

IRON DEPRIVATION MAY ENHANCE INSULIN RECEPTOR AND GLUT4 TRANSCRIPTION IN SKELETAL MUSCLE OF ADULT RATS

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Abstract: *Objectives:* Considering that phenotype related to iron overload associated with pathological conditions differs from that caused by dietary iron excess, our study set out to evaluate the impact of dietary iron restriction and dietary iron supplementation on oxidative stress and functional outcome in adult, healthy rats. *Methods:* adult rats were divided into the three groups and fed diets containing 10, 35 or 350 mg/kg iron (restricted-diet, control-diet and supplemented- diet groups, respectively) for 78 days. Hematological variables, fasting blood glucose, hepatic enzyme activity and C-reactive protein levels were analyzed. Iron and glycogen concentrations in liver and skeletal muscle were determined. The extent of tissue damage caused by either dietary iron restriction or iron supplementation was accessed by measuring malondialdehyde, carbonyl, NADPH oxidase, glutathione peroxidase, glutathione reductase and glutathione-s-transferase in various tissues. The mRNA expression levels of insulin receptor, glucose transporter 4 and p53 were also determined. *Results:* Fasting blood glucose values trended toward a decrease by dietary iron restriction, moreover, hepatic glycogen content decreased with concomitant increases in skeletal muscle. In addition, dietary iron restriction resulted in a twofold increase in mRNA expression of Insr and fourfold increase in Glut4 expression in skeletal muscle. Although the dietary iron restriction did not affect body iron status, it caused hepatic low oxidative damages. However, high liver NADPH oxidase activity and increased levels of protein oxidation in muscle were observed. Chronic feeding of high iron diet induces iron overload and resulted in elevated levels of stress markers in tissues. *Conclusion:* Dietary iron deprivation may improve insulin receptor and glucose transporter transcription in muscle; however, our results show that dietary iron restriction can prevent and/or promote oxidative damage in a tissue-specific manner, emphasizing the importance of maintaining optimal iron intake.

Key words: Dietary iron, oxidative stress, Glut4, Insr, adult rats.

Introduction

A reciprocal influence between iron metabolism and diabetes type 2 was first derived from the observation that the frequency of diabetes is increased in hereditary hemochromatosis (HH) (1-3). In human and mouse model of HH, iron overload results in β -cells oxidative stress and decreased insulin secretory capacity with a compensatory increase in insulin sensitivity that is reversed with iron depletion (4, 5). Indeed, iron depletion through phlebotomy and/or iron chelators reduced hyperinsulinemia, hyperglycemia, and improved liver function in patients with diabetes type 2 and non-alcoholic fatty liver diseases (6). Recently, phlebotomy from individuals with metabolic syndrome was shown to decrease fasting blood glucose, HbA1c, blood pressure and the ratio of LDL to HDL (7). Similar results are seen in the leptin-deficient model of diabetes wherein restricted iron diet or iron chelators result in significant protection from diabetes through enhancing insulin secretion and sensitivity (5). Besides, in apparently healthy people, frequent blood donation seems to have a protective effect against the development of diabetes type 2 (8). Albeit the possible mediators of the association between iron and diabetes risk are not known, oxidative stress and inflammation are thought to be involved in the interplay between iron overload and insulin-resistant disorders (2).

Insulin exerts a wide range of metabolic functions in target

tissue by interacting with specific, high-affinity insulin receptors (Insr), which are down regulated in diabetes, because of hyperinsulinemia and hyperglycemia (9, 10). Iron depletion by deferoxamine (DFO) has been shown to modulate InsR mRNA levels resulting in its increased binding and internalization activity in hepatocytes (11).

The tumor protein p53- a critical mediator of cell cycle arrest and apoptosis- is activated by cellular stress including an increase in reactive oxygen species production (12). Recent findings show that p53 is involved in iron homeostasis and may contribute to growth arrest by reducing the availability of intracellular iron through the inactivation of iron regulatory protein (13). Hence, iron chelation therapy has been suggested as potential antineoplastic treatment, since iron deprivation can inhibit cell proliferation and/or induce apoptosis (14). Several lines of evidence suggest that p53 negatively regulates insulin signaling (15). Because the glucose transporters mediate glucose uptake, they represent potential regulatory targets of oncogenes. It has been demonstrated that Glut1 and Glut4 promoters, are directly regulated by p53 in a cell type specific manner (16), and p53 can suppress the transcription of glucose transporters along with the insulin receptor to inhibit glucose uptake (17).

Considering that phenotype related to iron overload associated with pathological conditions differs from that caused by dietary iron excess, our study set out to evaluate

the impact of dietary iron manipulation on serum glucose levels; glycogen concentration and transcription of Glut4, Insr and p53 in muscle, in apparently healthy adult rats. Our data showed that iron deprivation may enhance insulin receptor and glucose transporter transcriptions in the skeletal muscle without inducing systemic iron deficiency. Moreover, dietary iron restriction can prevent and/or promote oxidative damage in a tissue-specific manner, emphasizing the importance of maintaining optimal iron intake.

Materials and methods

Animals

Adult male *Rattus norvegicus* rats (n=20), aged 15 months, were obtained from Biotecnologia Planalto (Planaltina DF, Brazil). The animals were housed individually in stainless – steel cages, inside a room at $22 \pm 2^\circ\text{C}$, with a 12-h light/dark cycle. Rats were provided standard Purina® chow for rodent containing about 200 mg iron/kg of diet (a diet corresponding to the iron present in the rodent chow), and deionized water ad libitum for acclimatization to the environment. All animal procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the Ethics Committee for the Use of Animals of the Institute of Biological Sciences, University of Brasilia, Brazil as UnB DOC No. 100.199/2009.

Experimental design:

Rats were divided into three groups with an average body weight of 523.18 ± 3.5 g. Rats were fed the American Institute of Nutrition (AIN-93M) [18] (Rhoder®) diet as follows: (i) iron-restricted diet [restricted-diet (RD), n=5] containing 10 mg of iron/kg of diet which was the lowest concentration possible to achieve due to iron contamination of the other diet ingredient; (ii) iron- supplemented diet [supplemented-diet (SD), n=8] by supplementing the AIN-93M with ferrous sulfate to reach a final concentration of 350 mg of iron/kg of diet that was estimated from the “tolerable upper limit intake” for humans (70 kg, adult), adjusting the value proportionally to the recommendation for rats, and (iii) as the control diet (CD, n=7) AIN-93M containing 35 mg of iron/kg of diet, an amount considered to meet the minimum requirement of iron for normal growth and hematopoiesis, was used [18]. Ferrous sulfate (Sigma Aldrich Co., St. Louis, MO, USA) was used as the iron source because it is highly bioavailable and often used in iron supplementation and for food fortification. These diets were fed to rats for 12 weeks and deionized water was freely available from nipple drinkers. The diet was prepared weekly, freeze-dried and stored at 4°C . Feeding took place between 16.00 h and 08.00 h. The amount of food offered daily was 30 g. The rats were weighed weekly, and the food intake was recorded daily throughout the treatment.

Sample collection

After the end of 12 weeks, the rats were fasted overnight. Then, anesthesia was induced and maintained with 1-2% isoflurane. Blood samples were collected with heparinized syringes into the tubes with and without EDTA 7.0% ($21 \mu\text{l/ml}$ blood), by cardiac puncture from all the animals and then, serum was harvested from centrifuged blood ($3,000 \times g$ for 15 min). Liver, spleen, heart, gut and tibialis anterior muscles were dissected out, washed with ice-cold saline, weighed and immediately plunged into liquid nitrogen, and then transferred to a -80°C freezer until assay.

Hematological variables

Blood was drawn for a complete blood count using an automated hematological analyzer. The iron parameters for serum iron, transferrin saturation and transferrin concentration were determined using the colorimetric ferrozine-based assay. To determine the hepatic enzymes activities, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) activities were determined by kinetic colorimetric assay. C-reactive protein concentration (CRP) was measured using an immunoturbidimetric method, which is based on the agglutination of latex particles coated with anti-CRP antibodies when they are mixed with serum containing CRP. Fasting blood glucose was determined by enzymatic procedure. All parameters were measured by the Sabin® Laboratory (Distrito Federal, Brazil).

Tissue iron determination

The concentration of iron in tissues was determined using the method described by Baranowska et al. (1995) [19]. Briefly, samples of liver, spleen, heart, gut and muscle were digested with 5 mL concentrated HNO_3 (Sigma Aldrich Co., St. Louis, MO, USA) and 2.5 mL H_2SO_4 (Sigma Aldrich Co., St. Louis, MO, USA) in a Provecto Analitica Microwave System (DGT 100 Plus, Jundiai, Sao Paulo, Brazil, 2003). After digestion, the samples were resuspended in 0.1 mol/L nitric acid to a final volume of 25 mL. The concentration of iron in the samples was determined by inductively coupled plasma atomic emission spectrometry (ICP – AES/Spectro, Kleve, Germany) using a 238 nm line. A calibration curve was obtained for the range from 0 to 10 ppm solution of Fe-Merck Titrisol (Merck, Darmstadt, Germany) to calculate the concentrations of iron in the tissues.

Lipid peroxidation

The formation of malondialdehyde (MDA) in liver, spleen, heart, gut and muscle homogenates were measured by a high-performance liquid chromatographic system (15 cm Shimadzu C18 CLC-ODS (M) column Shimadzu, Kyoto, Japan) as described previously (20). Briefly, 1 mL of 1% sulfuric acid (H_2SO_4) (Sigma, St. Louis, MO, USA) was added to 0.1 g of tissue and homogenized in an Ultra-Turrax homogenizer (Ultra-

IRON DEPRIVATION MAY ENHANCE INSULIN RECEPTOR, GLUT4 TRANSCRIPTION IN SKELETAL MUSCLE

Turrax T8, IKA - Werke, Staufen, Germany). The homogenate was centrifuged at $12,000 \times g$ for 15 min at 4°C . An aliquot of 250 μL of the supernatant was mixed with 375 μL of 440 mmol/L phosphoric acid (H_3PO_4) (Vetec, Rio de Janeiro, RJ, Brazil) and 125 μL of 42 mmol/L 2-thiobarbituric acid (TBA) (Sigma, St. Louis, MO, USA) and heated at 100°C for 1 h. After cooling, 500 μL of this mixture was mixed with 500 μL of a 91:9 mixture of MeOH and 1 mol/L NaOH (Sigma, St. Louis, MO, USA). The resulting precipitation solution was centrifuged at $12,000 \times g$ for 5 min at 4°C , and the supernatant was filtered using a 13 mm, 0.45 μm , Tuffryn membrane filter (Gelman Sciences). Aliquots of 20–30 μL of the tissues samples were injected onto a HPLC. The spectrofluorometric detector wavelengths were set at 532 nm (excitation) and 553 nm (emission). A four-point standard curve (0.81–16.16 nmol/mL) was made with tetraethoxy-*p*-propane (TEP) (Sigma, St. Louis, MO, USA) dissolved in 1% H_2SO_4 , as acid hydrolysis of TEP yields stoichiometric amounts of MDA (column 15 cm: $y = 7.1027620.132$; $r^2 = 0.9903$ and column 25 cm: $y = 7.10266 + 0.0473$; $r^2 = 0.9974$). The results were expressed as nmol MDA/mg total protein. The total protein concentration of the homogenates was determined by the method as previously described (21).

Protein oxidation

For determination of oxidatively modified proteins in liver, spleen, heart, gut and muscle, the carbonyl formation was assayed (22). The absorbance of the samples was measured at 376 nm (spectrophotometer Shimadzu – TCC 240A), and the carbonyl content was expressed as nmol carbonyl groups/mg total protein using an extinction coefficient of 22,000 $\text{mM}^{-1}\text{cm}^{-1}$. The total protein concentration of the homogenates was determined by the method as previously described (21).

Preparation of tissue homogenates for enzyme assays

Aliquots of liver, spleen, heart, gut and muscle were homogenized (Ultra-Turrax T8, IKA - Werke, Staufen, Germany) using 0.5 mol/L potassium phosphate buffer, pH 7.2, containing 50 mmol/L EDTA and 1 mmol/L phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA) at 4°C . The homogenate was centrifuged at $15,000 \times g$ for 20 min and the supernatant was used immediately to determine catalase, glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH Oxidase) activities.

Catalase activity

The activity of catalase was quantified by the consumption of 10 mmol/L of H_2O_2 at 240 nm (spectrophotometer Shimadzu – TCC 240A) in buffer containing 10–50 mL of tissue homogenates. Blanks were run without H_2O_2 . One unit of catalase was defined as the amount of enzyme required to decompose 1 mmol of H_2O_2 /min (23).

Glutathione Peroxidase activity

Glutathione peroxidase activity was quantified using H_2O_2 as a substrate in a coupled assay with Glutathione Reductase-catalyzed oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (spectrophotometer Shimadzu – TCC 240A). First, the basal consumption of 0.15 mmol/L of nicotinamide adenine dinucleotide phosphate was measured in buffer containing 2 mmol/L of azide, 5 mmol/L of glutathione (GSH), 1.5 U of glutathione reductase and 10–50 mL of tissue homogenates. Then, 20 mL of H_2O_2 was added to a final concentration of 0.2 mmol/L. Blanks were run without tissue homogenate. One unit of glutathione peroxidase was defined as the amount of enzyme required to oxidize 1 nmol of nicotinamide adenine dinucleotide phosphate/min (23).

Glutathione Reductase activity

Glutathione reductase (GR) activity was quantified by spectrophotometry (spectrophotometer Shimadzu - TCC 240A) at 340 nm, where the oxidation of nicotinamide adenine dinucleotide phosphate was monitored for 20 seconds. Tissue homogenate activity (75–250 mL) was measured in 50 mmol/L of potassium phosphate buffer (pH 7.2) containing 0.5 mmol/L of ethylenediaminetetra-acetic acid, 0.2 mmol/L of nicotinamide adenine dinucleotide phosphate and 1 mmol/L of glutathione oxidized (GSSG) (23). Blanks were run only with GSSG. One unit of glutathione reductase was defined as the amount of enzyme required to oxidize 1 nmol of nicotinamide adenine dinucleotide phosphate/min.

Glutathione-S-transferase activity

The Glutathione-S-transferase (GST) activity was measured by following the conjugation of 1 mmol/L of GSH with 1 mmol/L of 1-chloro-2, 4-dinitrobenzene at 340 nm (spectrophotometer Shimadzu - TCC 240A) in 50 mmol/L of potassium phosphate buffer (pH 7.2) containing 50 mL of tissue homogenate (24). One unit of GST was the amount of enzyme required to yield 1 nmol of product/min.

NADPH Oxidase activity

The Nicotinamide adenine dinucleotide phosphate oxidase activity was measured spectrophotometrically (Spectrophotometer, Shimadzu – TCC 240A) at 340 nm by following the reduction of NADPH through its consumption by the NADPH oxidase (25). The assay was monitored over 300 seconds and the assay buffer contained 50 mmol/L of potassium phosphate buffer (pH 7.2) with 0.5 mmol/L of ethylenediaminetetra-acetic acid, 0.1 mmol/L of nicotinamide adenine dinucleotide phosphate and 125–250 mL of tissue homogenate. The rate of NADPH oxidation was calculated using a molar extinction coefficient of 6.22 $\text{mM}^{-1} \cdot \text{cm}^{-1}$. One unit of NADPH oxidase was the amount of enzyme required to oxidize 1 nmol of NADPH/min.

Tissue glycogen determination

The concentration of glycogen in tissues was determined using the method described by Lo et al. (1970) (26). Samples of liver and tibialis anterior muscle were hydrolyzed in 1 mL of 30% (wt/vol) KOH solution in a boiling water bath for 30 min. At 20 min of the incubation, tubes were shaken by hand to facilitate the digestion. After cooling to room temperature, 2 mL of ethanol (95%) (Sigma Aldrich Co., St. Louis, MO, USA) were added, the samples were boiled again for 5 min to facilitate precipitation of glycogen and then centrifuged at $10,000 \times g$ for 5 min. The glycogen precipitates were dissolved in water and analyzed by the phenol sulfuric acid colorimetric method (26). The absorbance of the samples was measured at 490 nm (spectrophotometer Shimadzu – TCC 240A). A calibration curve was obtained for the range from 0 to 500 ppm solution of glycogen from bovine liver (Sigma Aldrich Co., St. Louis, MO, USA) to calculate the concentrations of glycogen in the tissues.

RNA extraction and reverse transcription-polymerase chain reaction analysis (qRT-PCR)

Total RNA was isolated from hepatic and skeletal muscle using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, 100 mg of tissue were homogenized in 1 mL of Trizol using an Ultraturrax homogenizer, (UKA, Deutschland, Germany). After extraction, RNA from the aqueous phase was precipitated using isopropyl alcohol, washed with 70% ethanol and then was dissolved in deionized water. The integrity of the RNA was assessed by electrophoretic profile on 1% agarose, 1,000 x GelGreen Nucleic Acid Gel Stain (Biotium Inc., Hayward, California, USA), and Tris/Boric acid/Ethylenediamine tetraacetic acid buffer solution 1x gels (Sigma, St. Louis, MO, USA). The gel was analysed using the software 1D LabImage (Kapelan Bio-Imaging Solutions, Leipzig, Germany) to confirm the absence of degraded genetic material. The RNA samples were quantified by measuring their absorbance at 230, 260 and 280 nm (UV-visible Ultrospec 3000, Pharmacia Biotech, Cambridge, England). The purity of RNA was verified by assessing the presence of proteins with the 260/280 nm ratio, while the presence of aromatic compounds, such as phenols, was assessed with the ratio 260/230 nm. The benchmark used to evaluate these ratios was greater than or equal to 1.8. Total RNA was then precipitated using anhydrous sodium acetate at 3 mol/L and pH 5.2 (0.1 vol) (Sigma, St. Louis, MO, USA) and 100% ethanol (2.5 vol) at 4°C (Sigma, St. Louis, MO, USA, 2.5 times the volume 825mL) and incubated at -20°C overnight. After incubation, the samples were centrifuged at $10,000 \times g$ at 4°C for 30 min (Centrifuge Eppendorf 5415R, Hamburg, Germany). Ethanol (1 mL) (Sigma, St. Louis, MO, USA) was added to the pellet and it was centrifuged at $10,000 \times g$ for 5 min at 4°C, dried at room temperature and diluted in 20 mL of deionized water. After this procedure, the absorbance at 230, 260 and 280 nm was determined (UV-visible Ultrospec 3000, Pharmacia Biotech, Cambridge, England) and the

A260/A280 and A260/A230 ratios were again determined to assess the purity and concentration of the RNA in the treated material. Total RNA (1 μ g) was used for the cDNA synthesis reactions (20 μ L final volume) using an ImProm-II Reverse Transcription System (Promega Corporation, Madison, USA). Oligo(dT) primers were added to the total RNA and denaturation was at 70°C for 5 min. ImProm-II Reverse Transcriptase was added, and the samples were incubated at 42°C for 50 min, followed by inactivation at 70°C for 15 min. An attempt at cDNA synthesis without the reverse transcriptase enzyme was performed for each sample (negative control). An aliquot of the reaction control (no reverse transcriptase) was subjected to RT-PCR using a reaction system that had been tested and the oligonucleotide sequences had previously been used by other authors and described in scientific articles to confirm the absence of amplified genetic material. Real-time PCR was carried out using the Fast SYBR Green Master Mix 2x reagent (Applied Biosystems, Foster City, CA, USA) with 2.0 μ L of cDNA (corresponding to 0.6 ng of total RNA) in a final volume of 10 μ L, 5 μ L Fast SYBR Green Master Mix and 0.2 μ mol/L (final concentration) of each primer. The following oligonucleotides were used as PCR primers: p53 (NM-030989), sense: 5' CCC AGG GAG TGC AAA GAG AG-3', antisense: 5'-TCT CGG AAC ATC TCG AAG CG-3'; Insr (NM-017071) sense: 5'- GGG ATG CAC TTG TTG TTG TG-3', antisense: TTG GCG CTG TGT AAA CTT CA-3'; Glut4 (NM-012751), sense: 5'- CTG GGC TGA TGT GTC TGA TGC-3', antisense: 5'- CCC CCG ATA CCT CTA CAT-3'; and β -actin (Actb), sense: GTC GTA CCA CTG GCA TTG TG; antisense: CTC TCA GCT GTG GTG GTG AA (NM_031144.3). Quantitative PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Cingapura) for 40 cycles at 95°C for 20 s, 60°C for 3 s and 60°C for 20 s. The amplification specificity of each amplified product was verified using a melting curve. The PCR amplification efficiency was evaluated by running standard curves for each amplicon in different template dilutions. A standard curve was plotted for each studied gene correlating the Δ CT (CT gene of interest – CT internal control) versus the log of the cDNA amount. A slope value of the regression line plot of Δ CT values vs. log of input nucleic acid < 0.1 was used as a general criterion to accept the validation of the experiment, and a slope value of the regression line plot of CT values vs. log of input nucleic acid ~ -3.32 was considered as an efficient reaction. The concentration of gene-specific mRNA in treated samples relative to untreated samples was calculated using the $2^{-\Delta\Delta CT}$ formula as follow (27):

$$2^{-\Delta\Delta CT} = \frac{[(C_T \text{ gene of interest} - CT \text{ internal control}) \text{ treatment group}]}{[(C_T \text{ gene of interest} - CT \text{ internal control}) \text{ control group}]}$$

Results are shown as fold changes compared to the control group, being expressed in arbitrary units.

IRON DEPRIVATION MAY ENHANCE INSULIN RECEPTOR, GLUT4 TRANSCRIPTION IN SKELETAL MUSCLE

Statistical analysis

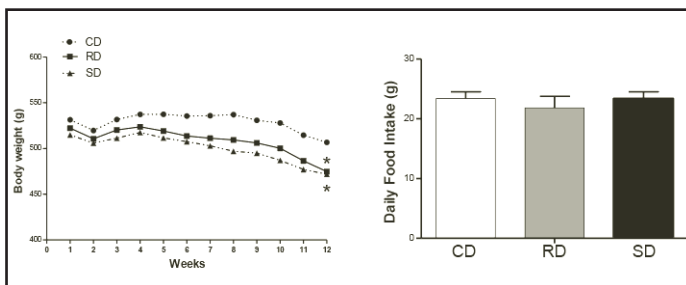
Treatment effects were analyzed by ANOVA followed by Bonferroni's post-tests using SPSS 19.0 software (SPSS Inc., Chicago, IL). Differences were considered significant if p values < 0.05 . Results are expressed as means \pm SD.

Results

Body weight and food intake: Body weights in both treatment groups were comparable to the control group. Rats fed iron-restricted diet (RD) as well as fed iron-supplemented diet (SD) lost, significantly, greater weight during the 12-week treatment period than those fed control diet (CD) ($p=0.002$ and $p<0.001$, respectively) (Figure 1A); although the daily food intake did not differ across groups (Figure 1B).

Figure 1

Body weight and daily food consumption in adult rats treated with control diet (CD) $n=7$, iron-restricted (RD) $n=6$, and iron-supplemented (SD) diets $n=8$, during 12 weeks; * significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$)



Hematological variables and serum iron status: Although the hemoglobin concentration did not differ statistically among the groups, its values trended toward an increase through the iron-restricted diet ($p=0.06$). Hematocrit levels were higher in the iron-restricted and iron-supplemented diet than in the control group ($p=0.021$ and $p=0.025$, respectively). No differences in numbers of red blood cells, mean corpuscular volume, and mean corpuscular hemoglobin value were found. The other parameters (numbers of lymphocytes, monocytes and leukocytes) did not alter across all groups. With regard to the circulating iron status, serum iron concentration significantly increased in iron-supplemented diet group ($p=0.034$); however, its concentration did not significantly altered in the rats fed iron-restricted diet. Although statistical difference was not reached in total iron binding capacity (TIBC) and in transferrin concentrations, a strong trend towards decreased values of transferrin saturations induced by iron-restricted diet was observed ($p=0.084$) (Figure 2).

Fasting blood glucose, hepatic enzymes activities and C-reactive protein levels : Even though significance was not reached in fasting blood glucose levels among the groups, nonetheless, its values trended toward a decrease through the

iron-restricted diet ($p=0.055$) (Table 1). The iron-supplemented diet induced higher levels of aspartate aminotransferase (AST) ($p=0.035$), however, neither ALT nor ALP showed significant difference. However, mean values of gamma-glutamyl transpeptidase (GGT) were significantly increased by iron-supplemented diet ($p=0.019$) (Table 1). Iron-restricted diet did not cause any alterations in hepatic enzymes activities. Lower levels of C-reactive protein (CRP) were found in rats fed with iron-supplemented diet relative to the control group ($p=0.047$) (Table 1).

Figure 2

Hemoglobin (Hb) (A), Hematocrit (Htc)(B), serum iron (C), transferrin saturation (TS)(D), total iron binding capacity (TIBC)(E) and transferrin (TfR)(F) levels consumption in rats treated with control diet (CD) $n=7$, iron-restricted (RD) $n=5$, and iron-supplemented (SD) diets $n=8$, during 12 weeks; * Significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$)

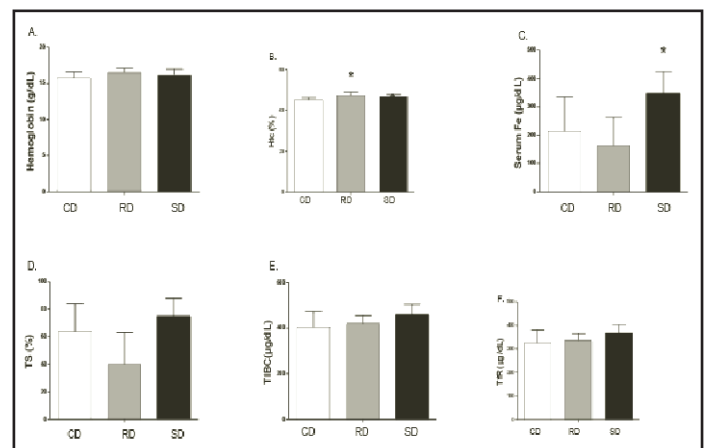


Figure 3

Concentration of iron in the tissues of adult rats treated with control diet (CD) $n=7$, iron-restricted (RD) $n=5$, and iron-supplemented (SD) diets $n=8$, during 12 weeks; * Significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$)

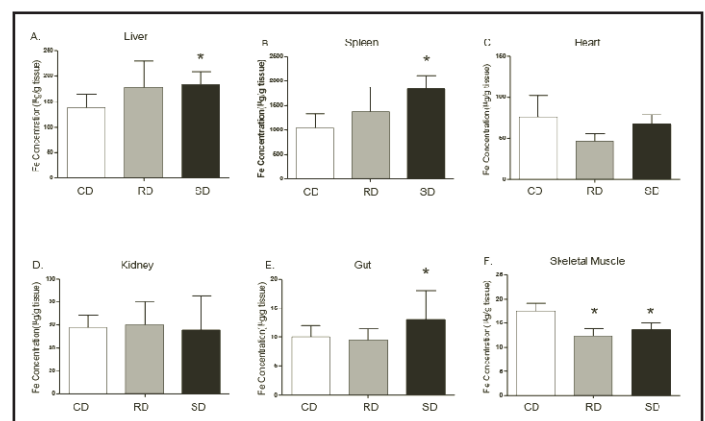


Table 1

Liver weight, hepatic enzymes activity and fasting blood glucose in rats treated with control diet (CD), iron-restricted (RD) and iron-supplemented (SD) diets during 12 weeks

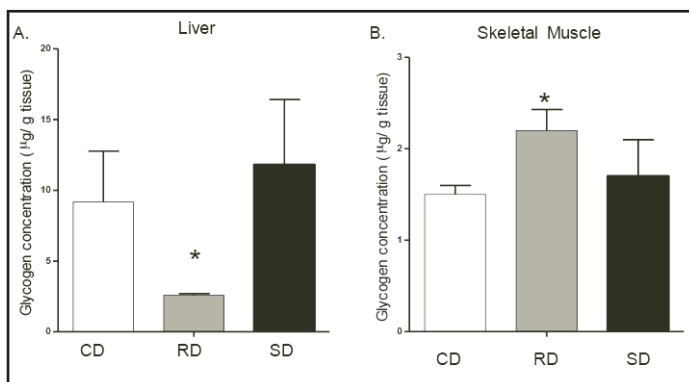
	CD	RD	SD
Liver wt (g)	12.7 ± 2.0	12.4 ± 1.3	11.1 ± 2.0
CRP (mg/dL)	0.48 ± 0.06	0.56 ± 0.12	0.38 ± 0.11
ALT (U/L)	92.2 ± 48.8	57.2 ± 19.5	156.5 ± 99.8
AST (U/L)	355.8 ± 140.1	440.6 ± 173.4	735.7 ± 328.3 *
ALP (U/L)	62.3 ± 15.6	61.0 ± 13.9	65.7 ± 21.5
GGT (U/L)	1.6 ± 1.1	1.4 ± 0.8	6.9 ± 5.3 *
Fasting blood Glucose (g/dL)	125.9 ± 6.9	117.6 ± 5.7	125.5 ± 19.7

Values are mean ± SD; CRP: C-reactive protein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase; * significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$).

Tissue iron status: Liver, spleen and gut iron concentration was significantly influenced by high iron diets, it was higher in rats fed iron-supplemented diet than in rats fed the control diet ($p=0.005$, $p=0.008$ and $p=0.042$, respectively). Iron-supplemented diet did not alter iron content in heart, however, in skeletal muscle its value was significantly lower compared to the rats fed the control diet ($p<0.001$) (Figure 3). Among the rats fed iron-restricted diet, reduced levels of iron concentration in the skeletal muscle was observed ($p=0.001$; Figure 3), nevertheless, low iron diets did not induce any significant reduction in

Figure 4

Concentration of glycogen in liver (A) and in skeletal muscle (B) of adult rats treated with control diet (CD) $n=7$, iron-restricted (RD) $n=5$, and iron-supplemented (SD) diets $n=8$, during 12 weeks; * Significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$)



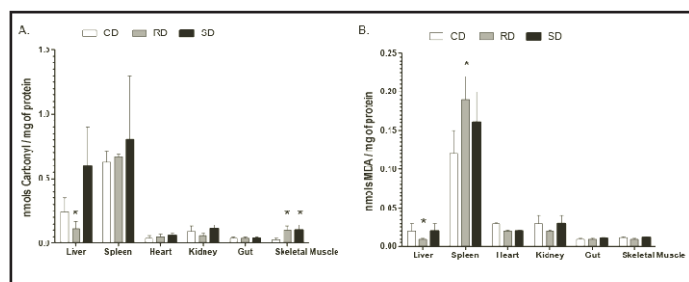
Glycogen content: Figure 4 shows the liver and muscle glycogen concentration. Iron-restricted diet markedly reduced hepatic glycogen concentration ($p=0.007$); while in muscle

its concentration was significantly augmented ($p<0.001$). In rats fed iron-supplemented diet, no significant difference was found.

Biomarkers of oxidative stress and antioxidant enzyme activity : Iron-restricted diet induced a significant reduction in MDA levels in liver, heart and gut and a significant increase in MDA levels in spleen compared to the control diet. Protein oxidation, as indicated by carbonyl formation, was decreased in liver, but increased carbonyl levels were observed in muscle (Figure 5). The iron-supplemented diet increased MDA levels in spleen, heart and gut compared to the control diet. The higher carbonyl levels were observed in liver, heart and muscle than the control diet (Figure. 4). Rats fed the iron-restricted diet had significantly greater activities of NADPH oxidized in the liver and spleen ($p=0.003$ and $p<0.001$, respectively) and also of glutathione peroxidase (GPx) in the spleen ($p<0.001$) than rats fed control diet. Rats fed iron-supplemented diet had significantly higher activities of catalase, glutathione-s-transferase (GST) in the gut than control rats. Decreased NADPH oxidized activity was observed in spleen as well as in the gut of the rats fed the iron-supplemented diet comparing to those fed the control diet (Figure 6).

Figure 5

Protein carbonyl concentration (A) and malondialdehyde (MDA)(B) in the tissues of adult rats treated with control diet (CD), iron-restricted (RD) and iron-supplemented (SD) diets during 12 weeks; * Significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$)



Glut4, Insr and p53 transcription in tissues

In liver, iron-restricted diet promotes p53 transcription (up to 1.5-fold, $p=0.006$); nevertheless, its level in iron-supplemented group remained unchanged (Figure 7A). Gene expression analysis in muscle showed up-regulation of Glut4 gene (up to 2-fold, $p<0.001$) and also Insr gene (up to 4-fold, $p=0.015$) in iron-restricted group (Figure 8). The p53 mRNA levels in iron-restricted group as well as in iron-supplemented group were significantly decreased ($p=0.01$ and $p<0.001$, respectively) (Figure 7B).

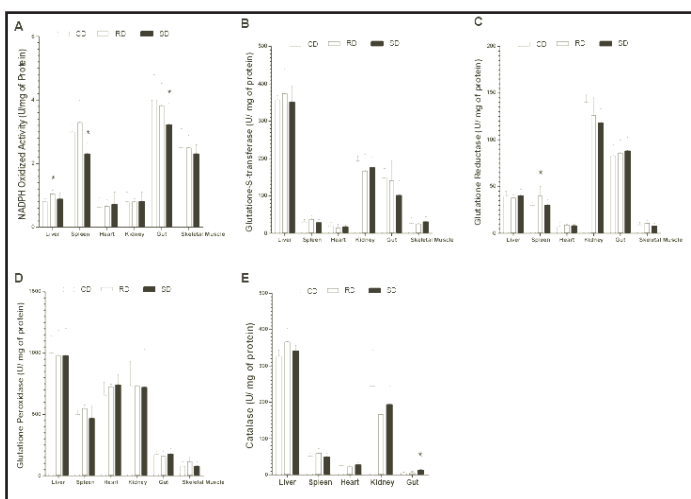
IRON DEPRIVATION MAY ENHANCE INSULIN RECEPTOR, GLUT4 TRANSCRIPTION IN SKELETAL MUSCLE

Discussion

Previously published data lead to the prediction that dietary iron excess may be associated with diabetes and decrease in iron stores may be beneficial to diabetes risk. In this study, we evaluated the impact of dietary high and low iron levels on the fasting blood glucose, glycogen levels, insulin receptor (Insr) and glucose transporter 4 (Glut4) transcriptions in skeletal muscle; and the redox status in adult rats. Because p53 is a redox regulated transcription factor (28) and also p53 is able to contribute to the regulation of insulin sensitivity (17); we then investigated whether those alterations were influenced by p53 transcription. This study shows that iron deprivation has the following effects without causing iron-deficiency anemia: (i) increased Insr and Glut4 transcription and increased glycogen content in the skeletal muscle; (ii) decreased slightly the fasting blood glucose; (iii) prevent and / or promote oxidative stress; and (iv) may modulate the expression of p53 in a cell type manner.

Figure 6

Specific activity of NADPH oxidized (A), GST (B), GR (C), GPx(D), and catalase (E) from tissue homogenates of adult rats treated with control diet (CD) n=7, iron-restricted (RD) n=5, and iron-supplemented (SD) diets n=8, during 12 weeks; * Significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$). GR, glutathione reductase; GPX, glutathione peroxidase; GST, glutathione-S-transferase; NADPH, Nicotinamide adenine dinucleotide phosphate oxidase



It is well known that skeletal muscle cells continuously generate ROS, and aging reduces the ability for muscles to buffer oxidants leading to the oxidative damages (29). It has been shown that iron deficiency causes oxidative stress and mitochondrial dysfunction in liver, heart, skeletal muscle and blood cells, however, the skeletal muscle was more severely affected than liver (30), and it might be caused by decreased

heme level and complex IV activity (31, 32). This fact may explain the increased oxidative damage to proteins observed in the skeletal muscle of rats fed either iron-restricted or iron-supplemented diet because both these groups had lost iron. Moreover, iron depletion induced- protein damage in both these groups may have damaged the integrity of the mitochondrial membrane, resulted in decreases of DNA synthesis and DNA repair (33).

Figure 7

Quantification of tumour protein 53 (Tp53) mRNA from liver (A) and skeletal muscle (B) of adult rats treated with control diet (CD) n=7, iron-restricted (RD) n=5, and iron-supplemented (SD) diets n=8, during 12 weeks; * Significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$). RT-PCR analyses were normalized against the mRNA expression of the β -actin gene in the same tissue sample

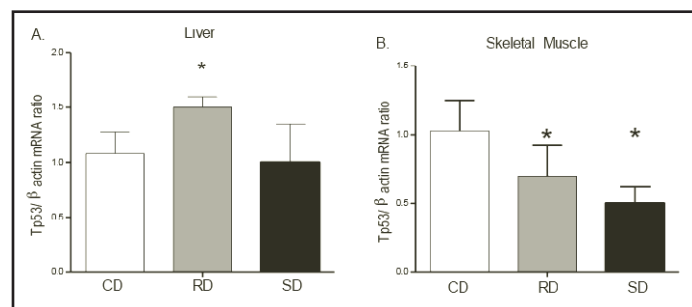
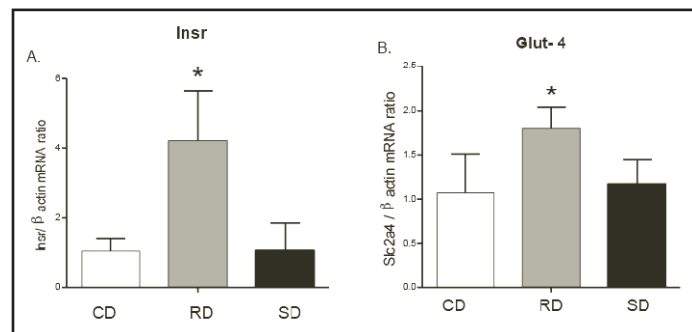


Figure 8

Quantification of insulin receptor (A) and Glut-4 (B) mRNA from skeletal muscle of adult rats treated with control diet (CD), iron-restricted (RD) and iron-supplemented (SD) diets during 12 weeks; * Significantly different from control diet (CD)-fed animals (ANOVA, Bonferroni's multiple comparison post hoc test $P < 0.05$). RT-PCR analyses were normalized against the mRNA expression of the β -actin gene in the same tissue sample



Earlier studies have been shown that, an iron chelator, deferoxamine, stabilizes the hypoxia- inducible factor 1 α expression (HIF-1 α), which increased glucose transporter expression (Glut1) in hepatic tissue (11). Furthermore,

iron depletion has been shown to increase nuclear HIF-1 α expression in the pancreas (34). Recent findings showed a significant increase in glycogen synthase activity and glycogen accumulation in myoblasts exposed to hypoxia (35). In the present study, the increase in the intracellular storage of glucose observed in the skeletal muscle of rats fed with iron-restricted diet may be attributable to the stabilization of HIF-1 α induced by iron depletion in skeletal muscle; nevertheless, it might seem paradoxical since anaerobic glycolysis is characterized by increased glucose demand. In accordance with our results, it has been proposed that glycogen synthase (GYS1) promoter is induced by HIF-1 α , suggesting that HIF-1 α stimulates glycogen synthesis and repress its degradation (35).

Iron depletion mediates cell arrest and apoptosis through the activation of p53 (36, 37), however, it has been demonstrated that monocytes of individuals homozygous for the C282Y HFE mutation that are relatively iron poor due to inability to retain iron (38) had lower p53 levels than their normal counterpart (39). It has also been suggested that p53 can repress the transcription of Glut4 along with the insulin receptor, leading to the inhibition of cellular glucose uptake (17). In the present study, a concomitant increase in insulin receptor and glucose transporter 4 transcription with decreasing p53 transcription observed in skeletal muscle of rats fed iron-restricted diet may strengthen the role of p53 in the promotion of insulin resistance and therefore, contributes to the metabolic disorders. Curiously, we also observed a significant decrease in iron concentration and p53 transcription in the skeletal muscle of SD group, but with regard to *Insr* and *Glut4* transcription no significant change was observed. It is worth noting that iron deficiency is associated with increased oxidative damages (32). Herein, down regulation of p53 induced by iron-restricted diet as well as by iron-supplemented diet might be interpreted as a mechanism to protect myocytes from stress damages. Quite the contrary, in the liver of rats fed with iron-restricted diet, we observed the up-regulation of p53, supporting the antiproliferative activity of iron chelators. Therefore, it seems reasonable to assume that p53 transcription responds to the iron deprivation in a cell-type specific manner.

Surprisingly, feeding the iron-supplemented diet (350 mg iron supplemented/Kg) increased the hepatic iron concentration only 30% compared with feeding the control diet (35 mg iron supplemented/Kg). Moreover, both treated group lost weight, even though, the daily food intake did not differ across all groups. The difference between our results and those observed in similar studies in which rats were fed high iron diets (40-42) might be attributable to the age of rats used in the present study. In our study, adult rats were used whereas, others (40-42) studied growing rats. As expected, chronic feeding of high iron diet led to the iron overload and increase oxidative stress in tissues. Iron supplementation also led to the liver injury as indicated by serum AST and GGT levels. However, no alteration in insulin-stimulated glucose transport, glycogen

concentration and fasting blood glucose was observed.

The concentrations of iron in serum, liver, spleen, heart and gut were not affected by iron restricted-diet. Decreasing iron supply may increase intracellular iron trafficking from iron store in ferritin and hemosiderin, catalyzing some of the oxidant-induced damage due to the increase iron availability (32). Moreover, concurrent increases in hematocrit levels with slight decrease in transferrin saturation suggest a reduction in iron mobilization in rats fed iron-restricted diet. However, the possibility of hemoconcentration should be considered because these animals lost more weight as compared to the control rats.

Curiously, in the liver of rats fed with iron-restricted diet, despite the low levels of oxidative stress, we found higher activity of NADPH oxidase. Considering that NADPH oxidase is a principal producer of reactive oxygen species (ROS) in many tissues (43-45) and superoxide anion and oxidant drives from it by complex chain reactions are main contributors in the defense of against invading foreign microorganisms (46), it seems reasonable to assume that the up regulation of p53 is a response to the higher activity of NADPH oxidase, even though dietary iron restriction did not induce overt liver injury, as indicated by serum aminotransferase levels and organ weight. Evidences have also been shown that high levels of ROS activate p53, which in turn leads to the p53-mediated apoptosis and senescence (15, 47, 48). This seems inconsistent with the low levels of lipid and protein oxidation markers found in the liver, however, the reason is not clear. Probably, other competitive antioxidant mechanism such as the effect of weight loss or apoptosis induced by activation of p53 may have been hidden the oxidative damage. Moreover, the lack of dramatic alteration in antioxidant enzymes activity in rats fed iron-restricted diet might also be due to the fact that this group lost more weight during the treatment than those rats fed the control diet.

Conclusion

Taken together and considering limitations in the present study, our results suggest that dietary iron restriction enhances insulin receptor expression and promotes glucose transporter transcription in skeletal muscle without causing iron deficiency anemia. Furthermore, the hepatic up-regulation of p53 induced by iron deprivation supports the antiproliferative activity of iron chelators as shown previously (12, 49, 50). These finding also suggest iron restriction can promote and/or prevent oxidative damage in a tissue-specific manner, because the response of cells to iron deprivation is complex with multiple molecules and signaling pathways being involved. In conclusion, our results imply that both iron deficiency and excess from daily supplementation are undesirable and emphasize the importance of maintaining optimal iron intake. Further studies are warranted to evaluate the functional and clinical outcomes of dietary iron restriction on adult healthy subjects.

Ethical Standards: All handling and management procedures were carried out in

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accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Brasilia.

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Conflict of Interest:

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