloric restriction and supplemented with vitamin E (RV). The data are the mean ± SEM (n = 8 rats); * $p < 0.05$ compared with group C; #, $p < 0.05$ comparing group R with group RV, and group C with group CV.



pared with the C group. The colonic mucosa was thicker in the RV and CV groups compared with the C group. The height of the crypt was higher in the R and CV groups compared with the C group. The height of the absorptive epithelium was similar for all 4 groups (Table 1; Fig. 2). These results (Table 1) corroborated previous findings of the systemic antioxidant effects of CR in rat models (do Amaral et al. 2011).

Influence of vitamin E supplementation and CR on glucose homeostasis during the Ip.GTT and the Ip.ITT

Glucose tolerance (Fig. 1C) was evaluated by calculating the area under the curve for all 4 groups. The area under the curve was smaller for the RV (9537 ± 1042), R (13 780 ± 1449), and CV (12 670 ± 666) groups, compared with the C group (16 610 ± 936) (Fig. 1D). The Ip.ITT (Fig. 1E) was used to directly evaluate insulin sensitivity in vivo. The glucose disappearance rates (Kitt) obtained for the R group and the animals treated with vitamin E for 21 days (RV, 5.3 ± 0.51%/min; R, 4.3% ± 0.13%·min^{-1}; CV, 4.5% ± 0.17%·min^{-1}) indicated higher insulin sensitivity in these groups, compared with the C group (2.5% ± 0.31%·min^{-1}) (Fig. 1F).

Static study of insulin secretion by isolated islets from rats submitted to vitamin E supplementation and CR

Once the animal model had been validated by biochemical and morphometric characterization, we analyzed the response of islets to stimulation using glucose and different secretagogues. Figure 3 shows the dose–response curves for static insulin secretion induced by different concentrations of glucose. Although the curves were shifted to the right in the case of the R and RV groups, the EC_{50} values did not differ significantly among groups C (11.18 ±

Fig. 2. Histological examination of the structure of the colon mucosa. C, colonic crypt; M, mucosa muscularis; m, mucosa height. Panel C, control group. Panel CV, control group supplemented with vitamin E [30 mg·(kg body mass)$^{-1}$·day^{-1}]. Panel R, rats subjected to caloric restriction. Panel RV, rats subjected to caloric restriction and supplemented with vitamin E. Stained with hematoxylin–eosin. Scale bar = 100 μm. [Colour online.]

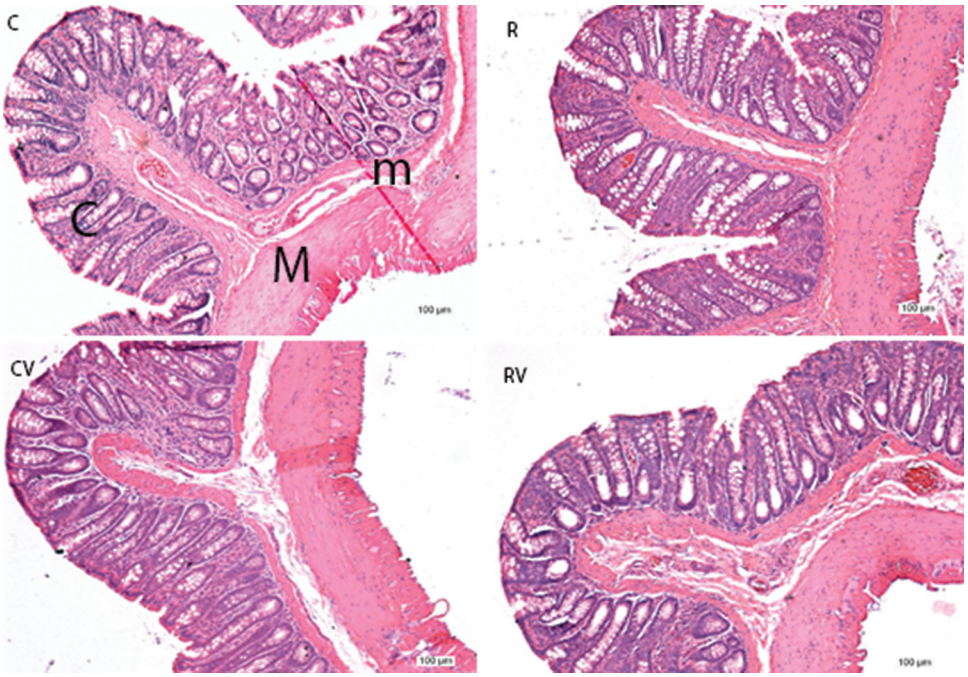
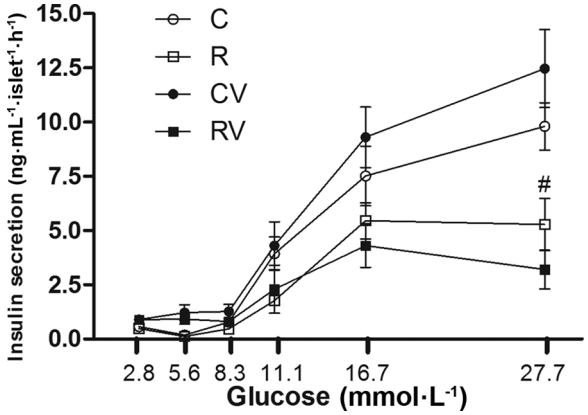


Fig. 3. Insulin secretion in response to increasing concentrations of glucose (2.8–27.7 mmol·L^{-1}) by isolated islets from the control group (C), control group supplemented with vitamin E [30 mg·(kg body mass)$^{-1}$·day^{-1}] (CV), rats subjected to caloric restriction (R), and rats subjected to caloric restriction and supplemented with vitamin E (RV). The data are the mean ± SEM (n = 12 groups of islets per group); #, $p < 0.05$ comparing groups R and RV with groups C and CV.



0.08 mmol·L^{-1} glucose), CV (13.71 ± 0.2 mmol·L^{-1} glucose), R (11.87 ± 0.1 mmol·L^{-1} glucose), and RV (11.10 ± 0.23 mmol·L^{-1} glucose). The maximum insulin responses to 27.7 mmol·L^{-1} glucose were 8.66 in group C, 10.89 in group CV, 5.36 in group R, and 3.7 in group RV (ng insulin·islet^{-1}·h^{-1}). Analysis of the insulin secretion stimulated by glutamine, leucine, arginine, and KIC showed an increase in the secretory capacity of islets of the CV group, compared with the other groups (Fig. 4). In contrast, group R showed decreased insulin secretion when stimulated with arginine and KCl (depolarizing agents), compared with the isolated islets from group C. The results suggest that insulin secretion in the RVgroup returned to the levels of group C after stimulation with KCl.

Effects of vitamin E supplementation and CR on the insulin signaling pathway

Increases of IR protein content in the basal state were observed in the epididymal fat pads and muscle tissue from groups CV, R, and RV, compared with group C (Fig. 5). IR and phospho-Akt protein contents were increased in the liver tissue from groups R and RV, compared with groups C and CV (Fig. 5). Similar results for phospho-Akt were found for epididymal fat pads, whereas for the muscle tissue, only group R showed an increase by comparison with the other groups.

Effect of vitamin E supplementation and CR on mRNA expression of HGF and IDE

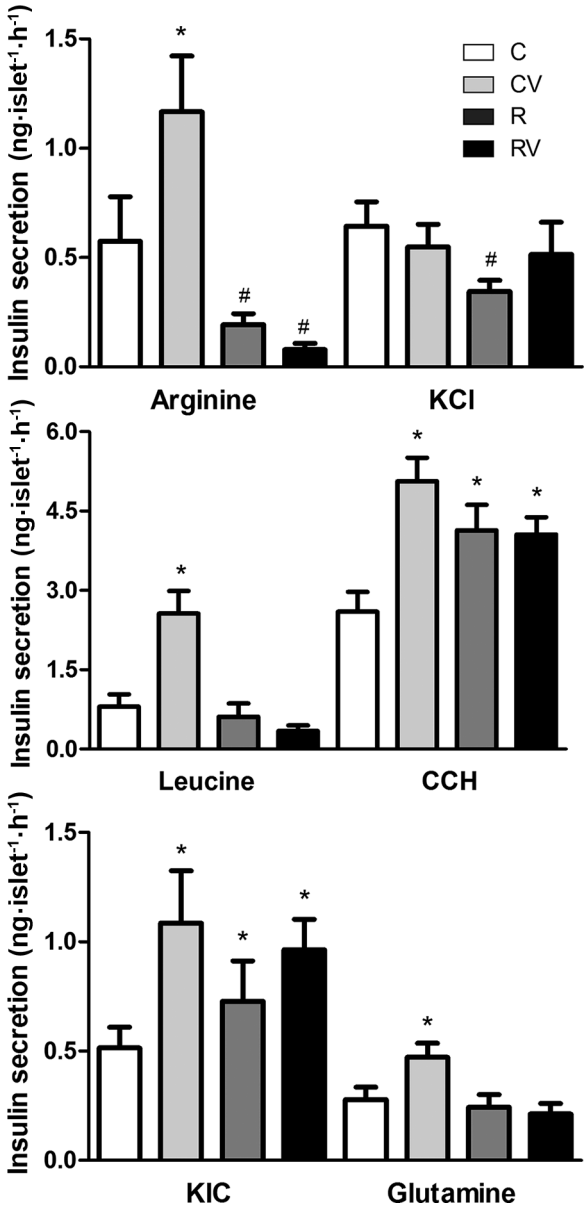
As shown in Fig. 6, there were no statistically significant differences in the gene expression of HGF among groups C (1.04 ± 0.06), CV (1.04 ± 0.06) and RV (0.94 ± 0.008), whereas there was a decrease in group R animals, which were subjected to caloric restriction (0.60 ± 0.04), compared with the other groups. The gene expression of IDE showed an increase in the CV group (0.86 ± 0.02) and a decrease in the R group (0.51 ± 0.01), compared with group C (0.65 ± 0.02). The expression of IDE in the RV group (0.92 ± 0.01) was similar to that in the CV group.

Discussion

The results show that vitamin E supplementation and CR can modulate insulin secretion, sensitivity, large intestine histology structure, and clearance of insulin. However, no metabolic changes were observed that would suggest synergism between vitamin E supplementation and CR (group RV).

The biochemical data are in agreement with other studies using rodents, where CR under different experimental protocols caused effects similar to those observed here (Masoro et al. 1992; Argentino et al. 2005; Varady and Hellerstein 2007; do Amaral et al. 2011). The higher levels of cholesterol and triglycerides in the CV group could also be explained by the increase in low-density lipoprotein cholesterol and overexpression of hydroxymethylglutaryl–CoA reductase (Hmgcr) observed by our research group (Santolim et al. 2017). The

Fig. 4. Insulin secretion in response to glutamine, arginine, leucine, KIC, KCl, and carbachol (CCH) by isolated islets from the control group (C), control group supplemented with vitamin E [30 mg·(kg body mass)$^{-1}$·day^{-1}] (CV), rats subjectd to caloric restriction (R), and rats subjected to caloric restriction and supplemented with vitamin E (RV). The data are the mean ± SEM (n = 12 groups of islets per group); *, $p < 0.05$ comparing group C with groups R, CV, and RV; #, $p < 0.05$ comparing groups R and RV with groups C and CV.



decreased levels of MDA found in the CV and RV groups were in agreement with the findings of other studies involving rodents and humans (Ihara et al. 2000), with evidence of the antioxidant effect of vitamin E supplementation in both animal groups. MDA is a marker of oxidative stress and many studies have validated its use to test the hypothesis that CR promotes longevity by reducing structural oxidative damage (Li et al. 2012). However, in this study, no difference was observed in the MDA level of the CR group, although our group has previously reported increased Mn-SOD mRNA expression in this same animal model (Santolim et al. 2017). Additionally, MDA levels are elevated in states of insulin resistance (Scott and King 2004; Cuerda et al. 2011), and in this study, decreased MDA levels in the animals with vitamin E supplementation could have been associated with the insulin sensitivity observed by GTT, ITT, and insulin signaling. The increase in crypt height in the CV and R groups may suggest an adaptive process of the large intestine to preserve the absorption of water and electrolytes caused by vitamin E and CR, as previously described by Schoffen et al. (2014). These results suggest that dehydration, hypovolemia, and hypotension are prevented when elevated glucagon levels occur during fasting (Allister et al. 2013). In addition, the data from measurements of the colonic walls in the CV and RV group indicate that the thickness of the muscular layer and lamina propria increased, which suggests an effect from the vitamin E supplementation; however, previous authors have examined the effects of supplementation with vitamin E without seeing any effect on the thickness of the intestinal wall or muscular tunic (Roldi et al. 2009). However, the incretin hormone cholecystokinin is released from enteroendocrine cells in the colon in response to nutrient intake (Fakhry et al. 2017), and cholecystokinin secretion contributes to insulin secretion from pancreatic β-cells (Hardikar 2004), which supports the results for insulin secretion observed in the CV group. In addition, cholecystokinin is known to be important in islet β-cell maturation and proliferation (Hardikar 2004). Our findings reveal increased sensitivity to insulin in animals with vitamin E supplementation, with or without CR, in agreement with the literature (Ihara et al. 2000; Robertson 2007; do Amaral et al. 2011). This finding is probably due to the antioxidant and beneficial effects of vitamin E and CR on the cells of the organism (Faure et al. 1997; Hiratsuka et al. 2005; Vistoropsky et al. 2008; Chiang et al. 2011; Cuerda et al. 2011; Li et al. 2012). IR, Akt protein, and HGF mRNA expression are involved in glucoregulatory tissue functions and are modulated by dietary changes, especially under CR conditions. Reduced HGF in group R indicates increased peripheral insulin sensitivity. However, supplementation with vitamin E induced an increase in insulin sensitivity without changes in the expression of the HGF gene. The results thus indicate that regulation of the insulin signaling pathway was tissue-specific.

According to the literature, the duration and percentage of CR modulate the insulin signaling pathway differently (Gazdag et al. 1999; Dean and Cartee 2000; Davidson et al. 2002; McCurdy et al. 2003; McCurdy and Cartee 2005; Arias and Cartee 2007; Park et al. 2008; Cerqueira et al. 2011). To elucidate the functional roles of vitamin E supplementation and CR in promoting insulin secretion, we studied the regulation of the cellular mechanisms activated by the insulin secretagogues. The study of these mechanisms is extremely important because they have insulinotropic properties and contribute to peripheral insulin sensitivity. Amino acids are important for stimulating β-cell electrical activity, essential for insulin secretion as leucine (KIC), glutamine, and arginine. Leucine stimulates insulin secretion in pancreatic β-cells by activating glutamate dehydrogenase (GDH) and thereby increasing mitochondrial metabolism, and increasing ATP production by transamination of leucine to α-ketoisocaproate (KIC) and subsequent entry into the Krebs cycle. Glutamine is converted by glutaminase to glutamate, which is oxidized by GDH in the mitochondria to produce α-ketoglutarate that also enters the Krebs cycle. Arginine stimulates the insulin secretion by transport of the cationic amino acid into the cell, resulting in membrane depolarization in pancreatic β-cells (Newsholme et al. 2007; Zou et al. 2014). On the other hand, carbachol (CCH) and KCl have different mechanisms to stimulate the insulin secretion. CCH is an acetylcholine (Ach) mimetic that binds to Ach muscarinic receptor stimulating phospholipase C (PLC) activation and subsequently, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). These events increase free cytosolic Ca^{2+} (Batista et al. 2012). KCl is a depolarizing agent of the pancreatic β-cell membrane that stimulates the insulin secretion (Chia et al. 2017). In our study, we found that insulin secretion, in response to different agents, was regulated differently among the 4 test groups. Vitamin E treatment increased glucose sensitivity, with a consequent increase in insulin secretion when stimulated with high concentrations of glucose, glutamine, leucine, arginine, KIC, and CCH, in agreement with literature reports for

Fig. 5. Insulin signaling in the epididymal fat pads, liver, and muscle of the control group (C), control group supplemented with vitamin E [30 mg·(kg body mass)$^{-1}$·day^{-1}] (CV), rats subjected to caloric restriction (R), and rats subjected to caloric restriction and supplemented with vitamin E (RV). Typical blots showing the protein content of insulin receptor (IR) and phospho-Akt in the liver, epididymal fat pad, and muscle. The relative expressions of IR and phospho-Akt were calculated. The data shown are the mean ± SEM (n = 4 rats); *, $p < 0.05$ compared with the C group.

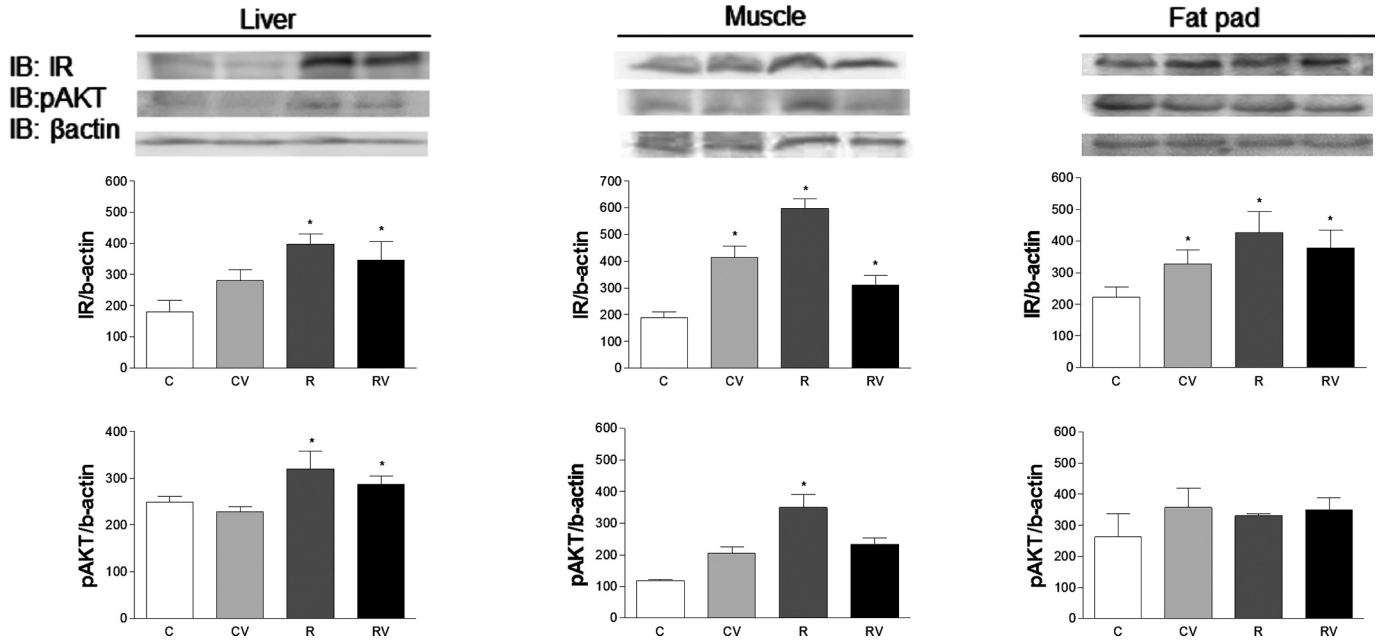
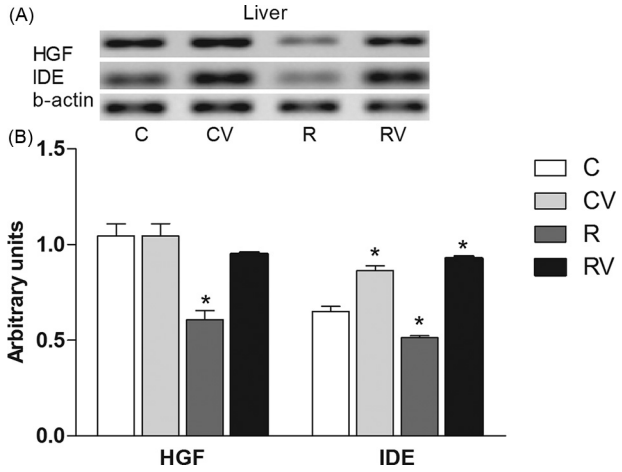


Fig. 6. Expression levels of hepatocyte growth factor (HGF) and insulin degrading enzyme (IDE) mRNA in rat liver. (A) Representative results from semiquantitative RT–PCR of HGF and IDE mRNA expression, and (B) densitometric analysis of HGF and IDE mRNA expression, for the control group (C), control group supplemented with vitamin E [30 mg·(kg body mass)$^{-1}$·day^{-1}] (CV), rats subjected to caloric restriction (R), and rats subjected to caloric restriction and supplemented with vitamin E (RV). Changes in mRNA are expressed in normalized densitometric units relative to β-actin mRNA. Values are shown are the mean ± SEM (n = 4 rats); *, $p < 0.05$ compared with group C.



different experimental conditions (Tajiri and Grill 1999; Sjöholm et al. 2000; Tsujinaka et al. 2005). This indicated the potential of vitamin E supplementation to attenuate glucose insensitivity in situations of diabetes and CR, in agreement with the literature (Williams et al. 2012). In contrast, CR led to lower insulin secretion in response to stimulation with glucose, arginine, and KCl, compared with the isolated islets from group C, and to an increase in insulin secretion when stimulated with CCH and KIC (do Amaral et al. 2011). Zawalich and Zawalich (2000) reported that in a study using isolated islets from rats that had fasted for 24 h, a 90% reduction in insulin secretion was observed, without any reduction in islet insulin content. Considering the results we obtained for group R, previous work found that there was no difference between the fasted and fed states in terms of insulin secretion stimulated by leucine and KIC (Newsholme et al. 2007). Such discrepancies between studies can be explained by differences in the ways that the β-cells process signals (do Amaral et al. 2011).

Because vitamin E supplementation resulted in an increase in insulin secretion, whereas CR had the opposite effect, at least with stimulation by depolarizing agents (arginine and KCl), we investigated insulin clearance in the liver of these animals. Removal of insulin from the physiological system is dependent on the binding of the peptide to its receptor, as well as on the IDE present in various tissues, predominantly the liver (Duckworth et al. 1998). It has been shown that IDE can preserve the means for peripheral insulin sensitivity and clearance (Ye 2013; Wei et al. 2014). In this study, reduced mRNA expression of IDE could provide an explanation for the reduced insulin secretion in group R. This mechanism acted to increase the peripheral insulin sensitivity in group R, suggesting that it was associated with decreased insulin clearance. Previous work by our group showed that CR increases serum levels of nitric oxide (Santolim et al. 2017), whereas Cordes et al. (2009) found that IDE activity was inhibited by nitric oxide. We cannot exclude the possible activity of IDE in the regulation of insulin secretion, as this was not examined in this study. In one study, it was found that animals treated with a cafeteria diet presented decreased levels of the IDE gene and increased insulin secretion (Brandimarti et al. 2013), whereas another study found the opposite (Castell-Auví et al. 2012). Elsewhere, the IDE activity in obese mice was optimized by pioglitazone, maintaining the homeostasis of insulin secretion (Wei et al. 2014).

Finally, we conclude that both vitamin E supplementation and CR were effective in increasing insulin sensitivity, although both the regulation of insulin secretion and glycemic homeostasis occurred through different mechanisms. Regulation by vitamin E seemed to involve its antioxidant action, with a reduction in MDA levels, changes to the structure of the large intestine, and an

increase in the levels of IDE mRNA, which accelerated insulin clearance and prevented hyperinsulinemia. On the other hand, the regulation of glycemic homeostasis by CR appeared to be associated with a decrease in IDE mRNA expression, insulin secretion, and delayed insulin clearance. However, based on the changes to insulin secretion and sensitivity in group RV, there was no evidence to suggest a synergistic interaction between vitamin E and CR in group RV. Vitamin E therapy and CR seem to be more effective applied individually for glycemic homeostasis.

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