Resveratrol reduces lipid peroxidation and increases sirtuin1 expression in adult animals programmed by neonatal protein restriction

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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 upregulates antioxidant enzymes, and cooper/zinc superoxide dismutase (Cu/Zn SOD) in adult offspring whose mothers were protein restricted during lactation. Lactating Wistar rats were divided in: control (C) group, fed a normal diet (23% protein) and low protein and high carbohydrate (LPHC) group, fed a diet containing 8% protein. After weaning (21 days), C and LPHC offspring were fed a normal diet until 180 days-old. At the 160th day, animals were separated in four groups: control, control+Res, LPHC and LPHC+Res. Resveratrol (30mg/kg/d by gavage) was given for 20 days. 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 seems to respond to Res treatment with a lower lipid peroxidation and higher hepatic SIRT1 expression that we did not observed in the Res-treated controls. It is probable that the protective effect can be attributed to Res activating SIRT1, only in LPHC programmed group.
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 2004, 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, Sichieri et al. 2000; Passos et al. 2004, Vicente et al. 2004). In developing countries, those previous malnourished populations during developmental periods could afford a normal protein and energy supply in their diets and could be ready to develop diseases, according to developmental origins of diseases hypothesis. Oxidative stress is implicated in most human diseases. Antioxidants may decrease the oxidative damage and its alleged harmful effects. Many people are taking antioxidant supplements, such as resveratrol, believing to improve their health and prevent diseases (Balluz et al. 2000, Radimer et al. 2004, Glauert et al. 2010).

Previously, we have demonstrated in rats, that low protein and high carbohydrate diet (LPHC) 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, Fagundes et al. 2009). Other studies reported a lower insulin secretion and its higher sensitivity in adult rats submitted to severe post-natal 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% of protein during lactation (Zambrano et al. 2006). Furthermore, offspring of dams fed a low protein diet during lactation had higher levels of SIRT1 at 12 weeks of age that may regulate the aging process (Chen et al. 2009a). Besides, protein restriction in lactation was 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 (Mc Cay et al. 1935, Barja 2000, Gredilla & Barja 2005) associated with an increase of sirtuin 1 (SIRT1), a histone deacetylase (Bordone & Guarente 2004). However, the mechanisms through 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 species of oxygen (ROS) generation in rodents (Gredilla & Barja, 2005). Differently to the chronic ER, studies in rats showed that maternal ER only during lactation program 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, Fagundes et al. 2009).

Early-life nutrition also seems to influence the longevity in rodents since protein restriction and slow growth during lactation leads to early life alterations in the expression of key molecules, such as SIRT, which may influence lifespan (Chen et al. 2009a, Chen et al. 2009b, Martin-Gronert et al. 2008). 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). Besides, restriction of dietary amino acids different from methionine decreases mitochondrial protein oxidative modification 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 (O$_2^-$) to H$_2$O$_2$. Cu/Zn superoxide dismutase (Cu/Zn SOD or SOD1) is one of the major isoforms of SOD. Non-
enzymatic antioxidants are represented by ascorbic acid (Vitamin C), alfa-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 (HDACs). In mammals, seven homologues (SIRT1-SIRT7) have been identified and SIRT1 is a central factor in the metabolic adaptation to energy restriction, 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, 40, 5-trihydroxystilbene), a natural phytoalexin found in grapes, mimics the positive effects of chronic energy restriction. Res is recognized for its wide range of biological effects, including anti-inflammatory, anticancer, anti-mutagenic and 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. 2001). Studies have shown that Res can extend the lifespan of S. cerevisiae (Howitz et al. 2003; Jarolim et al. 2004), Caenorhabditis elegans (Viswanathan et al. 2005; Wood et al. 2004), 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 programmed 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 better response to 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 adults control and LPHC-programmed 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 07:00 h, lights off 19:00 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, sixteen lactating rats were randomly assigned to each one of the following groups: control group (C; n=8), with free access to a standard laboratory diet (23% protein); low protein and high carbohydrate diet group (LPHC; n=8), 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 protein source. In order to make isoenergetic diets, a higher amount of starch was added to 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 d lactation), all pups received standard laboratory diet until they were 180 days old. The body weight of pups, during lactation, was monitored daily. From weaning until the 180th day, body weight and food intake were monitored every 4 days.

Three pups from each litter were randomly chosen and followed during the experimental period. At 160th days, one male offspring from each of eight litters was randomly assigned to receive resveratrol or vehicle solution, the other animal of this litter was discarded. Both treatments were administered by gavages during an experimental period of 20 days. Group I: Control (C) rats received 0.5% (w/v) aqueous methylcellulose (vehicle); group II: Control + resveratrol (C+Res) rats received Res (30 mg/kg/d); group III: Low protein and high carbohydrate diet (LPHC) rats received 0.5% (w/v) aqueous methylcellulose and group IV: Low protein and high carbohydrate diet resveratrol (LPHC+Res) rats received 30 mg/kg/d or resveratrol (Macarulla et al. 2009). Because of its low solubility in water, resveratrol was suspended into carboxymethylcellulose solution (Das et al. 2008). The suspension was prepared freshly and shaken vigorously before oral gavage. The amount used corresponded approximately to 5 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 euthanized with a non lethal dose of pentobarbital (0.06 g/Kg/b.w.) in order to collect blood, liver and visceral fat mass. The blood was collected by cardiac puncture and poured in a tube containing ethylene diamine tetra-acetic acid (EDTA). Visceral fat mass (VFM) was excised and weighed for evaluation of the central adiposity – mesenteric, epidydimal, 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 sample from the tail vein of fasting rats using a glucosimeter (ACCU-CHEK ® Advantage, Roche Diagnostics, Mannheim, Germany).

**Serum Insulin determination**

Blood samples were centrifuged (2 000 × 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 commercial RIA kit (ImmuChem TM 125 I, coated tube, ICN Biomedicals Inc., NY, USA). The intra-assay variation was 8.9 %.

**Insulin sensitivity evaluation**

We evaluated insulin sensitivity according to 3 formulas:

- I/ G ratio: Fasting Insulin (µ UI/ ml)/ Fasting Glucose (mmol/l)
- The homeostasis model assessment of insulin resistance (HOMA-IR):
  \[\text{HOMA-IR} = \frac{[\text{Insulin} (\mu IU/ml) \cdot \text{serum glucose (mmol/l)}]}{22.5}\] (Matthews et al. 1985).
- HOMA-β: \[\text{HOMA-β} = \frac{[\text{Insulin} (\mu IU/ml) \cdot 20]}{\text{serum glucose (mmol/l)} - 3.5}\] (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
**Sirtuin1 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 lyses buffer [50 mM HEPES, 1 mM MgCl2, 10 mM EDTA, and 1% Triton X-100 with the protease inhibitor cocktail Complete (Roche, Indianapolis, IN) (pH 6.4)]. After centrifugation, the homogenates were stored at -20°C. SIRT-1 and SOD-1 content was analyzed by Western blotting as described below.

The protein concentration was determined by the BCA™ protein kit assay (Pierce, Rockford, IL). Samples (30 µg total protein) were electrophoresed in 12 or 12.5% Tris-glycine sodium dodecyl sulfate (SDS) polyacrylamide gels. Proteins were transferred onto PVDF membranes (Hybond ECL; Amersham Pharmacia Biotech, London, UK), 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 hour, washed 3x with T-TBS and incubated with the primary antibodies (Cu/Zn SOD, 1:500; SIRT1, 1:500) for 1 hour at room temperature. Cu/Zn SOD and SIRT-1 antibodies were purchased from Santa Cruz Biotechnology Inc. (San Francisco, CA, USA). After washing 3x with T-TBS, the blots were incubated with secondary antibodies (SIRT1 - 1:6250, peroxidase-labeled antirabbit IgG, Amersham Biosciences Inc., Piscataway, NJ and Cu/Zn SOD - 1:1000, peroxidase-labeled antirabbit IgG, Santa Cruz Biotechnology, CA, USA) for 1 hour and then, incubated with streptavidin (Zymed, CA, USA) in the same dilution of the secondary antibody for 1 hour. The blots were then washed and developed with diaminobenzamidine (Sigma Chemical Co., St. Louis, MO) as chromogenic substrate or with enhanced chemiluminescence (ECL; Amersham Biosciences Inc., Piscataway, NJ). The SIRT1 membranes were stained with rouge ponceau to evaluate the relative amounts of transferred proteins (Almeida et al. 2009).

**Sirtuin1 RNA expression: RT-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, reverse transcription (RT) was carried out on 1µg of total RNA for all tissues using SuperScript III kit. Products were amplified on Applied Biosystems 7500 Real-Time PCR System (Life Technologies Co., Frederick, MD, USA) using SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA) according to the recommendations of the manufacturer. Briefly, after initial denaturation at 50°C for 2 min and 95°C for 10 min, reactions were cycled 40 times using the following parameters: 95°C for 15 sec, 60°C for 30 sec and 70°C for 45 sec. Product purity was confirmed by agarose gel analysis. Relative mRNA levels were determined by comparing the PCR cycle threshold (Ct) between groups. The housekeeping gene used was 36B4. Data are expressed as fold induction over control group, which was set to (1 or 100%). The sequences of the forward and reverse primers were, respectively: 5’-CAGGTPGAGAATCCAAA-3’ and 5’-CAAATCAGGCAAGATGCTGT-3’ for the Sirt1 and 5’-CCGAGGCAACAGTTGGGTA-3’ for 36B4 (Rodgers et al. 2005, Machado et al. 2009).

**Thiobarbituric acid-reactive substances (TBARS)**

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

Total antioxidant capacity was evaluated by measuring the reduction of 2, 20-diphenylpicrylhydrazyl (DPPH) stable free radical by blood plasma. The DPPH reduction assay was performed by adding a sample (20 µl of blood plasma) plus 10mM sodium phosphate buffer pH 7.4 (total volume of 400 µl) to 400 µl of 0.1mM methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH). After 30-min incubation at ambient temperature (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 ± SEM. 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 sacrifice (180 days) are shown in Figure 1. Offspring from mothers fed a low protein and high carbohydrate diet (LPHC) 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 into 160 to 180 days-old rats did not alter their body weight (Fig.2A). LPHC offspring showed lower visceral fat mass at 180 days of age (-36%, p<0.05). Oral administration of Res did not alter visceral fat mass, either in control or LPHC group (Fig. 2B).

As shown in Table 2, LPHC group presented lower glycemia (-11%, p<0.05) at 180 days-old. The treatment with resveratrol did not modify glucose serum concentration in any
group. Insulinemia was lower in LPHC group (-36% vs C, p<0.05) as in LPHC+Res group (-28.3% vs C+Res, p<0.05). Concerning insulin sensitivity, LPHC animals presented lower ratio insulin / glycemia (-33% vs C, p < 0.05), lower HOMA-IR (-47% vs C, p < 0.05) and lower HOMA-β (-32% vs 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% vs LPHC, -52% vs C; p<0.01 and -41% vs C+Res; p<0.01, Fig. 3A).

As shown in Fig. 3B, the adult progeny from LPHC mothers had a higher total antioxidant capacity (TAC) when compared with those 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 Figure 4. Res treatment only increased hepatic SIRT1 protein in LPHC group (+10%, p<0.05, Fig. 4A) and the increase in mRNA expression was more evident (3.5 fold vs 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 animals programmed by a low protein and high carbohydrate diet during lactation. The present
findings indicated that maternal protein restriction during lactation programmed the adult offspring for a higher total antioxidant capacity (TAC) than controls, not further incremented by resveratrol. However, resveratrol was able to decrease lipid peroxidation only in the LPHC programmed animals and this effect may be related to an increase of SIRT1 in the liver. To the best of our knowledge, there are no data reporting the effect of resveratrol in the adult offspring programmed by protein restriction during lactation. Resveratrol usually is ingested in the wine. Thus, in our model we choose an adult age to be compatible with the age of wine ingestion in humans and the period of twenty days seems to be a chronic treatment in most of 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 are increasingly recommended, the present result points that the use of Res are more useful in previously protein restricted individuals.

We confirmed our previous observations showing that maternal protein restriction during lactation programmed the adult offspring for lower body weight and lower visceral fat mass (Passos et al. 2000, Fagundes et al. 2007, Fagundes et al. 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, previously reported in these animals (Dutra et al. 2000, Fagundes et al. 2007, Lisboa et al. 2008, Fagundes et al. 2009). Resveratrol did not affect VFM in either group and there is no previous report on the effect of resveratrol upon VFM.

Accordingly with our previous report (Fagundes et al. 2009), LPHC animals had lower glycemia and insulinemia at 180 days-old. 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 seems to be not consequent to glucose homeostasis improvement. The ability of resveratrol in reducing insulin levels and hyperglycemia was reported in several studies including streptozotocin-nicotinamide induced experimental diabetic rats (Palsamy & Subramanian, 2008, Ramadori et al., 2009, Sharma et al. 2010). However, these effects of resveratrol seem to occur in animals that had impaired glucose homeostasis.

At the 180th day, 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 (Fagundes et al. 2009; Lisboa et al. 2008). Resveratrol did not alter serum lipid profile in any group. Although one study had demonstrated the ability of resveratrol in improve dyslipidemia in rodents (Mulvihill & Huff 2010), this seems to be controversial, because other studies in obese rats with a similar dose of our study for eight weeks was unable to alter lipid profile (Rivera et al. 2009, Aubin et al 2008). 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, bilirrurbin, detoxify ROS and minimize the damage caused to the biomolecules. Studies have shown that the stable free radical DPPH could be a useful, simple and inexpensive method in the estimation of the total antioxidant capacity in biological samples (Chrzczanowicz et al. 2008, Botelho et al. 2010). The higher TAC found in LPHC-programmed 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 at 3 months-old and glutathione peroxidase, glutathione reductase and manganese superoxide dismutase at one year-old rodents (Tarry-Adkins et al. 2007, Tarry-Adkins et al. 2008, Chen et al. 2009). On other hand, other studies had associated protein-restriction during neonatal life with increased oxidative stress and related detrimental effects (Theys et al 2009; Fetoui et al. 2009). It has been shown that low protein diets during gestation or gestation and lactation could predispose to pancreatic islet dysfunction later in life by unbalance 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 at 3 months-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 day 14 (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 LPHC group, no differences in the liver antioxidant enzyme Cu/Zn SOD was observed among the groups. Ungvari et al. (2007) showed that resveratrol upregulated 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 which are excreted in urine and seems to be well tolerated and non-toxic both in human 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) suggest that resveratrol levels in plasma could be seriously under-estimated since a large part of the molecule may be bound by cell membranes or lipophilic tissues. Here, we showed that oral administration of resveratrol increase TAC on control group. It is interesting to note the fact that resveratrol did not modify TAC in LPHC group. Since this group has already high levels of TAC, it is possible that TAC reached a saturable 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 malondialdehyde (MDA). A similar reduction in plasma TBARS concentration was observed in fructose-fed rats during echronic 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: 1. to 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 of superoxide radical’s production causing local oxidative stress (Soylemez et al. 2009, Spanier et al. 2009).

Along with 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 programmed by maternal protein restriction. This apparent discrepancy in the percentual 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 cardiomiopathy (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,
SIRT1 moderate overexpression 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.
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 (Pacholec et al. 2010; Beher et al. 2009).

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 protective effect against
lipid peroxidation.

In conclusion, maternal protein restriction during lactation programs the adult
offspring for a higher antioxidant capacity and these animals seems to respond to treatment
with resveratrol, which results in lower lipid peroxidation that may be mediated in part by
increased liver SIRT1 expression.
**Declarations of interest:** The authors declare no conflict of interest.

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**References**


Almeida NA, Cordeiro A, Machado DS, Souza LL, Ortiga-Carvalho TM, Campos-de-Carvalho AC, Wondisford FE & Pazos-Moura CC 2009 Connexin40 messenger ribonucleic acid is positively regulated by thyroid hormone (TH) acting in cardiac atria via the TH receptor. *Endocrinology* **150** 546-554.

Aubin MC, Lajoie C, Clément R, Gosselin H, Calderone A & Perrault LP 2008 Female rats fed a high-fat diet were associated with vascular dysfunction and cardiac fibrosis in the


Botelho PB, Fioratti CO, Abdalla DS, Bertolami MC & Castro IA 2010 Classification of individuals with dyslipidaemia controlled by statins according to plasma biomarkers of oxidative stress using cluster analysis. *Brazilian Journal of Nutrition* **103** 256-265.


Dutra SC, Passos MC, Lisboa PC, Santos RS, Cabanellas AP, Pazos-Moura CC & Moura EG 2003 Liver deiodinase activity is increased in adult rats whose mothers were submitted to malnutrition during lactation. *Hormone and Metabolic Research* **35** 268-270.


Passos MC, Vicente LL, Lisboa PC & de Moura EG 2004 Absence of anorectic effect to acute peripheral leptin treatment in adult rats whose mothers were malnourished during lactation. *Hormone and Metabolic Research* **36** 625-629.


Purushotham A, Schug TT, Xu Q, Surapureddi S, Guo X & Li X 2009 Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. Cell Metabolism 9 327-338.


Vicente LL, Moura EG, Lisboa PC, Costa AMA, Amadeu T, Mandarim-de-Lacerda CA & Passos MCF 2004 Malnutrition during lactation is associated with higher expression of leptin receptor in pituitary of the adult offspring *Nutrition* **20** 924-928.


Yu, PB 1994 Cellular defenses against damage from reactive oxygen species. *Physiological Reviews* 74 139-162.

Table 1: Composition of the control and low protein diets (g/Kg)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control (17% protein)</th>
<th>LPHC (8% protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (85g protein/100g)</td>
<td>200</td>
<td>94.1</td>
</tr>
<tr>
<td>Corn starch</td>
<td>529</td>
<td>635</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Mineral mix*</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix*</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

* Detailed composition given by Reeves et al.
Table 2: Glucose homeostasis and serum lipid profile of 180 days-old offspring whose mothers were fed with 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) * p < 0.05; n = 8 animals / group

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>C+Res</th>
<th>LPHC</th>
<th>LPHC+Res</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycemia (mg / dl)</strong></td>
<td>76.5±1.6</td>
<td>71.5±1.7</td>
<td>68.2±2.7*</td>
<td>71.4±1.2</td>
</tr>
<tr>
<td><strong>Serum insulin (µUI / ml)</strong></td>
<td>57.2±2.9</td>
<td>53.1±3.4</td>
<td>36.6±4.6*</td>
<td>38.0±3.6*</td>
</tr>
<tr>
<td><strong>I/G ratio</strong></td>
<td>13.4±1.0</td>
<td>12.1±0.8</td>
<td>9.0±1.2*</td>
<td>9.6±0.9*</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>10.9±1.2</td>
<td>9.0±0.5</td>
<td>5.8±0.9*</td>
<td>6.7±0.7*</td>
</tr>
<tr>
<td><strong>HOMA-β</strong></td>
<td>264.6±20</td>
<td>238.9±16</td>
<td>177.9±24*</td>
<td>188.6±18*</td>
</tr>
<tr>
<td><strong>Total cholesterol (mg / dl)</strong></td>
<td>71.3±4.4</td>
<td>61.4±3.3</td>
<td>57.4±2.6*</td>
<td>64.3±3.7</td>
</tr>
<tr>
<td><strong>HDL-c (mg / dl)</strong></td>
<td>30.3±1.5</td>
<td>31.5±1.4</td>
<td>26.9±0.6</td>
<td>30.0±1.2</td>
</tr>
<tr>
<td><strong>LDL-c (mg / dl)</strong></td>
<td>23.7±3.5</td>
<td>22.6±2.7</td>
<td>24.4±3.2</td>
<td>23.5±3.9</td>
</tr>
<tr>
<td><strong>VLDL-c (mg / dl)</strong></td>
<td>11.9±1.2</td>
<td>9.9±0.7</td>
<td>9.8±0.7</td>
<td>10.2±0.9</td>
</tr>
<tr>
<td><strong>Triglycerides (mg / dl)</strong></td>
<td>59.2±6.1</td>
<td>44.5±4</td>
<td>51±4.7</td>
<td>45.4±2.6</td>
</tr>
</tbody>
</table>
Figure 1

A

![Graph showing body weight against age for different groups.](image)

- C
- LPHC
Figure 1

B

Food intake (g/100gBW)

Days
Figure 3

A

![Bar chart showing MDA (nmoles/ml) for different conditions. The bars are labeled as C, C+Res, LPHC, and LPHC+Res. The chart includes statistical significance indicators (** and *) indicating differences between groups.]
Figure 3

Scavenging activity of DPPH (%)

C  C+Res  LPHC  LPHC+Res

*
Figure 4

A

C   C+Res   LPHC  LPHC+Res

SIRT1

Cyclophilin

Hepatic SIRT1 expression (relative to C group)

* *
Figure 4

B

Hepatic mRNA SIRT1 (SIRT1/36β4 a.u.)

C  C+Res  LPHC  LPHC+Res

*
Figure 4

[Diagram showing hepatic Cu/Zn-SOD expression relative to C group for different conditions: C, C+Res, LPHC, LPHC+Res. The graph indicates a comparison of Cu/Zn-SOD and Actin expression levels across these conditions.]
**Figure Legends**

**Figure 1:** Body weight and relative food intake evolution. A. Body weight of rats whose mothers were fed with C (△) or LPHC (□) diet during lactation. B. Food intake g/100 g BW after weaning until adulthood of rats whose mothers were fed a C (△) or LPHC (□) diet during lactation. Data are reported as mean ± SEM. Statistical significance was determined by Two-way ANOVA and Newman Keuls post-test. *p < 0.05; n = 8 animals/group.

**Figure 2:** Body weight evolution during the treatment with Res and visceral fat mass at 180 days old rats. A. Body weight from 160 to 180 days of age of Control rats that received Res (▲) or vehicle (△) and of LPHC rats that received Res (■) or vehicle (□). B. Visceral fat mass of Control, LPHC, C+Res and LPHC+Res rats. Data are reported as mean ± SEM. Statistical significance was determined by Two-Way ANOVA and Newman Keuls post-test. *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 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 Control, LPHC, C+Res and LPHC+Res rats. Data are reported as mean ± SEM. Statistical significance was determined by two-Way ANOVA and Newman Keuls post-test. *p < 0.05 **p<0.01.; n = 8 animals/group.
**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 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 ± SEM. Statistical significance was determined by two-Way ANOVA and Newman-Keuls post test. *p < 0.05; n = 8 animals / group.