Resveratrol reduces lipid peroxidation and increases sirtuin 1 expression in adult animals programed by neonatal protein restriction

Juliana Gastão Franco¹, Egberto Gaspar de Moura¹, Josely Correa Koury², Paula Affonso Trotta¹, Aline Cordeiro³, Luana Lopes Souza³, Norma Aparecida dos Santos Almeida³, Natália da Silva Lima¹, Carmen Cabanelas Pazos-Moura³, Patrícia Cristina Lisboa¹ and Magna Cottini Fonseca Passos^{1,4}

¹Department of Physiological Sciences, Roberto Alcântara Gomes Biology Institute and ²Department of Basic and Experimental Nutrition, Institute of Nutrition, State University of Rio de Janeiro, Rio de Janeiro 20550-030, RJ, Brazil

³Laboratory of Molecular Endocrinology, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro 21949-900, RJ, Brazil

⁴Department of Applied Nutrition, Institute of Nutrition, State University of Rio de Janeiro, Rio de Janeiro 20550-013, RJ, Brazil

(Correspondence should be addressed to E. G. de Moura who is now at Departamento de Ciências Fisiológicas, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, 5° andar, Avenue 28 de Setembro, 87, Rio de Janeiro 20550-030, RJ, Brazil; Email: egberto@pq.cnpq.br)

Abstract

Resveratrol (Res) has been associated with protective effects against oxidative stress. This study evaluated the effect of Res over lipid peroxidation, antioxidant defense, hepatic sirtuin 1 (SIRT1), which up-regulates antioxidant enzymes, and copper/zinc superoxide dismutase (Cu/Zn SOD) in adult offspring whose mothers were protein restricted during lactation. Lactating Wistar rats were divided into control (C) group, which were fed a normal diet (23% protein), and low-protein and high-carbohydrate (LPHC) group, which were fed a diet containing 8% protein. After weaning (21 days), C and LPHC offspring were fed a normal diet until they were 180 days old. At the 160th day, animals were separated into four groups as follows: control, control+Res, LPHC, and LPHC+Res. Resveratrol was given for 20 days (30 mg/kg per day by gavage). LPHC animals showed a higher total antioxidant capacity (TAC)

without change in lipid peroxidation and SIRT1 expression. The treatment with Res increased TAC only in the control group without effect on lipid peroxidation and SIRT1. LPHC animals treated with Res had lower lipid peroxidation and higher protein and mRNA expression of SIRT1 without any further increase in TAC. No significant difference in liver Cu/Zn SOD expression was observed among the groups. In conclusion, maternal protein restriction during lactation programs the offspring for a higher antioxidant capacity, and these animals seem to respond to Res treatment with a lower lipid peroxidation and higher hepatic SIRT1 expression that we did not observe in the Res-treated controls. It is probable that the protective effect can be attributed to Res activating SIRT1, only in the LPHC-programed group.

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Introduction

Environmental, nutritional, or hormonal influences in early life (gestation and lactation) may change some physiological parameters in adulthood, a phenomenon known as programming (Lucas 1994, Barker 2007, Moura & Passos 2005, Moura *et al.* 2008). Studies in human and animal models have shown that malnutrition during critical periods of neonatal life is associated with later metabolic disorders (Holness *et al.* 2000, Passos *et al.* 2000, 2004, Sichieri *et al.* 2000, Vicente *et al.* 2004). In developing countries, previously malnourished populations can afford a normal protein and energy supply in their diets during developmental periods and are at higher risk of developing diseases, according to developmental origins of disease hypothesis. Oxidative stress is implicated in most human diseases. Antioxidants may decrease the oxidative damage and its alleged harmful effects. Many people take antioxidant supplements, such as resveratrol, believing

them to improve their health and prevent diseases (Balluz *et al.* 2000, Radimer *et al.* 2004, Glauert *et al.* 2010).

Previously, we demonstrated in rats that a low-protein and high-carbohydrate (LPHC) diet during lactation programs for lower body weight, lower visceral and total fat mass, lower glycemia and insulinemia, and leptin resistance at adulthood (Passos *et al.* 2004, Fagundes *et al.* 2007, 2009). Other studies reported a lower insulin secretion and higher insulin sensitivity in adult rats submitted to severe postnatal protein restriction (0 or 4% protein content; Moura *et al.* 1997) and increased insulin sensitivity in adult rats whose mothers were fed a diet containing 10% protein during lactation (Zambrano *et al.* 2006). Furthermore, offspring of dams fed a low-protein diet during lactation had higher levels of sirtuin 1 (SIRT1) at 12 weeks of age that may regulate the aging process (Chen *et al.* 2009a). Protein restriction in lactation has been associated with nephroprotective effects in adult male rats and increased antioxidant expression (Tarry-Adkins *et al.* 2007).

It is widely accepted that chronic energy restriction (ER) can increase longevity in many organisms from yeast to mammals and delays the onset of several age-related diseases in rodents (McCay *et al.* 1935, Barja 2000, Gredilla & Barja 2005) associated with an increase of SIRT1, a histone deacetylase (Bordone & Guarente 2004). However, the mechanisms through which chronic ER results in longevity and robust health are still unclear. In recent years, it has been shown that long-term ER decreases the levels of oxidative damage to cellular macromolecules mainly by reducing the rate of mitochondrial reactive oxygen species (ROS) generation in rodents (Gredilla & Barja 2005). In contrast to chronic ER, studies in rats showed that maternal ER, during lactation only, programs the adult offspring to a higher body length and weight (Passos *et al.* 2004, Vicente *et al.* 2004), while maternal protein restriction seems to protect against obesity and insulin resistance (Fagundes *et al.* 2007, 2009).

Early-life nutrition also seems to influence longevity in rodents since protein restriction and slow growth during lactation lead to early-life alterations in the expression of key molecules, such as SIRT1, which may influence lifespan (Chen *et al.* 2009a,b). The dietary factor that may be responsible for part of the longevity extension effect occurring in chronic ER is the restriction of the specific amino acid methionine. Studies have shown that methionine restriction can decrease mitochondrial ROS production and oxidative stress (Caro *et al.* 2009a). Restriction of dietary amino acids other than methionine decreases mitochondrial protein oxidation and increases SIRT1 in rat liver (Caro *et al.* 2009b).

Cells are equipped with enzymatic and non-enzymatic antioxidant systems to eliminate ROS. Enzymatic antioxidant defenses involve a group of enzymes, such as superoxide dismutase (SOD) that converts accumulated anion superoxide (O₂⁻) to H₂O₂. Cu/Zn SOD (SOD1) is one of the major isoforms of SOD. Non-enzymatic antioxidants are represented by ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids, hemoglobin, and other antioxidants (Yu 1994, Devaraj & Jialal 2000, Shen *et al.* 2006).

Sirtuins are a family of class III histone/protein deacetylases. In mammals, seven homologues (SIRT1–SIRT7) have been identified and SIRT1 is a central factor in the metabolic adaptation to ER, triggering appropriate physiological responses via the modulation of the activity of key transcriptional factors involved in metabolism and oxidative stress (Bordone & Guarente 2004).

Resveratrol (Res, *trans*-3,4',5-trihydroxystilbene), a natural phytoalexin found in grapes, mimics the positive effects of chronic ER. Res is recognized for its wide range of biological effects, including anti-inflammatory, anticancer, and anti-mutagenic protection from atherosclerotic disease (Howitz *et al.* 2003, Baur *et al.* 2006). The effects of Res are mediated in part by its ability to activate SIRT1 (Lagouge *et al.* 2006). Studies have shown that Res can extend the lifespan of *Saccharomyces cerevisiae* (Howitz *et al.* 2003, Jarolim *et al.* 2004),

Caenorhabditis elegans (Wood *et al.* 2004, Viswanathan *et al.* 2005), *Drosophila melanogaster* (Bauer *et al.* 2004), and the vertebrate fish *Nothobranchius furzeri* (Valenzano *et al.* 2006). However, in rodents, Res can delay age-related deterioration in mice without extending lifespan (Pearson *et al.* 2008).

As mentioned before, adult rats programed by low-protein diets during lactation presented a favorable metabolic phenotype, with lower adiposity and higher insulin sensitivity. We hypothesized that this phenotype may also be associated with lower oxidative stress and a better response to the anti-oxidative effects of resveratrol, together with a higher expression of SIRT1. Thus, our aim was to evaluate the effect of Res on lipid peroxidation, antioxidant defense, and the expression of SIRT1 in the liver of both adult controls and LPHC-programed animals.

Materials and Methods

Experimental model

Wistar rats were kept in a temperature-controlled room (25 ± 1 °C) with artificial dark–light cycles (lights on 0700 h, lights off 1900 h). Adult female rats were caged with male rats at the proportion of 3:1. After mating, each female was placed in an individual cage with free access to water and food until delivery. Our experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEA/189/2007), which based their analysis on the principles adopted and promulgated by the Brazilian Law issued on November 8, 2008, which concerns the rearing and use of animals in teaching and research activities in Brazil (Marques *et al.* 2009).

At birth, 16 lactating rats were randomly assigned to each one of the following groups: control (C; *n*=8) group, with free access to a standard laboratory diet (17% protein); LPHC diet (*n*=8) group, with free access to an isoenergetic and protein-restricted diet (8%). Table 1 shows the composition of the diets, which follow recommended standards (Reeves *et al.* 1993). The LPHC and control diets were made in our laboratory using casein as the protein source. In order to make isoenergetic diets, a higher amount of starch was added to

Table 1 Composition of the control and low-protein diets (g/kg)

Ingredients	Control (17% protein)	LPHC (8% protein)
Casein (85 g protein/100 g)	200	94.1
Corn starch	529	635
Sucrose	100	100
Soybean oil	70	70
Cellulose	50	50
Choline bitartrate	2.5	2.5
L-Cystine	3	3
Mineral mix ^a	35	35
Vitamin mix ^a	10	10

^aDetailed composition is given by Reeves *et al.*

the LPHC diet so as to make up for the decrease in energy content due to protein reduction (Fagundes *et al.* 2007). To avoid the influence of litter size on the programming effect, only mothers whose litter size was 10–12 offspring were used. At birth, all litters were adjusted to six males to each dam to maximize lactation performance. The diets started at birth, which was defined as day 0 (d0) of lactation, and ended at weaning (d21).

After weaning (21 days lactation), all pups received the standard laboratory diet until they were 180 days old. The body weight of pups was monitored daily, during lactation. From weaning until day 180, body weight and food intake were monitored every 4 days.

Three pups from each litter were randomly chosen and followed during the experimental period. On day 160, one male offspring from each of the eight litters was randomly assigned to receive resveratrol or vehicle solution, whereas the other animals of this litter were discarded. Both treatments were administered by gavage during an experimental period of 20 days. In group I, control (C) rats received 0.5% (w/v) aqueous methylcellulose (vehicle); in group II, control + resveratrol (C+Res) rats received Res (30 mg/kg per day); in group III, LPHC diet rats received 0.5% (w/v) aqueous methylcellulose; and in group IV, LPHC + Res rats received 30 mg/kg per day of resveratrol (Macarulla *et al.* 2009). Because of its low solubility in water, resveratrol was suspended in carboxymethylcellulose solution (Das *et al.* 2008). The suspension was prepared freshly and shaken vigorously before oral gavage. The amount used corresponded approximately to five times the amount consumed by a person who drinks 300 ml of red wine a day containing 2.0 mg of *trans*-resveratrol, a dose that is not harmful to rats (Juan *et al.* 2002).

At the end of the experimental period, the rats were killed with a lethal dose of pentobarbital (0.06 g/kg body weight) in order to collect blood, liver, and visceral fat mass (VFM). The blood was collected by cardiac puncture and poured in a tube containing EDTA. VFM was excised and weighed for evaluation of the central adiposity – mesenteric, epididymal, and retroperitoneal. Plasma samples were frozen at -80°C until analysis, and samples of liver and lipid tissues were frozen in liquid nitrogen and stored at -70°C .

Glucose measurement

Glycemia was determined in blood samples from the tail vein of fasting rats using a glucometer (ACCU-CHEK Advantage, Roche Diagnostics).

Serum insulin determination

Blood samples were centrifuged (2000 *g* for 20 min at 4°C) to obtain serum, which was individually kept at -20°C until assay. All measurements were performed in one assay. Insulin was determined by a commercial RIA kit (ImmuChem TM 125 I, coated tube, ICN Biomedicals, Inc., New York, NY, USA). The intra-assay variation was 8.9%.

Insulin sensitivity evaluation

We evaluated insulin sensitivity according to three formulas:

- I/G ratio: fasting insulin ($\mu\text{IU/ml}$)/fasting glucose (mmol/l)
- The homeostasis model assessment of insulin resistance (HOMA-IR): (insulin ($\mu\text{IU/ml}$) $\times$ serum glucose (mmol/l))/22.5 (Matthews *et al.* 1985).
- HOMA- β : (insulin ($\mu\text{IU/ml}$) $\times$ 20)/serum glucose (mmol/l) – 3.5 (Matthews *et al.* 1985).

Lipid profile

Serum levels of total cholesterol, triglycerides, high-density lipoprotein (HDL-c), low-density lipoprotein (LDL-c), and very low-density lipoprotein (VLDL-c) were analyzed using Biosystem commercial test kits. LDL-c and VLDL-c were obtained using Friedewald calculations:

- 1) LDL-c (mg/dl) = total cholesterol – (triglycerides/5) – HDL-c
- 2) VLDL-c (mg/dl) = triglycerides/5

SIRT1 and Cu/Zn SOD protein expression: western blotting analysis

Hepatic tissues were excised and homogenized in an Ultra-Turrax T25 basic (IKA Werke GmbH & Co. KG, Staufen, Germany) in lysis buffer (50 mM HEPES, 1 mM MgCl_2 , 10 mM EDTA, and 1% Triton X-100 with the protease inhibitor cocktail Complete (Roche; pH 6.4)). After centrifugation, the homogenates were stored at -20°C . SIRT1 and SOD1 content was analyzed by western blotting as described below.

The protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples (30 μg total protein) were electrophoresed in 12 or 12.5% Tris-glycine SDS polyacrylamide gels. Proteins were transferred onto polyvinylidene fluoride membranes (Hybond ECL; Amersham Pharmacia Biotech), blocked in 5% dry milk in T-TBS (0.02 M Tris/0.15 M NaCl, pH 7.5, containing 0.1% Tween 20) at room temperature for 1 h, washed 3 $\times$ with T-TBS, and incubated with the primary antibodies (Cu/Zn SOD, 1:500; SIRT1, 1:500) for 1 h at room temperature. Cu/Zn SOD and SIRT1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (San Francisco, CA, USA). After washing 3 $\times$ with T-TBS, the blots were incubated with secondary antibodies (SIRT1 – 1:6250, peroxidase-labeled anti-rabbit IgG, Amersham Biosciences, Inc., and Cu/Zn SOD – 1:1000, peroxidase-labeled anti-rabbit IgG, Santa Cruz Biotechnology) for 1 h and then incubated with streptavidin (Zymed, San Francisco, CA, USA) in the same dilution of the secondary antibody for 1 h. The blots were then washed and developed with diaminobenzamidine (Sigma Chemical Co.) as a chromogenic substrate or with enhanced chemiluminescence

Figure 1 Body weight and relative food intake evolution. (A) Body weight of rats whose mothers were fed with C (open triangle) or LPHC (open square) diet during lactation. (B) Food intake (g/100 g body weight) after weaning until adulthood of rats whose mothers were fed a C (open triangle) or LPHC (open square) diet during lactation. Data are reported as mean $\pm$ S.E.M. Statistical significance was determined by two-way ANOVA and Newman-Keuls post-test. * $P < 0.05$; $n = 8$ animals/group.

(Amersham Biosciences, Inc.). The SIRT1 membranes were stained with rouge ponceau to evaluate the relative amounts of transferred proteins (Almeida *et al.* 2009).

SIRT1 RNA expression: reverse transcription-PCR analysis

Total RNA was isolated from hepatic tissue using commercially available and standard methodology (TRIZOL reagent, Invitrogen) respectively. For quantitative real-time reverse transcription PCR (real-time RT-PCR) analysis, RT was carried out on 1 μ g of total RNA for all tissues using SuperScript III kit. The products were amplified on Applied Biosystems 7500 Real-Time PCR System (Life Technologies Co.) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the recommendations of the manufacturer. Briefly, after initial denaturation at 50 °C for 2 min and at 95 °C for 10 min, reactions were cycled 40 times using the following parameters: 95 °C for 15 s, 60 °C for 30 s, and 70 °C for 45 s. Product purity was confirmed by agarose gel analysis. Relative mRNA levels were determined by comparing the PCR cycle threshold (C_t) between groups. The housekeeping gene used was 36B4. Data are expressed as fold induction over the control group, which was set to 1 or 100%. The sequences of the forward and reverse primers were respectively 5'-CAGGTPGCAGGAATCCAAA-3' and 5'-CAAAT-CAGGCAAGATGCTGT-3' for Sirt1 and 5'-CCGAGG-CAACAGTTGGGTA-3' for 36B4 (Rodgers *et al.* 2005, Machado *et al.* 2009).

Thiobarbituric acid-reactive substances

As an index of lipid peroxidation, we used the formation of thiobarbituric acid-reactive substances (TBARS) during an acid-heating reaction as previously described (Draper *et al.* 1993). Briefly, plasma was mixed with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid (Sigma Chemical Co.); subsequently, they were heated in a boiling water bath for 30 min. TBARS were determined by the absorbance at 532 nm in a spectrophotometer and were expressed as malondialdehyde (MDA) equivalents (nm/ml).

Total antioxidant capacity

Total antioxidant capacity (TAC) was evaluated by measuring the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable-free radical by blood plasma. The DPPH reduction assay was performed by adding a sample (20 μ l of blood plasma) plus 10 mM sodium phosphate buffer, pH 7.4 (total volume of 400 μ l), to 400 μ l of 0.1 mM methanol solution of DPPH. After 30 min incubation at ambient temperature

Figure 2 Body weight evolution during the treatment with Res and visceral fat mass in 180-day-old rats. (A) Body weight from 160 to 180 days of age of control rats that received Res (filled triangle) or vehicle (open triangle) and of LPHC rats that received Res (closed square) or vehicle (open square). (B) Visceral fat mass of control, LPHC, C+Res, and LPHC+Res rats. Data are reported as mean $\pm$ S.E.M. Statistical significance was determined by two-way ANOVA and Newman-Keuls post-test. * $P < 0.05$; $n = 8$ animals/group.

(21 °C), absorbance of the samples at 520 nm was measured and compared with that of a reference sample containing only DPPH solution and phosphate buffer (Janaszewska & Bartosz 2002).

Statistical analysis

Results are reported as mean $\pm$ S.E.M. Differences between the C and LPHC groups were analyzed by two-way ANOVA followed by Newman–Keuls post-test. Differences were considered significant at $P < 0.05$.

Results

Body weight and relative food intake from weaning (21 days) to the killing (180 days) are shown in Fig. 1. Offspring from mothers fed a LPHC diet had lower body weight from the 11th day of lactation until adulthood (Fig. 1A), but no change in food intake (Fig. 1B). Resveratrol, administered by gavage during 20 days to 160- to 180-day-old rats, did not alter their body weight (Fig. 2A). LPHC offspring showed lower VFM at 180 days of age (-36% , $P < 0.05$). Oral administration of Res did not alter VFM either in control group or in LPHC group (Fig. 2B).

As shown in Table 2, LPHC group presented lower glycemia (-11% , $P < 0.05$) in 180-day-old rats. The treatment with resveratrol did not modify glucose serum concentration in any group. Insulinemia was lower in the LPHC group (-36% versus C, $P < 0.05$) and in the LPHC + Res group (-28.3% versus C + Res, $P < 0.05$). Concerning insulin sensitivity, LPHC animals presented lower ratio insulin/glycemia (-33% versus C, $P < 0.05$), lower HOMA-IR (-47% versus C, $P < 0.05$), and lower HOMA- β (-32% versus C, $P < 0.05$), and resveratrol did not alter this profile in these rats.

Lower total cholesterol was observed in LPHC animals (-19% , $P < 0.05$; Table 2). The Res treatment did not alter the serum lipid profile in any group.

Figure 3 shows lipid peroxidation, evaluated by plasma TBARS measurement. Rats whose mothers were fed with LPHC diet during lactation showed no difference in plasma TBARS concentration. Res induced a significant reduction of plasma TBARS only in LPHC group (-48% versus LPHC, -52% versus C; $P < 0.01$ and -41% versus C + Res; $P < 0.01$, Fig. 3A).

As shown in Fig. 3B, the adult progeny from LPHC mothers had a higher TAC when compared with that from C mothers ($+25\%$, $P < 0.05$). Res administration induced a significant increase of TAC only in the control group ($+16\%$, $P < 0.05$).

Hepatic protein and mRNA expression of SIRT1 and protein expression of Cu/Zn SOD are shown in Fig. 4. Res treatment only increased hepatic SIRT1 protein in the LPHC group ($+10\%$, $P < 0.05$, Fig. 4A), and the increase in mRNA expression was more evident (3.5-fold versus LPHC, $P < 0.05$, Fig. 4B).

No significant difference in Cu/Zn SOD expression was observed among the groups (Fig. 4C).

Discussion

The main focus of our present study was to evaluate the effect of resveratrol over the oxidative stress and the expression of SIRT1 in the liver both in control and in animals programed by a LPHC diet during lactation. The present findings indicate that maternal protein restriction during lactation programed the adult offspring for a higher TAC than controls, which was not further incremented by resveratrol. However, resveratrol was able to decrease lipid peroxidation only in the LPHC-programed animals, and this effect may be related to an increase of SIRT1 in the liver. To the best of our

Table 2 Glucose homeostasis and serum lipid profile of 180-day-old offspring whose mothers were fed with a normal diet during lactation and treated with Res (C + Res) or vehicle (C) and in offspring whose mothers were fed with a low-protein and high-carbohydrate (LPHC) diet during lactation and treated with Res (LPHC + Res) or vehicle (LPHC)

	C	C + Res	LPHC	LPHC + Res
Glycemia (mg/dl)	76.5 $\pm$ 1.6	71.5 $\pm$ 1.7	68.2 $\pm$ 2.7*	71.4 $\pm$ 1.2
Serum insulin (μ UI/ml)	57.2 $\pm$ 2.9	53.1 $\pm$ 3.4	36.6 $\pm$ 4.6*	38.0 $\pm$ 3.6*
I/G ratio	13.4 $\pm$ 1.0	12.1 $\pm$ 0.8	9.0 $\pm$ 1.2*	9.6 $\pm$ 0.9*
HOMA-IR	10.9 $\pm$ 1.2	9.0 $\pm$ 0.5	5.8 $\pm$ 0.9*	6.7 $\pm$ 0.7*
HOMA- β	264.6 $\pm$ 20	238.9 $\pm$ 16	177.9 $\pm$ 24*	188.6 $\pm$ 18*
Total cholesterol (mg/dl)	71.3 $\pm$ 4.4	61.4 $\pm$ 3.3	57.4 $\pm$ 2.6*	64.3 $\pm$ 3.7
HDL-c (mg/dl)	30.3 $\pm$ 1.5	31.5 $\pm$ 1.4	26.9 $\pm$ 0.6	30.0 $\pm$ 1.2
LDL-c (mg/dl)	23.7 $\pm$ 3.5	22.6 $\pm$ 2.7	24.4 $\pm$ 3.2	23.5 $\pm$ 3.9
VLDL-c (mg/dl)	11.9 $\pm$ 1.2	9.9 $\pm$ 0.7	9.8 $\pm$ 0.7	10.2 $\pm$ 0.9
Triglycerides (mg/dl)	59.2 $\pm$ 6.1	44.5 $\pm$ 4	51 $\pm$ 4.7	45.4 $\pm$ 2.6

* $P < 0.05$; $n = 8$ animals/group.

Figure 3 Plasma levels of thiobarbituric acid-reactive substances and total antioxidant capacity. (A) Plasma levels of TBARS at 180 days in offspring whose mothers were fed with a normal diet during lactation and treated with Res (C+Res) or vehicle (C) and in offspring whose mothers were fed with a LPHC diet during lactation and treated with Res (LPHC+Res) or vehicle (LPHC). (B) Scavenging activity of DPPH in plasma at 180 days of age of control, LPHC, C+Res, and LPHC+Res rats. Data are reported as mean $\pm$ S.E.M. Statistical significance was determined by two-way ANOVA and Newman-Keuls post-test. * $P < 0.05$ and ** $P < 0.01$; $n = 8$ animals/group.

knowledge, there are no data reporting the effect of resveratrol in the adult offspring programed by protein restriction during lactation. Resveratrol is usually ingested in wine. Thus, in our model, we chose an adult age to be compatible with the age of wine ingestion in humans, and the period of 20 days seems to be a chronic treatment in most rat studies. Besides wine, resveratrol is present in other food sources such as peanuts, and could be used as food supplements. Since protein malnutrition is still highly prevalent in human population and the use of antioxidants is increasingly recommended, the present result points that Res is more useful in previously protein-restricted individuals.

We confirmed our previous observations showing that maternal protein restriction during lactation programed the adult offspring for lower body weight and lower VFM (Passos *et al.* 2000, Fagundes *et al.* 2007, 2009). Since the food intake was similar among the groups, the lower adiposity suggests

lower lipogenesis or higher lipolytic activity, probably caused by changes in key hormones, such as higher thyroid hormones, catecholamine, and glucocorticoid, which are previously reported in these animals (Dutra *et al.* 2003, Fagundes *et al.* 2007, 2009, Lisboa *et al.* 2008). Resveratrol did not affect VFM in either group, and there is no previous report on the effect of resveratrol on VFM.

In accordance with our previous report (Fagundes *et al.* 2009), LPHC animals had lower glycemia and insulinemia in 180-day-old rats. These findings, together with lower I/G ratio, HOMA-IR, and HOMA- β , indicate that these animals had higher insulin sensitivity. Resveratrol did not modify this profile in both LPHC and control groups. Thus, the antioxidant effect of resveratrol in LPHC does not seem to be consequent to glucose homeostasis improvement. The ability of resveratrol to reduce insulin levels and hyperglycemia was reported in several studies including streptozotocin- and nicotinamide-induced experimental diabetic rats (Palsamy & Subramanian 2008, Ramadori *et al.* 2009, Sharma *et al.* 2010). However, these effects of resveratrol seemed to occur in animals that had impaired glucose homeostasis.

On day 180, LPHC offspring showed lower total cholesterol. This finding was in accordance with our previous study and may be associated with higher insulin sensitivity in liver or higher cholesterol biliary excretion caused by the higher serum thyroid hormones in these animals (Lisboa *et al.* 2008, Fagundes *et al.* 2009). Resveratrol did not alter serum lipid profile in any group. Although one study had demonstrated the ability of resveratrol to improve dyslipidemia in rodents (Mulvihill & Huff 2010), this seems to be controversial because other studies in obese rats given a similar dose to that in our study for 8 weeks reported unaltered lipid profiles (Aubin *et al.* 2008, Rivera *et al.* 2009). Thus, similar to the unchanged glucose homeostasis, the better antioxidant effect in LPHC group cannot be associated with changes in lipid profile.

Non-enzymatic antioxidants present in plasma, such as glutathione, vitamin C, vitamin E, and bilirubin, detoxify ROS and minimize the damage caused to biomolecules. Studies have shown that the stable free radical DPPH could be a useful, simple, and inexpensive method of estimation of the TAC in biological samples (Chrczanowicz *et al.* 2008, Botelho *et al.* 2010). The higher TAC found in LPHC-programed animals is in agreement with previous studies showing that protein restriction during lactation induced an up-regulation of antioxidant defense capacity at adulthood, represented by increased expression of kidney antioxidant enzymes: glutathione peroxidase and glutathione reductase in 3-month-old rats and glutathione peroxidase, glutathione reductase, and manganese SOD in 1-year-old rodents (Tarry-Adkins *et al.* 2007, 2008, Chen *et al.* 2009a,b). On the other hand, other studies had associated protein restriction during neonatal life with increased oxidative stress and related detrimental effects (Fetoui *et al.* 2009, Theys *et al.* 2009). It has been shown that low-protein diets during gestation or gestation and lactation could predispose to pancreatic islet

Figure 4 Hepatic protein and mRNA expression of SIRT1 and protein expression of Cu/Zn SOD at 180 days in offspring whose mothers were fed with a normal diet during lactation and treated with Res (C+Res) or vehicle (C) and in offspring whose mothers were fed with a LPHC diet during lactation and treated with Res (LPHC+Res) or vehicle (LPHC). (A) Hepatic SIRT1 protein expression. (B) Hepatic mRNA SIRT1 expression. (C) Hepatic Cu/Zn SOD protein expression. Data are reported as mean $\pm$ S.E.M. Statistical significance was determined by two-way ANOVA and Newman-Keuls post-test. * $P < 0.05$; $n = 8$ animals/group.

dysfunction later in life by an imbalance between higher concentration of superoxide radical-inactivating enzymes (SOD) and very low concentration of hydrogen peroxide-inactivating enzymes (CAT and GPX) in islets of the progeny in 3-month-old rats (Theys *et al.* 2009). In addition, protein-restricted diet given to mothers during late pregnancy and early postnatal periods induced oxidative stress in their pups on postnatal d14 (Fetoui *et al.* 2009). These findings indicate that the time window of protein restriction seems to be of critical importance for modulation of antioxidant defense capacity in adult life.

In the present study, although TAC is higher in the LPHC group, no differences in the liver antioxidant enzyme Cu/Zn SOD were observed among the groups. Ungvari *et al.* (2007) showed that resveratrol up-regulated the expression of other antioxidant enzymes such as glutathione peroxidase, catalase, and heme oxygenase-1 in cultured arteries, whereas it had no significant effect on the expression of SOD isoforms.

Since epidemiological studies have shown an inverse relation between red wine consumption and incidence of cardiovascular disease, the compounds present in grapes, mainly resveratrol, have been of great interest to researchers (Frémont 2000). Resveratrol is rapidly absorbed and metabolized, mainly as sulfo- and glucuro-conjugates that are excreted in urine, and seems to be well tolerated and non-toxic both in humans and in rats (Juan *et al.* 2002, Williams *et al.* 2009, Cottart *et al.* 2010). Although studies have shown that plasma resveratrol concentration is low, raising doubts about its efficiency (Goldberg *et al.* 2003, Vitaglione *et al.* 2005). Soleas *et al.* (2001) suggests that resveratrol levels in plasma could be seriously underestimated since a large part of the molecule may be bound by cell membranes or lipophilic tissues. Here, we showed that oral administration of resveratrol increases TAC in the control group. It is interesting to note that resveratrol did not modify TAC in the LPHC group. Since this group has already high levels of TAC, it is possible that TAC reached a saturated level. However, lipid peroxidation, evaluated by plasma TBARS concentration, decreased only in the LPHC+Res group. Lipid peroxidation refers to the oxidative degradation of lipids whereby free radicals pick up electrons from the lipids in cell membranes, resulting in cell damage. TBARS assay is a simple and useful method to quantify the end products of lipid peroxidation, specifically MDA. A similar reduction in plasma TBARS concentration was observed in fructose-fed rats during chronic administration of resveratrol (Miatello *et al.* 2005). It is postulated that Res probably reduces the deleterious effect of oxidative stress in living cells due to its ability to 1) compete with co-enzyme Q and decrease mitochondrial ROS production, 2) scavenge superoxide radicals, 3) inhibit lipid peroxidation induced by Fenton reactions, and 4) regulate the expression of antioxidant co-factors and enzymes (Pervaiz & Holme 2009). In addition, some studies demonstrated that Res inhibits NADPH oxidase activity and expression, which is a major contributor to

superoxide radical production causing local oxidative stress (Soylemez *et al.* 2009, Spanier *et al.* 2009).

Along with a decrease in lipid peroxidation, resveratrol also induced a small but significant increase in protein and a higher increment (3.5-fold) in mRNA expression of SIRT1 in the liver only in the group programed by maternal protein restriction. This apparent discrepancy in the percentage of increment between protein and mRNA expression is due, at least in part, to the more variable values in mRNA expression and also by a higher post-translational metabolism of the protein during processing. Other authors have shown that resveratrol treatment caused a significant increase in SIRT1 expression in association with anti-oxidative and anti-inflammatory effects in experimental models of colitis (Singh *et al.* 2010) and diabetic cardiomyopathy (Sulaiman *et al.* 2010). Moreover, the effect of resveratrol, attenuating the oxidative metabolism induced by high glucose on endothelial cells, was prevented by the ablation of SIRT1 expression (Ungvari *et al.* 2009). In addition, moderate overexpression of SIRT1 protected cells from oxidative stress (Csiszar *et al.* 2006, Alcendor *et al.* 2007, Lee *et al.* 2009), and hepatic SIRT1 action attenuated hepatic steatosis, inflammation, and oxidative stress (Purushotham *et al.* 2009). Therefore, it is likely the involvement of high liver SIRT1 in the reduction of lipid peroxidation in the LPHC+Res group. However, studies have shown that resveratrol is not a direct activator of SIRT1 since this compound interacts with multiple unrelated targets including receptors, enzymes, ion channels, and transporters (Behr *et al.* 2009, Pacholec *et al.* 2010).

It seems that to decrease lipid peroxidation, Res has to be acting in an animal with some increased level of non-enzymatic or enzymatic antioxidant capacity. Maybe the lower body weight present in these animals associated with lower adipogenesis (Fagundes *et al.* 2009) and higher insulin sensitivity are facilitating factors for resveratrol's protective effect against lipid peroxidation.

In conclusion, maternal protein restriction during lactation programs the adult offspring for a higher antioxidant capacity, and these animals seem to respond to treatment with resveratrol, which results in lower lipid peroxidation that may be mediated in part by increased liver SIRT1 expression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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