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Oxidative stress programming in a rat model of postnatal early overnutrition — role of insulin resistance☆,☆☆

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Abstract

Postnatal early overfeeding (EO) is related to later development of overweight and other metabolic disorders. As oxidative stress is implicated in most human diseases, as obesity and diabetes, we decided to study some parameters related to oxidative stress and insulin signaling in liver from EO animals in adult life. To induce EO, litter size was reduced to three pups per litter (SL: small litter) and groups with normal litter size (NL:10 pups per litter) were used as control. After weaning, rats had free access to standard diet and water. Body weight and food intake were monitored daily and offspring were killed at 180 days-old. Significant differences had $P<.05$ or less. As expected, SL rats had hyperphagia, higher body weight and higher visceral fat mass at weaning and adulthood. In liver, postnatal EO programmed for lower catalase (−42%), superoxide dismutase (−45%) and glutathione peroxidase (−65%) activities. The evaluation of liver injury in adult SL group showed lower nitrite content (−10%), higher liver and plasma malondialdehyde content (+25% and 1.1-fold increase, respectively). No changes of total protein bound carbonyl or Cu/Zn superoxide dismutase protein expression in liver were detected between the groups. Regarding insulin signaling pathway in liver, SL offspring showed lower IRβ (−66%), IRS1 (−50%), phospho-IRS1 (−73%), PI3-K (−30%) and Akt1 (−58%). Indeed, morphological analysis showed that SL rats presented focal areas of inflammatory cell infiltrate and lipid drops in their cytoplasm characterizing a microsteatosis. Thus, we evidenced that postnatal EO can program the oxidative stress in liver, maybe contributing for impairment of the insulin signaling.

Keywords: Small litter; Reactive oxygen species; Insulin signaling

1. Introduction

In recent decades, the prevalence of childhood obesity has greatly increased worldwide [1]. It is known that nutritional, environmental and/or hormonal influences during critical periods early in life can permanently change the structure and function of body tissues and systems; this association is denominated metabolic programming [2], and it was confirmed by epidemiological and experimental data [3,4]. Studies in animal models have shown that excess of nutrition in perinatal life represents a risk factor for obesity and associated metabolic disturbances in adulthood [5–7]. Recently, in a systematic review and meta-analysis, Risnes et al. [8] showed a strong association between higher birth weight and increased risk of cancer deaths.

Rats raised in "small litters" (SL) are an established animal model to study short- and long-term consequences of childhood obesity [9]. This model of postnatal early overnutrition (EO) was associated with hyperphagia, obesity, hypertension and hyperinsulinemia in adult life [10–14]. Other studies have suggested that oxidative stress, the imbalance between cellular production of reactive oxygen species (ROS) and antioxidant defenses in cells, could be an early event in the development of obesity-related chronic diseases, such as cardiovascular diseases, diabetes mellitus and cancer [15,16]. Nutrient overload and obesity increase ROS generation and oxidative stress. Excessive nutrient in the metabolic pathways leads to an increased electron flux through mitochondrial electron transfer chain. The consequent electron leak from respiratory complex I and III of electron transfer chain leads to an increased production of ROS from the mitochondria, such as superoxide and hydrogen peroxide [15].

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Previously, we have shown in adult SL rats, the programming for overweight, higher total and visceral fat mass, lower high-density lipoprotein cholesterol, hyperphagia, central leptin resistance and thyroid hypofunction in adult life [6,7]. At weaning, SL rats have insulin resistance characterized by an increase in fasting glucose levels and hyperinsulinemia, while at 6 months old, these animals showed a slight impairment in glucose tolerance test, 60 and 120 minutes after glucose load, suggesting insulin resistance, despite basal normoglycemia and normoinsulinemia [7]. Other reports showed that older (8 months old) SL rats present insulin resistance, suggesting that insulin resistance in this experimental model seem to be age dependent [10,11].

Since ROS have been proposed as an unifying mechanism linking nutrient excess and obesity-associated disturbances, in the present study we evaluated some parameters related to oxidative stress in adult rats programmed by EO. In addition, considering that there are two-way association between excessive ROS and insulin resistance, we studied the insulin signaling in liver.

2. Methods and materials

The use of the animals according to our experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/184/2007; CEUA/006/2009), which based their analysis on the principles adopted and promulgated by the Brazilian Law issued on November 8, 2008 [17,18]. Wistar rats were housed in a room with controlled temperature (25±1°C) and artificial dark–light cycles (lights on 07:00 h, lights off 19:00 h). Adult virgin female rats were caged with male rats (3:1) and after mating, each female was placed in an individual cage with free access to water and food until delivery.

2.1. Experimental model of postnatal EO

To induce EO during lactation, 3 days after birth, the litter size was adjusted to three male rats per litter (SL) [6,11]. Litter containing 10 pups per mother was used as control (NL). The rats analyzed were randomly chosen from 16 different litters (8 SL litters and 8 NL litters). After postnatal day 21 (PN21) that corresponds to weaning period, both groups have free access to water and standard diet. During lactation, body weight (BW) gain was daily monitored and from weaning until PN180, body weight and food intake (g/100g BW) were monitored every 4 days.

At PN180, rats were killed after to be anaesthetized with pentobarbital (0.06 g/kg BW) 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. The VFM (mesenteric, epididymal and retroperitoneal white adipose tissue) was excised and immediately weighed for evaluation of central adiposity. Plasma and liver samples were frozen at −80°C until analysis.

2.2. Determination of antioxidant enzyme activities in liver

Liver samples of 200 mg were homogenized in potassium phosphate buffer with EDTA in mechanical homogenizer (CT-136 model from Cientec–laboratory equipment, Campinas, SP, Brazil). After centrifugation, homogenates were stored at −80°C until analysis. The total protein content was determined by the Bradford method [19].

Total superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation as absorbance at 480 nm [20]. Catalase (CAT) activity was measured by the rate of decrease in H_2O_2 at 240 nm according to the method of Aebi [21]. Glutathione peroxidase (GPx) activity was evaluated according to Flohé & Günzler [22] by measuring the oxidation of NADPH at 340 nm in the presence of H_2O_2.

2.3. Nitrite assay

The yield of radical nitric oxide (NO) an indirect measurement of nitric oxide content was evaluated by Griss reaction through quantification of nitrite (NO_2^-) in liver at 540 nm [23].

2.4. Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was measured by malondialdehyde (MDA) concentration using the TBARS method as previously described [24,25]. Briefly, plasma and liver homogenates were mixed with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid (Sigma Chemical, St. Louis, MO, USA); subsequently they were heated in a boiling water bath for 30 min. The absorbance of the organic phase containing the pink chromogen was measured spectrophotometrically at 532 nm. MDA equivalents were expressed in nMol/mg protein.

2.5. Protein oxidation

Protein oxidation was evaluated in liver accordingly Levine et al. [26] as carbonyl groups reacting with 2,4-dinitrophenyl-hydrazine (Sigma). Values of absorbance were obtained at 380 nm and expressed in nmol of carbonyl by 0.5 mg of protein.

2.6. Western blotting analysis

Liver samples were homogenized in cold lysis buffer (50 mM Hepes, pH 6.4, 1 mM MgCl2, 10 mM EDTA and 1% Triton X-100) containing protease inhibitors (10 μg/μl aprotinin, 10 μg/μl leupeptin, 2 μg/μl pepstatin and 1 mM phenylmethylsuphonic fluoride, Sigma-Aldrich, St. Louis, MO, USA) using a Ultra-Turrax homogenizer (IKA Werke, Staufen, Germany). After centrifugation, homogenates were stored at −20°C. The total protein content was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA).

Samples (30 μg total protein) were electrophoresed in 10–12% Tris-glycine sodium dodecyl sulfate polyacrylamide gels. Proteins were transferred for polyvinylidene fluoride membranes (Hybond ECL; Amersham Pharmacia Biotech, London, UK), blocked in 5% dry milk in Tween-20 tris buffered saline (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× with T-TBS and incubated with the primary antibodies (Cu/Zn SOD, IR β, IRS1, phospho-IRS1, PI3-K, Akt1 and phospho-Akt1 at 1:500 concentration) overnight at 4°C. Primary antibodies were purchased from Santa Cruz Biotechnology (San Francisco, CA, USA). After washing 3× with T-TBS, blots were incubated with appropriate secondary antibodies at 1:5000 concentration (Santa Cruz Biotechnology) for 1 h and then incubated with streptavidin (Zymed, Carlsbad, CA, USA) in the same dilution of the secondary antibody for 1 h. Blots were developed with diaminobenzamidine (DAB; Sigma Chemical) as chromogenic substrate or with enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ, USA).

2.7. Liver histology

Liver samples were fixed in formalin (freshly prepared 1.27 mol/L formaldehyde, 0.1 M phosphate-buffered saline, pH 7.2) and embedded in paraffin to non-serial sections of 5 μm. Sections were placed in glass slides to stain in hematoxylin/eosin. The morphological study was performed utilizing digital images, acquired at random (TIFF format, 36-bit color, 1360x1024 pixels) with an Olympus DP71 camera and an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan).

2.8. Statistical analysis

Data are reported as mean±S.E.M. The *GraphPad Prism 4* program (GraphPad softwares, La Jolla, CA, USA) was used for statistical analyses and graphics. Two-way analysis of variance and Bonferroni post test were used to analyze body weight and food intake changes. Cu/Zn SOD expression and insulin signaling were analyzed by the non-parametric Mann–Whitney *U* test. The other experimental observations were analyzed by unpaired Student's *t* test, with significance level set at $P<.05$.

3. Results

3.1. Body weight, food intake and visceral fat mass

Body weight and food intake from weaning (PN21) to the sacrifice (PN180) are shown in Fig. 1. Offspring overfed during lactation (SL) had higher body weight than NL rats from PN7 until the end of lactation (+10%, $P<.0001$, Fig. 1A). SL rats remained overweight until PN180 (+15%, $P<.0005$, Fig. 1B). SL group presented a higher relative food intake from weaning until adulthood (PN180: +7%, $P<.05$, Fig. 1C). Also VFM was higher (+92%, $P<.0001$, Fig. 1D) in SL rats compared to NL rats.

3.2. Evaluation of oxidative stress parameters

As shown in Fig. 2, adult SL offspring showed lower CAT (−42%, $P<.0001$; Fig. 2A), SOD (−45%, $P<.0001$, Fig. 2B) and GPx activities (−65%, $P<.0001$, Fig. 2C) than the NL group. Despite the lower SOD activity, Western blot analysis showed that Cu/Zn SOD content was not different between the groups (NL:101.49±6.36 vs. SL:86.60±5.43).

As depicted in Fig. 3, liver nitrite bioavailability was lower in SL than NL group (−10%, $P<.0001$, Fig. 3A). Oxidative damage

assessed by MDA quantification was higher in SL group both in liver (+ 25%, $P<.05$; Fig. 3B) and in plasma (1.1 fold-increase; $P<.05$; Fig. 3C).

No significant difference in liver total protein bound carbonyl was observed between groups (Fig. 3D).

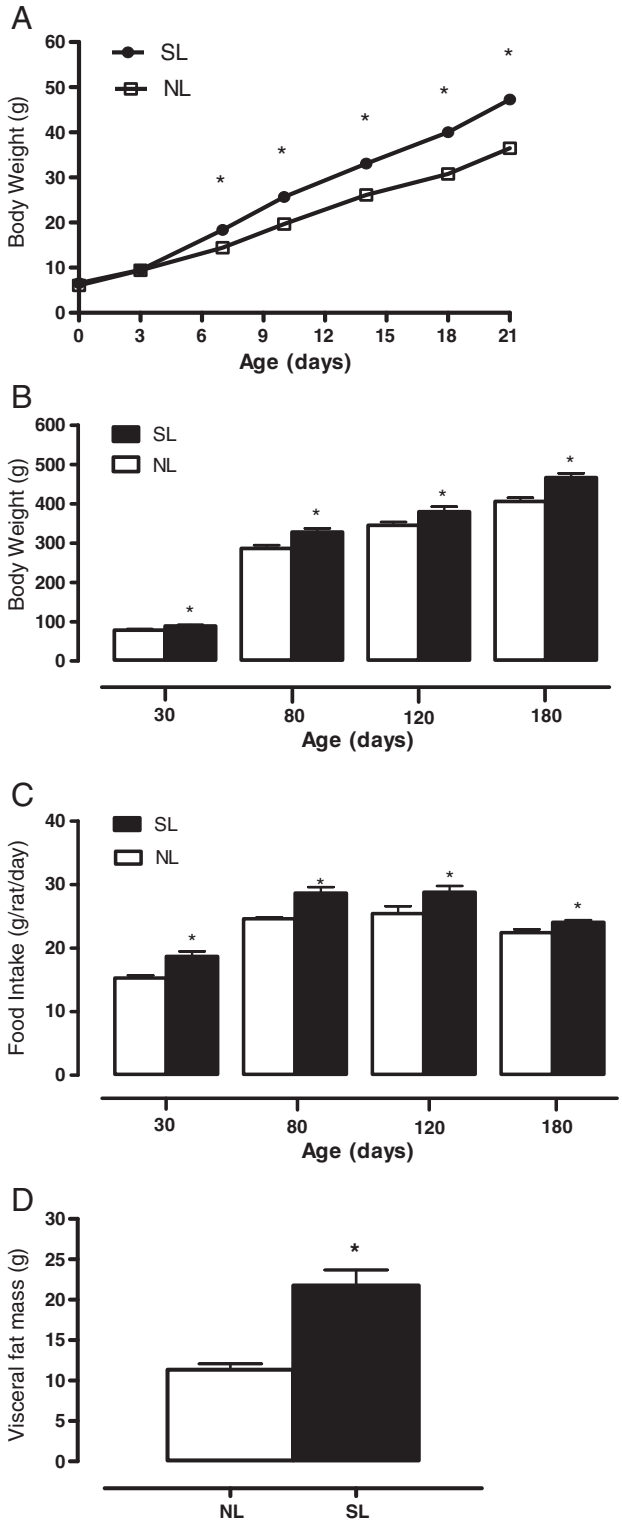

Fig. 1. Body weight evolution of SL (●) and NL (□) rats during lactation (A) and after weaning (B) until 180 days old. Food intake at 30, 80, 120 and 180 days of NL and SL rats (C). Visceral fat mass of NL and SL rats (D). Values are reported as mean±S.E.M. *$P<.05$; $n=8$ animals/group.

3.3. Insulin signaling

Liver content of insulin signaling molecules IRβ, IRS1, phospho-IRS1, PI3-K, Akt1 and phospho-Akt1 are shown in Fig. 4. The content of IRβ, phospho-IRS1, IRS1, PI3-K, Akt1 were lower in SL compared to NL group: IRβ (−66%; $P<.05$); Fig. 4A; phospho-IRS1 (−73%; Fig. 4B); IRS1 (−50%; $P<.05$; Fig. 4C), PI3-K (−30%; $P<.05$; Fig. 4D) and Akt1 (-58%; $P<.05$; Fig. 4E). We did not find differences in the content of phospho-Akt1 between the groups (Fig. 4F).

3.4. Liver histology

The morphological analysis showed a dysfunction in the hepatic tissue of adult SL offspring. As demonstrated in Fig. 5, SL rats presented focal areas of inflammatory cell infiltrate and drops of lipids in their cytoplasm characterizing a microsteatosis, differently of the NL rats that demonstrated a liver with preserved architecture.

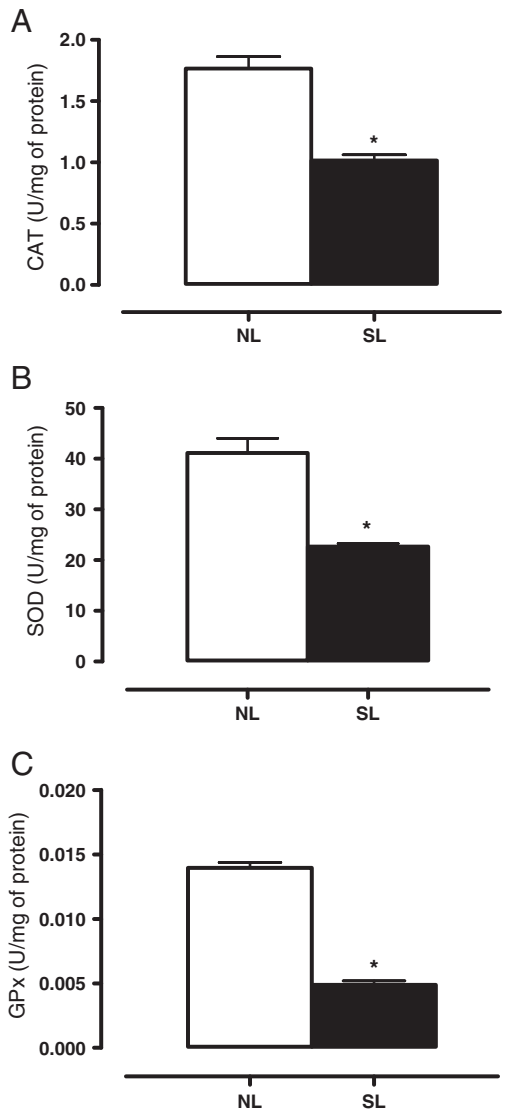

Fig. 2. Liver catalase activity (A), superoxide dismutase activity (B) and glutathione peroxidase activity (C) in adult SL (black) and NL (white) rats. Values are reported as mean ±S.E.M. *$P<.001$, $n=8$ animals/group.

4. Discussion

In the present study, we observed that EO induced by small litter size causes an increase in body weight gain during lactation and programs for hyperphagia and overweight in adult life, confirming previous reports [11,27,28,14] and also our previous results showing that SL rats presented higher central adiposity as well as central leptin resistance at 180 days old [6,7]. Since nutrient overload and obesity were associated with increased ROS generation the main focus of this study was to evaluate the oxidative stress in rats programmed by EO during lactation.

Obesity is associated with an unbalance of both lipid and carbohydrate metabolisms. These nutrients in excess also increase the demand on the mitochondria and the utilization of the electron transport chain leading to an increased generation of ROS [29,25]. Oxidative stress can occur as a result of increased ROS generation and/or failure of antioxidant system. The antioxidant system involves several nonenzymatic compounds and antioxidant enzymes such as SOD, CAT and GPx. SOD is the first line of antioxidant defense system. The two main isoforms of SOD, manganese SOD in mitochondria and copper-zinc SOD (Cu/Zn SOD) in cytosol converts superoxide radical into H_2O_2. H_2O_2, in turn, is converted to oxygen and H_2O by CAT or GPX [30]. Our present findings reveal that SOD, CAT and GPx activities are significantly decreased in adult SL rats, suggesting a reduced antioxidant defense, although differences in Cu/Zn SOD content was not observed. Rector et al. [31] have demonstrated a reduced hepatic activity of the free radical scavenger SOD and increased oxidized glutathione in obese rodent model of nonalcoholic fatty liver disease. This negative imbalance between reduced antioxidant defense and increased oxidative damage likely predisposes hepatocytes and hepatic mitochondria to progressive injury.

In this study, the lipid oxidative damage assessed by plasma and liver levels of MDA, one of the key end products of lipid peroxidation was increased in SL rats. To our knowledge, this is the first evidence showing an increase of MDA levels associated with a deficient antioxidant defense in adult rats programmed by postnatal early overnutrition. The underlying causes for increased MDA levels in this model probably associated with increased ROS production such as O_2^- are not yet established, but a decreased activity of the enzymatic antioxidant defense system represented by SOD, CAT and GPx enzymes can be implicated.

The ROS or peroxynitrite are powerful oxidizing agents that might cause depletion of sulfhydryl groups and oxidation of many molecules causing damage [32]. They can also cause DNA damage such as breaks, protein oxidation, and nitration of aromatic amino acid residues in proteins [33]. Measurement of NO content through quantification of nitrite in the liver showed a decreased nitrite bioavailability in SL rats. One of the most important reactions under physiological conditions is that O_2^- and NO radicals result in peroxynitrite. It is well known that O_2^- is important in the breakdown of NO to peroxynitrite, thereby depleting NO [34]. Therefore, this decrease in nitrite levels presumably represents enhanced NO degradation by O_2^- in the presence of a deficient antioxidant mechanism of defense. On the other hand, insulin resistance is associated with lower NO generation [35].

The deficiency of activity of antioxidant enzymes and the higher plasma and liver MDA concentrations in SL rats can indicate a higher oxidative stress in these animals. Some studies have associated oxidative stress and its role in the development of insulin resistance. ROS have been shown to activate the stress-sensitive serine/threonine kinase c-jun N-terminal kinase (JNK), which in turn phosphorylates IRS at serine residues and thus attenuate insulin signaling [36]. In the present study, SL rats presented an impairment of the insulin signaling in the liver, confirmed by reduction of IR, IRS1, p-IRS1, PI-3K and Akt1 content. Previously, Rodrigues et al. [14] have demonstrated, in the 90-day-old SL rats, lower IRS1, PI-3K and GLUT-4 expressions and lower Akt activity in adipocytes. Also,

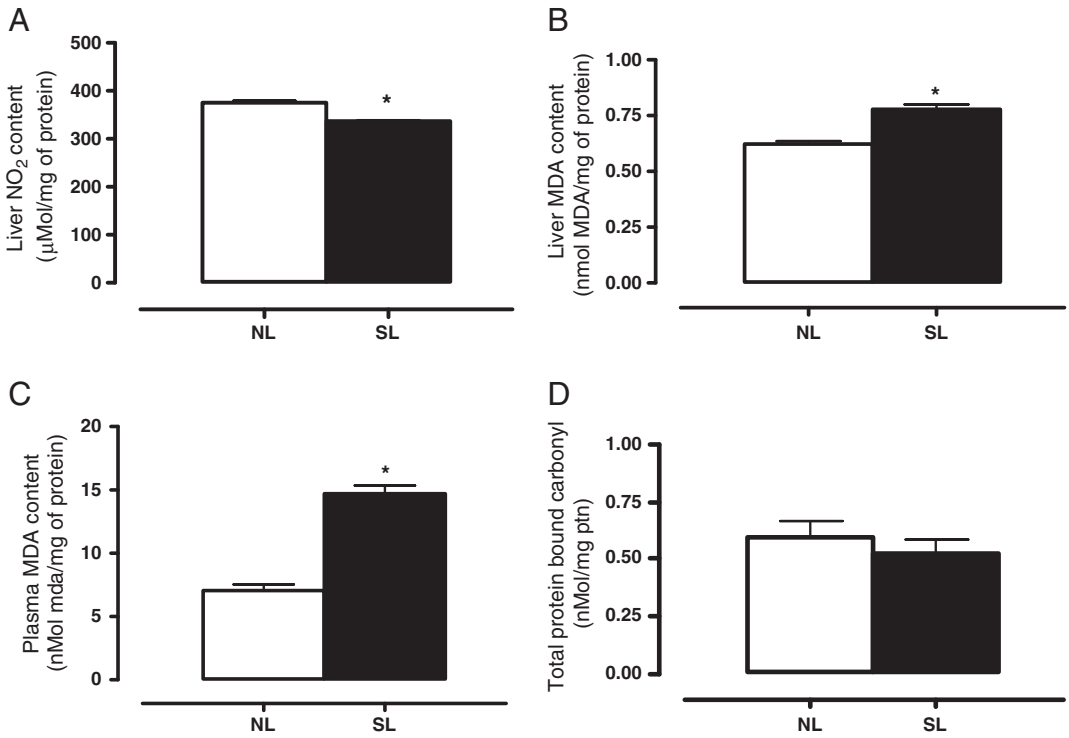

Fig. 3. Liver nitrite content (A), liver TBARS (B), plasma TBARS (C) and liver total protein bound carbonyl content (D) in adult SL (black) and NL (white) rats. Values are reported as mean±S.E.M. *$P<.05$; $n=8$ animals/group.

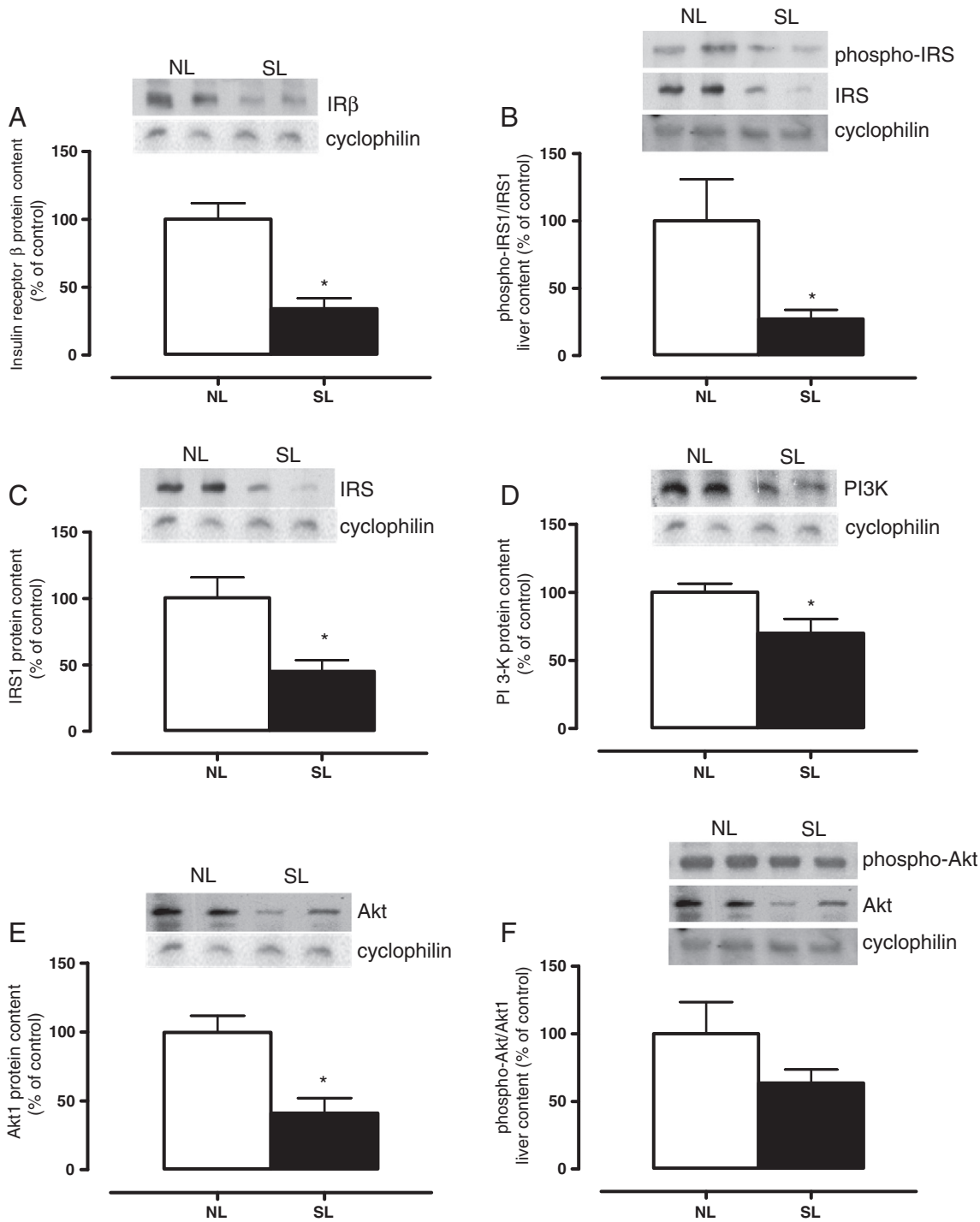

Fig. 4. Liver IRβ (A), phospho-IRS-1 (B), IRS-1 (C), PI3K (D), Akt (E) and phospho-Akt1 (F) protein content in adult SL (black) and NL (white) rats. Values are reported as mean±S.E.M. *P<.05; n=8 animals/group.

Martins et al. [37] have found that 150 days-old SL Swiss mice had decreased insulin sensitivity in the heart. However, in 1-year-old SL rats, no changes in liver and heart insulin pathway signaling were observed [38].

It is possible that the excessive fat tissue or the inabilities of fat storage, common on obesity, links nutrient excess to insulin resistance. The food intake reduction found in both C and SL groups at 180 days old compared with 120 days old is probably due to ageing, since orexigenic hypothalamic peptides are reduced during ageing [39]. Also, the higher body weight compared to the lower food intake could be explained by the lower rest metabolic rate associated with ageing. We know that even in humans, ageing is associated with a higher visceral fat mass gain compared to total body mass, especially in men [40]. The increased free fat acids (FFA) flux into circulation causes ectopic accumulation of fat in tissues such as muscle and liver [41,42]. Besides, adipose tissue not only releases FFA but also produces several inflammatory molecules including tumor necrosis factor α (TNF-α) and interleukin (IL)-6 which may have local effects on adipose metabolism and also systemic effects on other tissues [43,44]. In liver, TNF-α inhibits insulin signaling by mechanisms

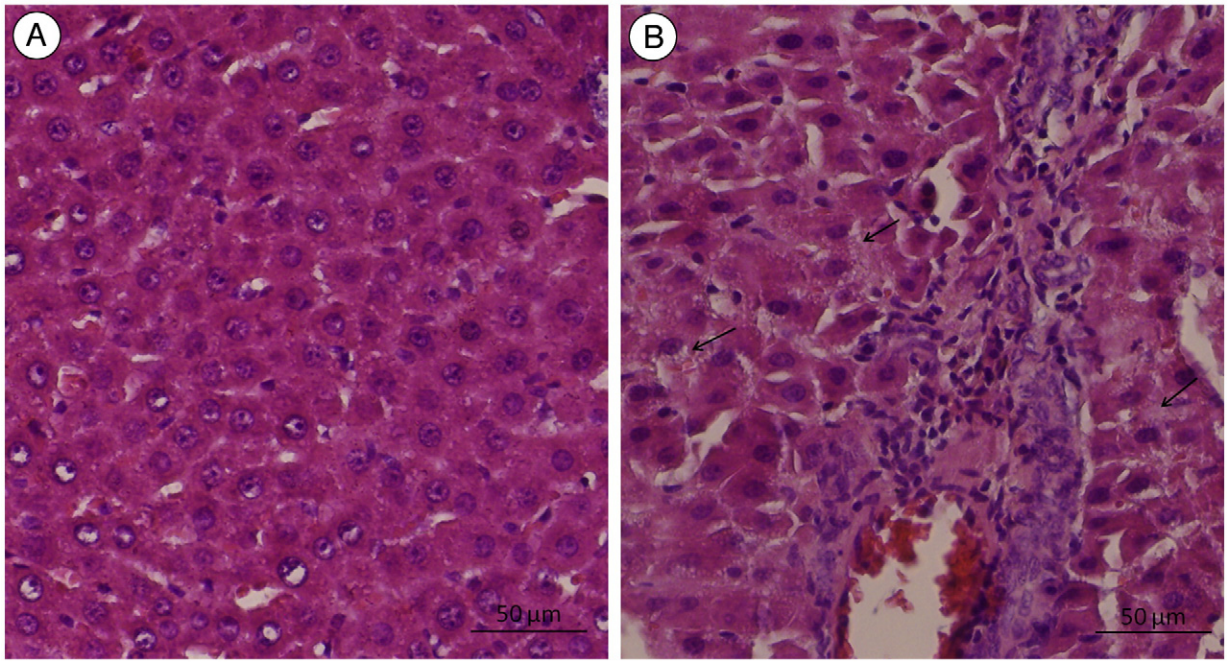

Fig. 5. Liver histology. Photomicrographs of the liver with same magnification (×40) and stained with hematoxylin-eosin. (A) Typical architecture of a NL offspring. (B) Liver of SL offspring with microsteatosis (arrow) and inflammatory cell infiltrate.

including the activation of serine kinases such as JNK-1 and induction of suppressor of cytokine signaling (SOCS) proteins [45]. Likewise, IL-6 impairs insulin signaling in liver through serine phosphorylation of IRS-1 and activating SOCS proteins [43]. Furthermore, IL-6 induces very low-density lipoprotein secretion and hypertriglyceridemia and it could directly affect liver lipid metabolism [46–48].

The higher oxidative stress evidenced by the lower activity of antioxidant enzymes CAT, SOD and GPx and the higher MDA liver and plasma content could be responsible for the impairment in liver insulin signaling in the SL group. Kathirvel et al. [49] demonstrated the relation between the higher liver oxidative stress and impairment of insulin signaling in transgenic mouse model of nonalcoholic fatty liver disease. In vitro oxidative stress in mammalian skeletal muscle leads to loss of IRS-1and IRS-2 proteins, increased relative IRS-1Ser^{307}phosphorylation and decreased phosphorylation of Akt Ser ^{473} [16].

The oxidative stress is recognized as a promoter of important hepatic injury [50]. This damage is associated to an inflammatory response and microsteatosis (nonalcoholic steatosis) as demonstrated in postnatal EO offspring. Additionally, hepatic injury could be suggested in SL offspring through reduction of serum albumin and increased serum globulin demonstrated in our previous report [7]. Several studies in different experimental models have shown that the diminished ratio between albumin and globulin (A/G) could be considered as marker of hepatic tissue lesion [51–53].

In general terms, epigenetic mechanisms, such as DNA methylation or hystone acethylation/deacethylation, induced by neonatal imprinting factors (diets, hormones, pollutants) may lead to an increased risk of metabolic disorders in the adult offspring [4]. Studies correlate the visceral obesity to DNA hypermethylation of important enzymes involved in mitochondrial fatty acid oxidation, gluconeogenesis, and lipogenesis in the liver causing a silencing of your expression and contributing to obesity-induced liver insulin resistance [54,55]. Thus, this explanation may help to understand the mechanism involved in the permanent changes of oxidative stress parameters and insulin signaling induced by overnutrition during lactation. Whether this alteration can turn overfed children more susceptible to cell damage caused by higher ROS generation in adult life, which deserves epidemiological and prospective studies.

In conclusion, our present findings evidenced that postnatal EO can program the oxidative stress in liver, maybe contributing for impairment of the insulin signaling.

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