Biomedicine & Pharmacotherapy 149 (2022) 112820

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Social isolation triggers oxidative status and impairs systemic and hepatic insulin sensitivity in normoglycemic rats

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ARTICLE INFO

Keywords:
Insulin
Social isolation
Oxidative stress
Liver
Hypothalamus

ABSTRACT

Drug-naïve psychotic patients show metabolic and hepatic dysfunctions. The rat social isolation model of psychosis allows to investigate mechanisms leading to these disturbances to which oxidative stress crucially contributes. Here, we investigated isolation-induced central and peripheral dysfunctions in glucose homeostasis and insulin sensitivity, along with redox dysregulation. Social isolation did not affect basal glycemic levels and the response to glucose and insulin loads in the glucose and insulin tolerance tests. However, HOMA-Index value were increased in isolated (ISO) rats. A hypothalamic reduction of AKT phosphorylation and a trend toward an increase in AMPK phosphorylation were observed following social isolation, accompanied by reduced GLUT-4 levels. Social isolation also induced a reduction of phosphorylation of the insulin receptor, of AKT and GLUT-2, and a decreased phosphorylation of AMPK in the liver. Furthermore, a significant reduction in hepatic CPT1 and PPAR-α levels was detected. ISO rats also showed significant elevations in hepatic ROS amount, lipid peroxidation and NOX4 expression, whereas no differences were detected in NOX2 and NOX1 levels. Expression of SOD2 in the mitochondrial fraction and SOD1 in the cytosolic fraction was not altered following social isolation, whereas SOD activity was increased. Furthermore, a decrease of hepatic CAT and GSH amount was observed in ISO rats compared to GRP animals. Our data suggest that the increased oxidant status and antioxidant capacity modifications may trigger hepatic and systemic insulin resistance, by altering signal hormone pathway and sustaining subsequent alteration of glucose homeostasis and metabolic impairment observed in the social isolation model of psychosis.

1. Introduction

Prescription of antipsychotic medication has significantly increased in the last decades, given the significant burden of the psychotic disease on health and socio-economic systems [1]. A consistent number of studies showed a significant correlation between pharmacological treatments with both typical and atypical antipsychotics and dysfunctions of glucose and lipid metabolism in terms of impaired glucose homeostasis, altered insulin sensitivity and insulin resistance [2–4], enhanced amount of adipose tissue, weight gain and higher body mass index [5–8], as well as steatosis and hepatic alterations [9–11]. Of note, increasing evidence demonstrated the presence of metabolic and hepatic disturbances in drug-free and drug-naïve psychotic patients [12,13], supporting the hypothesis that antipsychotic medications may not be the main underlying cause of these seeming adverse effects. However, "drug-free" or "drug-naïve" conditions are difficult to be observed in

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https://doi.org/10.1016/j.biopha.2022.112820
Received 10 December 2021; Received in revised form 28 February 2022; Accepted 9 March 2022
Available online 12 March 2022

human subjects diagnosed with psychosis. Thus, in this context, non-pharmacological animal models of this disease represent a very useful tool to investigate mechanisms leading to drug-independent metabolic and hepatic impairments. Among them, the post-weaning social isolation rearing provides a reliable method to induce in rodents behavioural and neuropathological dysfunctions reminiscent of what is observed in psychotic patients [14–18]. Our previous data showed that the rearing condition, characterized by the total deprivation of social interactions, was significantly associated with neuroendocrine [19] and metabolic dysfunctions, in terms of total and visceral fat increase [20].

Many cells, as well as those of the brain, use glucose as a primary substrate and its cellular uptake requires insulin, therefore the hormone signaling is critical for these tissues [21]. Insulin, in addition to regulating glucose metabolism, supports cognition and improves the outgrowth of neurons; alterations in insulin signaling cause inter alia insulin resistance, and may accelerate brain aging, affect emotional regulation and conceivably neurodegeneration [22,23].

Oxidative stress, defined as an alteration in the balance between reactive oxygen species (ROS) generation and antioxidant buffering capacity, has been implicated in both metabolic and hepatic dysfunctions [24] and insulin resistance [25]. In this context, enhanced levels of one of the final products of lipid peroxidation, malondialdehyde (MDA), have been reported in different peripheral organs involved in the modulation of metabolic pathways, including liver [26,27]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases NOX enzymes, as well as mitochondria, represent an important source of ROS production in the liver [28]. In particular, several lines of evidence have reported a key involvement of NOX4 enzyme in the development of different hepatic dysfunctions and pathological conditions, including viral infections, hepatic steatosis, inflammation and fibrosis [28–31]. Dysfunctions of antioxidant enzymes have also been reported to be involved in the development of metabolic impairment [32,33] and hepatic alterations [34–37]. In particular, A reduced amount of catalase (CAT), one of the most relevant antioxidant enzymes acting in the liver, has been described as crucially involved in the onset and progression of hepatic dysfunctions [38]. Moreover, specific CAT polymorphisms have also been involved in the development of diseases related to metabolic dysfunctions [39–41]. In addition, decreased glutathione (GSH) levels, as well as dysregulation of GSH-related pathways, have been described in metabolic disturbances [42] and liver injuries [43].

Social isolation is a huge psychosocial stressor, because of the related hyperactivation of the hypothalamic-pituitary-adrenocortical axis [44], whose effects could reflect on the loss of metabolic homeostasis. Indeed, social isolation may increase the risk of amplification of metabolic alterations. In humans, social isolation has been associated with abdominal obesity, altered lipid profile, and insulin resistance [45].

Here, we investigated the possible isolation-induced dysfunctions in glucose homeostasis and insulin sensitivity by the insulin signalling pathway determination in rat hypothalamus and liver. The involvement of redox dysregulation in this pathogenic process was also assessed, by evaluating hepatic ROS amount and lipid peroxidation, as well as levels of ROS producing (NOX4, NOX2 and NOX1) and degrading (SOD2, SOD1, CAT and GSH) enzymes.

2. Materials and methods

2.1. Animals

Adult male and female (10–12 weeks of age, 220–250 g) Wistar rats (Envigo, San Pietro al Natisone, Italy) were housed and mated at constant room temperature (22 ± 1 °C) and relative humidity (55 ± 5%) under a 12 h light/dark cycle (lights on from 7:00 AM to 7:00 PM). They had free access to food and water. All procedures that involved animals were conducted in conformity with the institutional guidelines of the Italian Ministry of Health (D.L. 26/2014), the Guide for the Care and Use of Laboratory Animals: Eighth Edition, the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2004) and the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes. The experimental protocol was approved by the Italian Ministry of Health (approval number 485/2019-PR, protocol n. B2EF8.22) and was conducted in accordance to ARRIVE guidelines. During the entire period of experiments, animal welfare was monitored every day to check for any sign of distress. All efforts were made to reduce the number and the pain of used animals.

2.2. Social isolation protocol

The social isolation protocol was performed as previously described [46,47]. Briefly, one male and two females were mated to obtain litters. At weaning (postnatal day 21), male pups were randomly assigned to control group housing (GRP, 3–4/cage) or social isolation single housing (ISO, 1/cage) for 7 weeks, thus until postnatal day 70. To elude a litter effect, one subject for each litter was placed into the GRP and one subject into the ISO. The cages were cleaned once a week. Both ISO and GRP rats were located in the same room, thus visual, auditory and olfactory contacts were maintained. Behavioral dysfunctions (locomotor hyperactivity and cognitive impairment) induced by social isolation were confirmed in the set of animals used in the present manuscript (Supplementary Material 1).

2.3. Glucose tolerance test

The Glucose Tolerance Test (GTT) was carried out for GRP and ISO rats at the end of the social isolation period, as previously described [48]. Briefly, after 12 h of fasting, blood samples were collected from the caudal vein immediately before an intraperitoneal (i.p.) injection of 50% glucose solution (time point 0) and 30, 60, 90, 120 min later. Blood glucose levels (mg/dL) at these same time points were measured by using commercially available strips and glucometer (Accu-chek, Roche).

2.4. Insulin tolerance test

The Insulin Tolerance Test (ITT) was performed in GRP and ISO rats, at the end of the social isolation period, after overnight fasting, as previously described [48,49]. Briefly, insulin (0.75 UI/kg) was subcutaneously administered. Blood samples were collected from the caudal vein immediately before (time point 0) and after 30, 60, 90, 120 min from insulin administration. Blood glucose levels (mg/dl) at these same time points were measured by using commercially available strips and glucometer (Accu-chek, Roche). Results were also expressed as area under the concentration-time curve (AUC) calculated for each animal.

2.5. Plasma biochemical parameters and insulin quantification

Plasma samples were obtained from GRP and ISO rats and used to measure the levels of total bilirubin, AST, ALT, Alkaline Phosphatase, Total, HDL and LDL Cholesterol, and Triglycerides by standardized methods purchased from Beckman-Coulter and automatically analyzed using the AU 5800 instrumentation following the manufacturer's recommendations (Beckman Coulter s.r.l., Milan, Italy). Furthermore, all procedures have been verified through internal quality controls analyzed daily to verify precision and accuracy. The coefficients of variation were $< 6\%$ for AST, ALT, and Alkaline Phosphatase, $< 4\%$ for HDL Cholesterol, LDL Cholesterol, total Cholesterol, Triglycerides, and total Bilirubin.

Levels of insulin in plasma were assessed by Enzyme-Linked Immunosorbent Assay (ELISA), using a commercially available kit (ThermoFisher Scientific, USA, ERINS), according to the manufacturer's instructions. For the analysis, each sample and standard were used in duplicate to prevent intra-assay variations.

2.6. Homeostatic Model Assessment (HOMA) index

As the index of insulin resistance (IR), the HOMA-IR was calculated by using the following formula: fasting insulin (μU/L) x fasting glucose (nmol/L) /22.5 [50].

2.7. Liver collection

At the end of the 7 weeks of social isolation, GRP and ISO rats were euthanized by decapitation following anesthesia with ketamine (50 mg/kg i.p.) and xylazine (10 mg/kg) and livers were removed. Fresh livers were immediately used for ROS and MDA assays. For Western Blotting and ELISAs procedures, livers were put on dry ice, then frozen and stored at − 80 °C, until analyses were performed (24–48 h later).

2.8. ROS and MDA assays

The levels of ROS were determined as previously reported [51]. An appropriate volume of freshly liver homogenate was diluted in 100 mM potassium phosphate buffer (pH 7.4) and incubated with a final concentration of 5 μM dichlorofluorescein diacetate (Sigma-Aldrich, Milan, Italy) in dimethyl sulfoxide for 15 min at 37 °C. The dye-loaded samples were centrifuged at 12500g for 10 min at 4 °C. The pellet was mixed at ice-cold temperatures in 5 mL of 100 mM potassium phosphate buffer (pH 7.4) and again incubated for 60 min at 37 °C. The fluorescence measurements were performed with GloMax® Discover Microplate Reader (Madison, WI, USA) at 488 nm for excitation and 525 nm for emission wavelengths. ROS were quantified from the dichlorofluorescein standard curve in dimethyl sulfoxide (0–1 mM).

The MDA assay was performed as previously described [51]. Livers were homogenized in a 1.15% KCl solution. An aliquot (200 μL) of the homogenate was added to a reaction mixture containing 200 μL of 8.1% SDS, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% thiobarbituric acid, and 600 μL of distilled water. Then, the samples were boiled for 1 h at 95 °C and centrifuged at 3000g for 10 min. The supernatant absorbance was measured by GloMax® Discover Microplate Reader (Madison, WI, USA) at 550 nm and the concentration of MDA was expressed as micromolar MDA per milligram protein of cell homogenate. A standard curve was prepared using MDA bis-(dimethyl acetal) as the source of MDA.

2.9. Western blotting

Liver and hypothalamus tissues were lysed with specific buffer (20 mM Tris–HCl, pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, leupeptin, and trypsin inhibitor 10 μg/mL). After 40 min, protein lysates (cytosolic fraction) were obtained by centrifugation at 14 000g for 15 min at 4 °C. The remaining pellet was dissolved with a specific buffer (1 M Tris–HCl, pH 6.8, 500 mM NaF, 10% sodium dodecyl sulfate (SDS), 1 mM Na3VO4, and trypsin inhibitor 10 μg/mL) and centrifugated at 14000 rpm for 30 min at 4 °C to obtain mitochondrial fractions. Protein concentrations were estimated by the Bradford protein assay using BSA as standard and determined using Life Science UV/Vis spectrophotometer DU730 (Beckman Coulter, Brea, USA). The equal amount of protein (50 μg) were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad, Segrate, Italy) using a Bio-Rad Transblot Turbo (Bio-Rad, Segrate, Italy). The filters were probed with antibody anti-phospho insulin receptor (InsR) (Thermo Scientific, Rockford, USA, catalogue number 44–800 G), anti-InsR (Cell Signaling Technology, Danvers, MA, USA, catalogue number #3025), anti-phospho protein kinase B (AKT, Cell Signaling Technology, Danvers, MA, USA, catalogue number #4060) or anti-AKT (Cell Signaling Technology, Danvers, MA, USA, catalogue number #2920), anti-phosphoAMPK (Cell Signaling Technology, Danvers, MA, USA, catalogue number #2535) or anti-AMPK (Cell Signaling Technology, Danvers, MA, USA, catalogue number #2793), anti-SOD2 (Cell Signaling Technology, Danvers, MA, USA, catalogue number #13141), anti-GLUT2 (Millipore Corporation, Billerica, MA, USA, catalog number #07–1402), anti-PPAR-α (Sigma-Aldrich, Milan, Italy, catalogue number P0369), anti-CPT1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, catalogue number sc-20669) and anti-SOD1 (Abcam, Cambridge, UK, catalogue number ab51254). Anti-β-Actin (Sigma-Aldrich, Milan, Italy, catalogue number A5441 or Abcam, Cambridge, UK, catalogue number ab8226) and anti-GAPDH (Sigma-Aldrich, Milan, Italy, catalogue number G9545) were used as housekeeping to ensure equal sample loading. Bands were detected by ChemiDoc imaging instrument (Bio-Rad, Segrate, Italy). Whole uncropped images of the original western blots from which figures were derived are shown in Supplementary Material 2.

2.10. Quantification of ROS producing and degrading enzymes in the liver

Liver samples were homogenized in PBS (10X w/V) with protease inhibitors, as previously described [52]. ELISAs commercially available were performed according to the manufacturer's instructions. Levels of NOX4 (MyBioSource, San Diego, California, USA, catalogue number MBS1600298), NOX2 (MyBioSource, San Diego, California, USA, catalogue number MBS7232989), NOX1 (MyBioSource, San Diego, California, USA, catalogue number MBS2508743), Catalase (MyBioSource, San Diego, California, USA, catalogue number MBS726781), and GSH (Biomatik, Ontario, Canada, catalogue number EKU04473) were quantified in rat liver samples. For the analysis, each sample and standard were used in duplicate to prevent intra-assay variations.

2.11. Measurement of SOD activity

SOD specific activity was evaluated in mitochondrial fraction of liver tissues, as previously described [53]. Briefly, SOD activity was measured in a medium containing 0.1 mM EDTA, 2 mM KCN, 50 mM KH_2PO_4, pH 7.8, 20 mM cytochrome c, 5 mM xanthine, and 0.01 U of xanthine oxidase. Enzyme activity was measured spectrophotometrically (550 nm) at 25 °C, by monitoring the decrease in the reduction rate of cytochrome c by superoxide radicals, generated by the xanthine–xanthine oxidase system. One unit of SOD activity was defined as the concentration of enzyme that inhibits cytochrome C reduction by 50% in the presence of xanthine and xanthine oxidase.

2.12. Statistical analysis

Statistical analysis was performed by using GraphPad 5.0 software for Windows (GraphPad Software, San Diego, CA, USA). Results were expressed as means ± standard error of the mean (SEM). Data were checked for normality and nonparamentric test or Geisser-Greenhouse's correction was carried out, as required. Two-way ANOVA for repeated measures was performed to analyze GTT and ITT tests results. Student's t-test or Mann Whitney test for were performed to analyze results from all the other experiments. For all statistical tests, a p value < 0.05 was considered statistically significant.

3. Results

3.1. Social isolation affects glucose homeostasis and insulin sensitivity

GTT for GRP and ISO rats was performed to assess possible differences in glucose homeostasis. While basal glycemic levels of ISO rats were comparable to the ones of GRP animals (Fig. 1D, Students' t-test, $p = 0.3475$), Two-way ANOVA for repeated measures revealed a significant effect only in time (Fig. 1A, for time: $F_{1,51} = 38.62$, $p < 0.0001$; for rearing: $F_{1,51} = 0.4477$, $p = 0.5124$). The same pattern was observed for insulin sensitivity in GRP and ISO rats after ITT test (Two-way ANOVA for repeated measures, Fig. 1B, for time: $F_{1,36} = 16.31$,

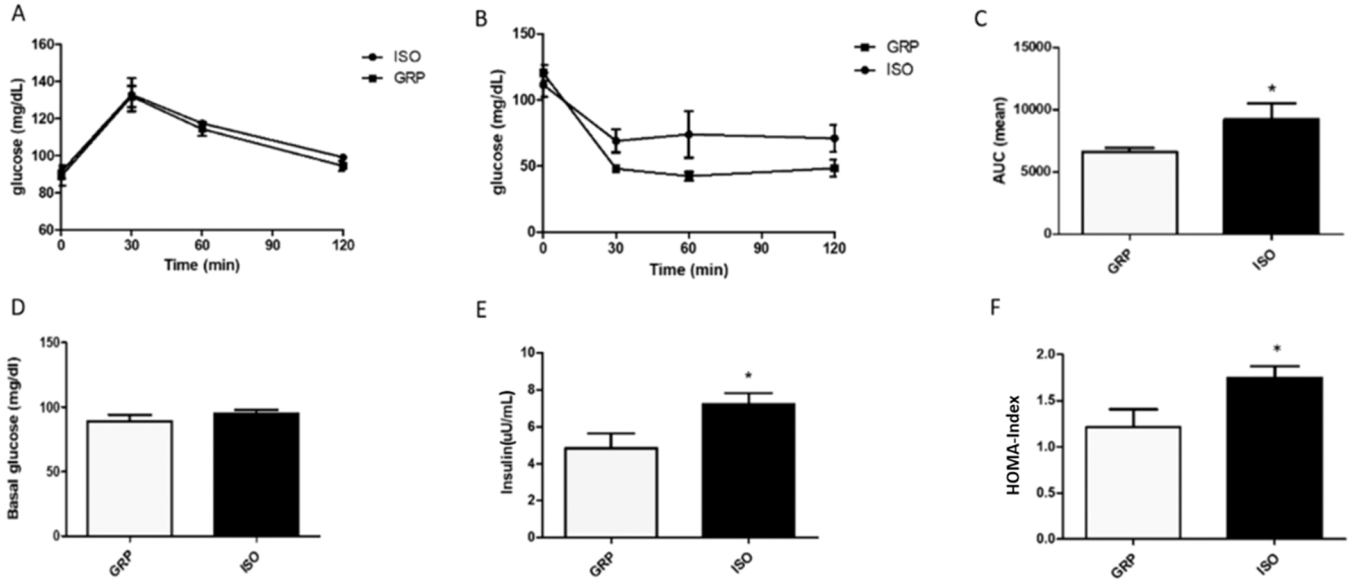

Fig. 1. Glucose homeostasis and insulin sensitivity in GRP and ISO rats. A) Glucose levels (mg/dL) in the GTT of GRP (n = 9) and ISO (n = 10) rats. Two-way ANOVA for repeated measures, for time: $F_{1,51} = 38.62$, $p < 0.0001$; for rearing: $F_{1,51} = 0.4477$, $p = 0.5124$. B) Glucose levels (mg/dL) in the ITT of GRP (n = 5) and ISO (n = 9) rats. Two-way ANOVA for repeated measures, for time: $F_{1,36} = 16.31$, $p < 0.0001$; for rearing: $F_{1,36} = 2.090$, $p = 0.1739$. C) AUC (mean) in GRP (n = 5) and ISO (n = 9) rats. Mann-Whitney test, * $p < 0.05$. D) Basal glucose levels (mg/dL) in GRP (n = 9) and ISO (n = 8) rats. Students' t-test, $p > 0.05$. E) Insulin levels (uU/mL) in GRP (n = 4) and ISO (n = 7) rats. Students' t-test, * $p < 0.05$. F) Homeostatic model assessment (HOMA) Index in GRP (n = 4) and ISO (n = 7) rats. Students' t-test, * $p < 0.05$.

$p < 0.0001$; for rearing: $F_{1,36} = 2.090$, $p = 0.1739$). However, AUC calculated for ISO rats showed a statistically significant increase compared to GRP animals (Fig. 1C, Students' t-test, $p = 0.0290$). Same results were found for either plasmatic insulin levels (Fig. 1E, Students' t-test, $p = 0.0393$), and HOMA-IR value (Fig. 1F, Students' t-test, $p = 0.0378$).

No significant differences were found between GRP and ISO rats in the following plasmatic biochemical parameters: total bilirubin, AST, ALT, Alkaline Phosphatase, total HDL and LDL Cholesterol, and Triglycerides (Table 1).

3.2. Social isolation impairs insulin signalling pathway in the rat hypothalamus

In the hypothalamus, as the managing centre of food intake, social isolation caused a reduction of AKT phosphorylation (Fig. 2A, Students' t-test, $p = 0.0115$) and a trend toward an increase in AMPK phosphorylation (Fig. 2B, Students' t-test, $p = 0.0667$). Furthermore, impairment of glucose transporter (GLUT)−4 was detected (Fig. 2C, Students' t-test, $p = 0.0086$).

Table 1
Values of plasmatic biochemical parameters in GRP and ISO rats.

PLASMATIC BIOCHEMICAL PARAMETER	VALUE (MEAN±SEM)	p VALUE (Student's t-test)
Total Bilirubin (mg/dL)	GRP = 0.1417 ± 0.01470 ISO = 0.1367 ± 0.01406	0.8108
AST (IU/L)	GRP = 226.5 ± 23.15 ISO = 273.3 ± 33.51	0.2770
ALT (IU/L)	GRP = 57.67 ± 3.648 ISO = 61.33 ± 4.631	0.5479
Alkaline Phosphatase (IU/L)	GRP = 21.60 ± 6.653 ISO = 20.00 ± 2.781	0.8178
Total Cholesterol (mg/dL)	GRP = 87.50 ± 5.239 ISO = 92.50 ± 5.421	0.5222
HDL Cholesterol (mg/dL)	GRP = 51.17 ± 3.005 ISO = 54.00 ± 3.055	0.5234
LDL Cholesterol (mg/dL)	GRP = 21.67 ± 2.092 ISO = 25.00 ± 2.033	0.2798
Triglycerides	GRP = 194.0 ± 27.44 ISO = 154.4 ± 13.03	0.2546

n = 5 GRP and n = 6 ISO.

3.3. Social isolation alters hepatic insulin sensitivity in rat

To study the molecular mechanism of hepatic insulin resistance induced by 7 weeks of isolation, liver samples were examined by western blot analysis. Indeed, ISO rats showed an impairment of insulin pathway compared to GRP rats as evidenced by the significant reduction of the phosphorylation of InsR (Fig. 3A, Students' t-test, $p = 0.0476$) and AKT (Fig. 3B, Students' t-test, $p = 0.0461$), and the protein expression of GLUT-2 (Fig. 3C, Students' t-test, $p = 0.0426$).

3.4. The hepatic lipid metabolism is altered by social isolation

In the liver of ISO rats, a reduction of the phosphorylation state of AMPK was observed (Fig. 4A, Students' t-test, $p = 0.0279$). Hepatic fatty acid oxidation was markedly altered, as shown not only by a significant decrease in hepatic CPT1 (Fig. 4B, Students' t-test, $p = 0.0084$), a key transporter involved in the influx of fatty acids into the mitochondria, but also by the reduction of PPAR-α levels (Fig. 4C, Students' t-test, $p = 0.0467$).

3.5. Social isolation increases ROS production and lipid peroxidation in the liver

In order to verify the impact of social isolation on oxidative stress and lipid peroxidation, we quantified ROS and MDA levels in the liver of GRP and ISO rats. Rats exposed to social isolation showed significant elevations in hepatic ROS amount compared to animals reared in group (Fig. 5A, Student's t-test, $p = 0.0205$). Accordingly, MDA levels showed a similar pattern (Fig. 5B, Student's t-test, $p = 0.0439$). Moreover, to confirm the oxidative damage induced by social isolation, the expression of ROS-producing enzymes in the liver was determined. The levels of the

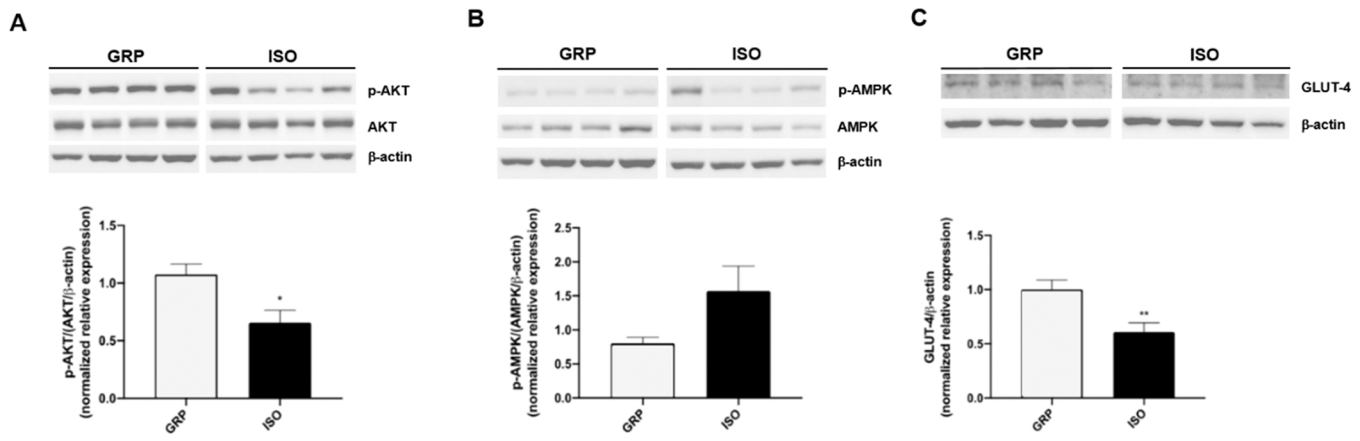

Fig. 2. The effects of isolation on insulin signaling in the hypothalamus. The protein expression of A) p-AKT (GRP n = 7, ISO n = 7), Student's t-test, * $p < 0.05$ B) p-AMPK (GRP n = 7, ISO n = 7), Student's t-test, $p > 0.05$ and C) GLUT-4 (GRP n = 7, ISO n = 7), Student's t-test, ** $p < 0.01$ was evaluated in GRP and ISO rats. Cropped Western blots are shown for each parameter.

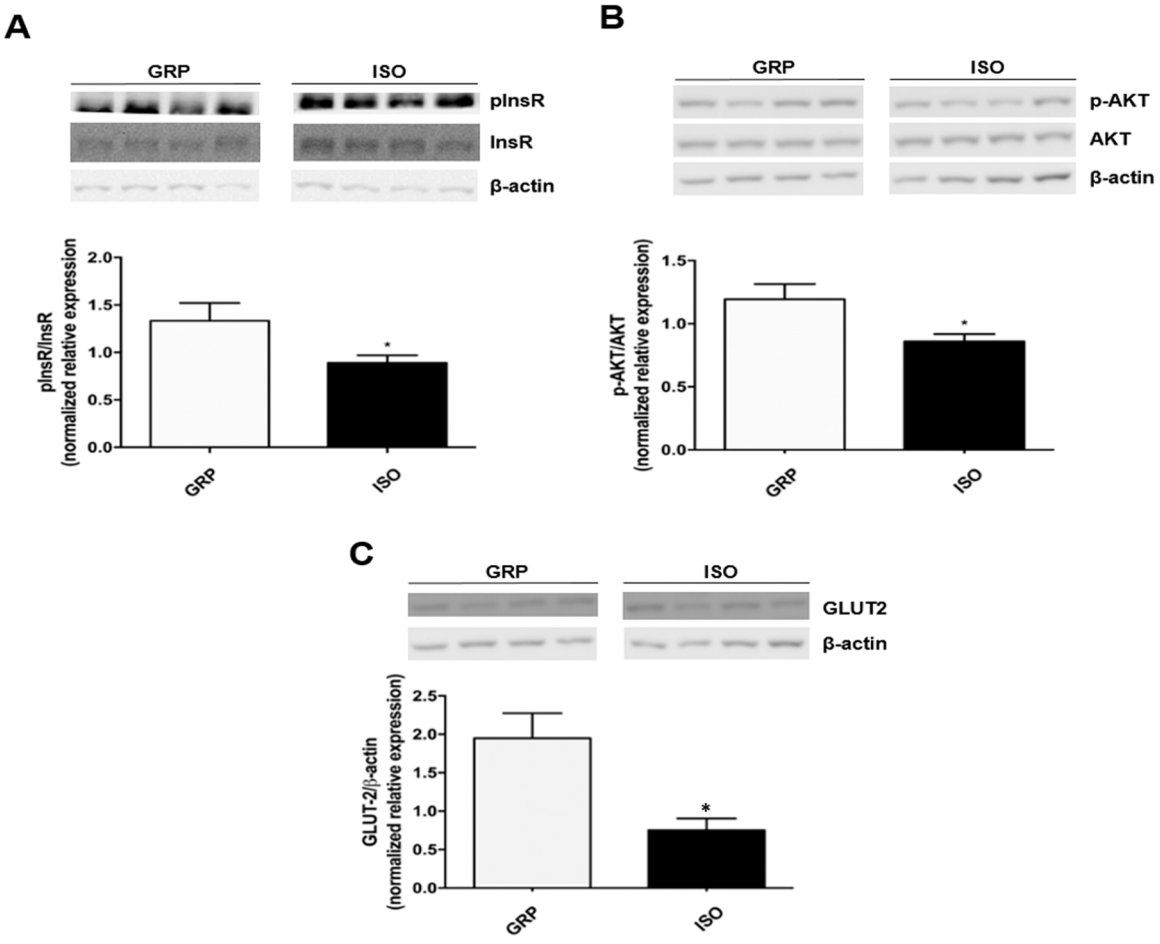

Fig. 3. The effects of isolation on insulin signaling in the liver. The protein expression of A) p-InsR (GRP n = 7, ISO n = 7), Student's t-test, * $p < 0.05$, B) p-AKT (GRP n = 5, ISO n = 7), Student's t-test, * $p < 0.05$ and C) GLUT-2 (GRP n = 5, ISO n = 7) Student's t-test, * $p < 0.05$ was evaluated in GRP and ISO rats. Cropped Western blots are shown for each parameter.

NADPH oxidase NOX4, NOX2 and NOX1 were determined. A significant increase of hepatic NOX4 expression was found in ISO rats compared to GRP animals (Fig. 6A, Student's t-test, $p = 0.0239$), whereas no differences were detected in NOX2 (Fig. 6B, Student's t-test, $p = 0.7870$) and NOX1 (Fig. 6C, Student's t-test, $p = 0.0870$) levels between the two experimental groups.

3.6. Social isolation alters hepatic antioxidant defense mechanisms

To assess the effects of social isolation on ROS-degrading enzymes in the liver, levels of SOD2, SOD1, CAT and GSH were determined for GRP and ISO rats. Hepatic protein expression of SOD2 and SOD1 in the mitochondrial and cytosolic fractions, respectively, did not differ

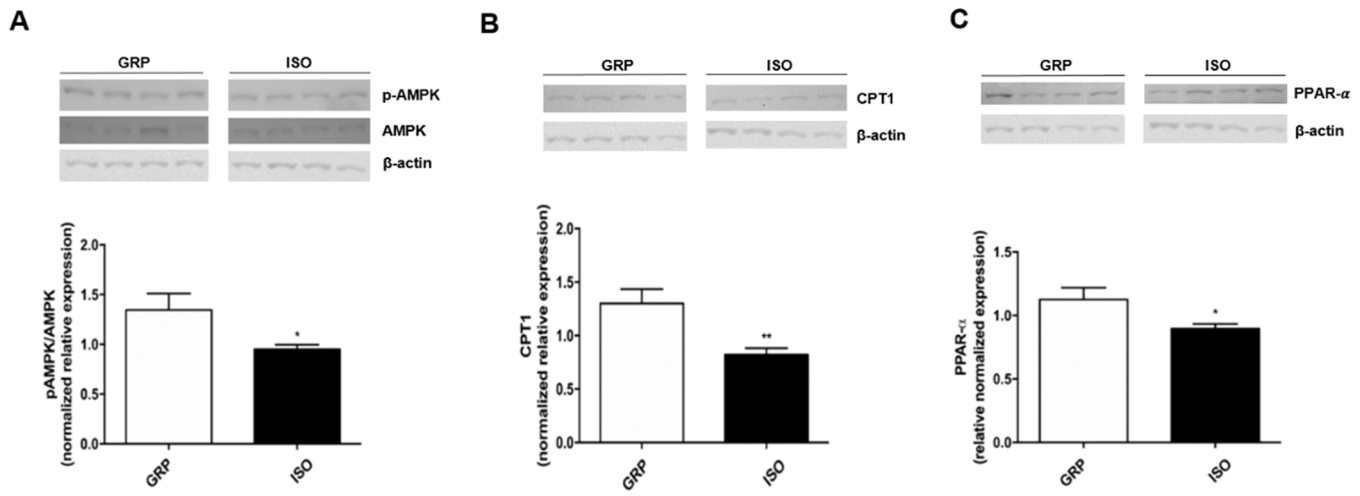

Fig. 4. The isolation impairs the pathway of hepatic lipid metabolism. The protein expression of A) p-AMPK, B) CPT1 and C) PPAR-α in GRP (n = 7) and ISO (n = 7) rats. Student's t-test, * $p < 0.05$, ** $p < 0.01$. Cropped Western blots are shown for each parameter.

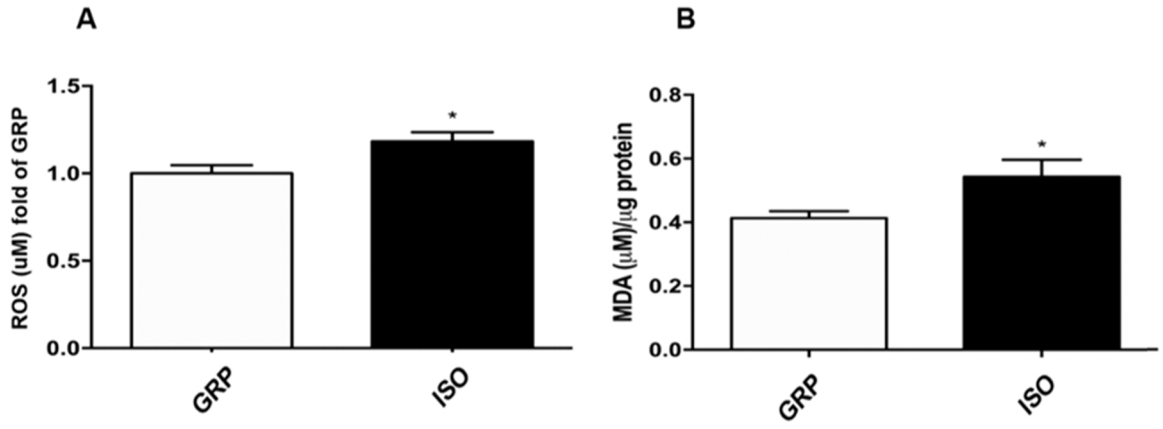

Fig. 5. ROS and MDA levels in the liver of GRP and ISO rats. A) ROS levels (μM, fold of GRP) in the liver of GRP (n = 8) and ISO (n = 8) rats. Student's t-test, * $p < 0.05$. B) MDA levels (μM/μg protein) in the liver of GRP (n = 7) and ISO (n = 7) rats. Student's t-test, * $p < 0.05$.

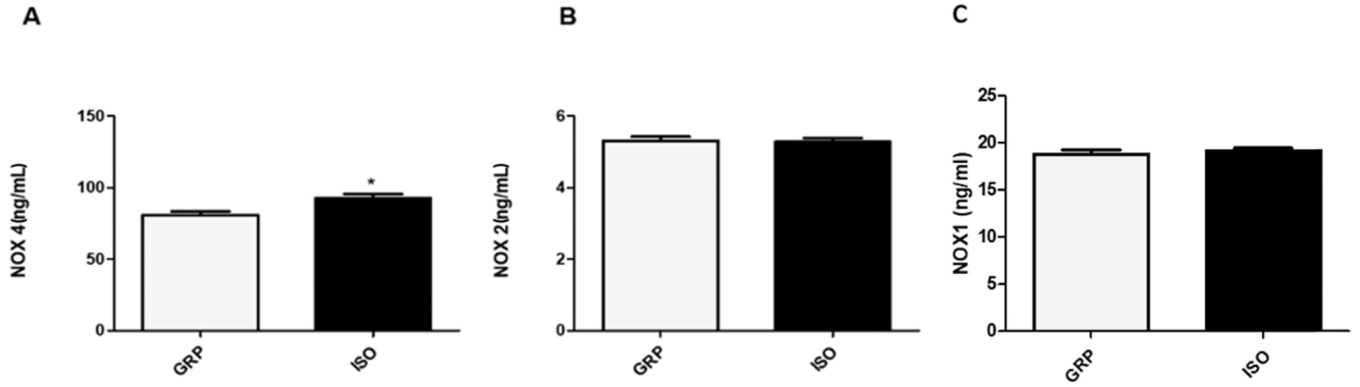

Fig. 6. NADPH oxidase NOX enzymes expression in the liver of GRP and ISO rats. A) NOX4 levels (ng/mL) in the liver of GRP (n = 4) and ISO (n = 4) rats. Student's t test, * $p < 0.05$. B) NOX2 levels (ng/mL) in the liver of GRP (n = 4) and ISO (n = 4) rats. Students' t-test, $p > 0.05$. C) NOX1 levels (ng/mL) in the liver of GRP (n = 5) and ISO (n = 7) rats. Students' t-test, $p > 0.05$.

between GRP and ISO animals (Figs. 7A and C, Student's t-test, $p = 0.7985$, and Figs. 7B and D, Student's t-test, $p = 0.5610$). Otherwise, the activity of SOD was significantly increased following social isolation (Fig. 7E, Student's t-test, $p = 0.0089$). A reduction of hepatic CAT (Fig. 7F, Student's t-test, $p = 0.0389$) and GSH (Fig. 7G, Student's t-test, $p = 0.0185$) levels was found in ISO rats compared to GRP animals.

4. Discussion

In this paper, we demonstrated that both peripheral and central

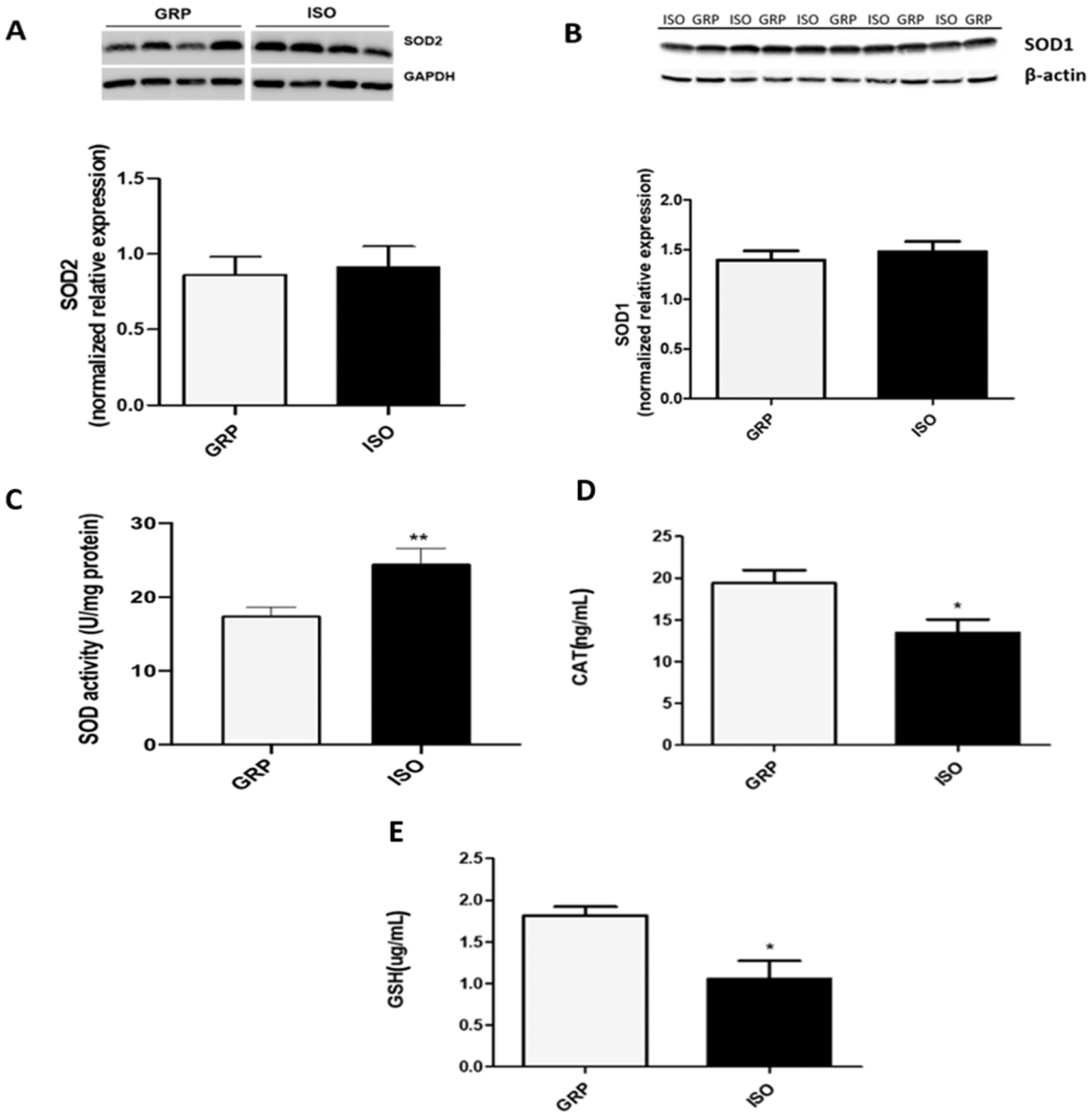

Fig. 7. Antioxidant enzymes in the liver of GRP and ISO rats. A) Representative blot and relative normalized expression of SOD2 in the liver (mitochondrial fraction) of GRP (n = 5) rats and ISO (n = 7) rats. Students' t-test, $p > 0.05$. B) Representative blot of SOD1 and relative normalized expression in the liver (cytosolic fraction) of GRP (n = 5) and ISO (n = 5) rats. Students' t-test, $p > 0.05$. C) SOD activity (mitochondrial fraction) in GRP (n = 7) rats and ISO (n = 7) rats. Students' t-test ** $p < 0.01$ D) CAT levels (ng/mL) in the liver of GRP (n = 4) and ISO (n = 4) rats. Students' t-test, * $p < 0.05$ E) GSH levels (ug/mL) in the liver of GRP (n = 4) and ISO (n = 4) rats. Students' t-test, * $p < 0.05$.

insulin signaling and sensitivity are altered in a non-pharmacological rat model of psychosis, obtained by exposure to chronic environmental stress (social isolation), that resembles a "drug-free" or "drug-naïve" condition of psychiatric disease. Plasmatic insulin levels and HOMA-IR index were found increased in ISO rats, suggesting impaired insulin sensitivity and enhanced insulin resistance without evident alteration of glucose homeostasis as evidenced by oral glucose tolerance test. In our experimental condition, glucose homeostasis requires the production of higher insulin amounts to maintain normal basal levels of glucose and physiological response to a glucose load. In line with our findings, rats exposed to cumulative stress, such as periodic maternal separation combined with post-weaning social isolation, showed comparable basal glucose levels than non-stressed animals, associated with increased insulin amount [54]. Accordingly, it has been reported that isolation stress may progressively affect glucose metabolism by inducing a condition of insulin resistance [55]. Furthermore, insulin resistance has been described in patients diagnosed with the first episode of psychosis (FEP), drug-naïve for antipsychotic medication [56,57], highlighting that the psychotic condition per se, regardless of the pharmacological treatment, may be directly implicated in the development of metabolic alterations, including diabetes, often observed in the later phases of the psychotic disease [58–60]. Our preclinical data are in agreement with clinical evidence obtained in drug-naïve FEP patients that showed a significant increase of HOMA-Index compared to matched control individuals [61,62].

Despite hyperinsulinemia and increased HOMA-IR Index, plasmatic levels of biochemical parameters related to hepatic functioning and lipid metabolism were not affected after seven weeks of social isolation. Based on our findings, we might hypothesize that seven weeks of post-weaning social isolation were not sufficient to unveil overt hepatic dysfunctions and alteration of lipid metabolism, which, instead, may appear at later time points. Supporting our hypothesis, a longer social isolation period (twelve weeks) was able to significantly alter hepatic biochemical parameters, such as AST and ALT [63]. Furthermore,

deprivation of social contacts in rodents lasting more than seven weeks has been described to induce alterations in biochemical markers related to lipid metabolism [64].

The autonomic outflow through the vagal innervation of the liver underlies the remarkable physiological activity of insulin within the CNS. Indeed, a key role of the "brain-liver axis" in controlling glucose production has been described [65,66]. Some brain regions, including the hypothalamus and choroid plexus, are considered sites of more fast entry for peripheral insulin into the CNS [67]. In particular, the hypothalamus can control liver functions by different neural and neuroendocrine connections [68]. Most glucose uptake in neurons takes place via GLUT 3, which is co-expressed with GLUT 4 [22]. GLUT4 expression is insulin-dependent, as well as its translocation from the cytosol to the plasma membrane that facilitates the uptake and utilization of glucose, regulating neuronal energy necessary for cognition and memory [69]. Induction of GLUT 4 translocation to the neuron cell membrane occurs via the AKT pathway [70] and improves insulin transport into the neurons during periods of high metabolic demand. These results suggest that the deregulation of insulin-dependent glucose transport in several brain regions may cause cognitive impairment. Here, we clearly demonstrated that social isolation in rats reduces AKT phosphorylation and GLUT4 expression in the hypothalamus. Moreover, social isolation partially affects brain AMPK, which is a canonical regulator of energy balance, increasing enzyme phosphorylation. Notably, antipsychotic drugs, such as olanzapine, well known for their orexigenic and obesity-prone properties, activate hypothalamic AMPK [71]. Thus, the alterations of the hypothalamic insulin signaling is accompanied by impaired hepatic insulin sensitivity in isolated rats, supporting the detrimental interconnection induced by chronic stress between brain and liver. Oxidative stress has been widely associated with different types of severe life stress, including social isolation [72]. Indeed, redox imbalance, at both central and peripheral levels, has been previously reported in socially isolated rats [19,20,46,47,73,74]. Here, increased hepatic oxidative stress has been shown in rats kept in social isolation for seven weeks, in terms of increased tissue ROS overproduction and enhanced lipid peroxidation. A close interaction between long-duration stressors and oxidative-dependent liver dysfunctions has been previously described in different preclinical studies [75,76]. Moreover, it has been demonstrated that the beneficial effects of a treatment with olanzapine, an atypical antipsychotic drug, in socially isolated rats were mediated by the modulation of hepatic oxidative stress [77]. In good agreement with our data, increased hepatic MDA levels following social isolation have been previously observed [77]. Importantly, increased oxidative stress in the liver of drug-free FEP patients has been described [78], as well as a correlation between insulin resistance, peripheral oxidative stress and the severity of the psychotic symptoms [79].

Mitochondria, as well as the NADPH oxidase NOX enzymes have been described as the major sources of ROS production in the liver, crucially contributing to the development of different hepatic physiopathological conditions [28]. In this regard, an important finding of our study regards the hepatic levels of the NOX4 enzyme, which were increased following exposure to social isolation, whereas no alterations were detected in the levels of NOX2 and NOX1 in the same organ.

At least in our experimental conditions, the increase of NOX4 enzyme observed in periphery following social isolation appears to be specific in the liver, as it was not observed in other peripheral sites of the same rat model, such as the adipose tissue, where, instead, an enhanced expression of NOX1 has been previously described [20]. Intriguingly, by using NOX4 knock-out mice, it has been demonstrated that NOX4-derived ROS are crucially implicated in the insulin signaling pathways and the prevention of insulin resistance, thus being required for the physiological glucose metabolism [80]. Moreover, in the same line, cultured liver cells deficient in NOX4 showed altered insulin signaling leading, finally, to hyperinsulinemia [81]. Thus, it can be speculated that the increase of NOX4 levels observed in the liver of socially isolated rats might represent a compensatory response to hyperinsulinemia and to the onset of insulin resistance as demonstrated by the increase of HOMA index observed following social isolation exposure. However, we cannot exclude that, in this compensatory process, other NADPH oxidase isoforms and/or other ROS sources may also be implicated.

Here, the increase in hepatic ROS amount and lipid peroxidation observed in ISO animals might be due to social isolation-induced dysfunctions of antioxidant enzymes, such as catalase and glutathione, whose levels were decreased in the organ. Our data are in agreement with previous outcomes on the reduced expression and function of these ROS-degrading enzymes in the CNSfollowing chronic exposure to social isolation [82], and their depletion in metabolic dysfunctions characterized by visceral fat accumulation [83,84], a peripheral hallmark of the rat social isolation model [20]. Interestingly, in our experimental conditions, hepatic SOD1 and SOD2 expression did not change following social isolation, whereas SOD activity was increased. The relevance of these findings is due not only to the different cellular locations (SOD1 in the cytosol, SOD2 in the mitochondria) but also to their different expression and regulation during rat liver ontogeny. Indeed, whereas SOD1 expression and activity significantly increase after birth, SOD2 levels reached functional levels at weaning [85], i.e. at the moment of the beginning of the social isolation procedure. Hence, the increase of SOD activity observed in ISO rats might be interpreted as an attempt of the mitochondrial antioxidant system in counteracting the increase in hepatic ROS amount and lipid peroxidation observed in this animal model to control ROS-induced damage [86]. Thus, the concomitant lack of differences in hepatic SOD protein expression observed in our experimental conditions might be explained as a possible isolation-induced downstream dysfunction of the above-mentioned attempt. Moreover, it has been demonstrated that SOD2 deletion impairs insulin function in rodents, resulting in impaired glucose-stimulated insulin secretion and hyperinsulinemia [87]. In the same line, it has been reported that SOD2 overexpression decreased hepatic ROS amount, protecting from the development of hepatic insulin resistance [88]. However, at this stage, we cannot exclude that SOD expression and activity might be altered at different time points of the social isolation procedure. Consistently with this hypothesis, it has been demonstrated that in the animal models of acute or chronic stressors, a different modulation of the antioxidant enzymes was shown [76]. In support of our evidence, SODs, as the first line of antioxidant defense, did not change following 6 weeks of chronic social isolation [77,89]. Otherwise, chronically isolated animals showed a marked decrease in GSH content [89]. Therefore, further investigations are warranted to elucidate the contribution of the activity of the mitochondrial antioxidant system against the alterations of peripheral redox homeostasis observed following chronic psychosocial stress.

5. Conclusions

In conclusion, we argue that the increased oxidant status and the modification of antioxidant capacity can trigger hepatic and systemic insulin resistance, by altering signal hormone pathway and sustaining subsequent alteration of glucose homeostasis and metabolic impairment observed in the social isolation model of psychosis.

Fundings

This study was supported by PRIN 2017 code 2017AY8BP4 to SS from the Italian Ministry of Education, University and Research (MIUR), Italy; PRIN 2017 code 2017YZF7MA to LT from MIUR and Research for Innovation (REFIN) from Apulia Region, Italy, code OE9C2692 to MB.

Conflict of interest statement

The Authors declare no conflict of interests.

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contribution statement

MB, AL, SS, MGM and LT designed the research. MB, AL, SS, CP, PT, GT, GC, VS and MGM performed the research. MB, AL, SS, PT and MGM analyzed the data. MB, AL, SS, MGM and LT wrote the manuscript. All authors revised and contributed to the article and approved the submitted version.

Data availability statement

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112820.

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