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N-Acetyl-L-Cysteine treatment efficiently prevented pre-diabetes and inflamed-dysmetabolic liver development in hypothalamic obese rats

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ABSTRACT

Aim: Hypothalamic obese rats are characterized by pre-diabetes, dyslipidemia, hyperadiposity, inflammation and, liver dysmetabolism with oxidative stress (OS), among others. We studied endocrine-metabolic dysfunctions and, liver OS and inflammation in both monosodium L-glutamate (MSG)-neonatally damaged and control litter-mate (C) adult male rats, either chronically treated with *N*-Acetyl-L-Cysteine since weaned (C-NAC and MSG-NAC) or not.

Methodology: We evaluated circulating TBARS, glucose, insulin, triglycerides, uric acid (UA) and, aspartate and alanine amino-transferase; insulin sensitivity markers (HOMA indexes, Liver Index of Insulin Sensitivity –LISI-) were calculated and liver steps of the insulin-signaling pathway were investigated. Additionally, we monitored liver OS (protein carbonyl groups, GSH and iNOS level) and inflammation-related markers (COX-2 and TNFα protein content; gene expression level of *Il1b*, *Tnfα and Pai-1*); and carbohydrate and lipid metabolic functions (glucokinase/fructokinase activities and, mRNA levels of *Srebp1c*, *Fas* and *Gpat*).

Key Findings: Chronic NAC treatment in MSG rats efficiently decreased the high circulating levels of triglycerides, UA, transaminases and TBARS, as well as peripheral (high insulinemia and HOMA indexes) and liver (LISI and the P-AKT:AKT and P-eNOS:eNOS protein ratio values) insulin-resistance. Moreover, NAC therapy in MSG rats prevented liver dysmetabolism by decreasing local levels of OS and inflammation markers. Finally, NAC-treated MSG rats retained normal liver glucokinase and fructokinase activities, and *Srebp1c*, *Fas* and *Gpat* (lipogenic genes) expression levels.

Significance: Our study strongly supports that chronic oral antioxidant therapy (NAC administration) prevented the development of pre-diabetes, dyslipidemia, and inflamed-dysmetabolic liver in hypothalamic obese rats by efficiently decreasing high endogenous OS.

1. Introduction

It is known that neonatal monosodium L-glutamate (MSG) i.p. administration in rodents induces morphological, behavioral and endocrine abnormalities, such as stunted growth, hyperadiposity and hypogonadism [1–3], rendering a phenotype designated as hypothalamic obesity [4]. Moreover, other authors have reported severe loss of neurons (e.g. catecholaminergic and peptidergic) in the MSG rat hypothalamic arcuate nucleus (ARC) [3–6], a central pivot structure involved in regulation of energy balance (storage/expenditure). At this level, leptin secreted by adipose tissue binds, in turn, to Ob-RB then triggering the hypothalamic leptin-signaling pathway, a key mechanism to maintain homeostasis [7]. Additionally, once the adult age is reached, MSG rodents are characterized by an inflamed endogenous environment (evinced by high circulating and tissue levels of specific inflammation markers) [4,8–13]. As a consequence, obese rats are highly prone to develop neuroendocrine-metabolic dysfunctions, such as hypophagia [4], high glucocorticoid production [4], enlarged white adiposity mass (hypertrophic adipocytes) [14], adipo-insular axis resistance [14,15], and testicular dysfunction [16], among others.

We earlier demonstrated [17] that adult male MSG rats are pre-diabetic (normal glycemia accompanied by compensatory hyperinsulinemia and thus higher insulin resistance index (HOMA-IR) and beta cell function (HOMA-β), with increased oxidative stress (OS) at both peripheral and liver levels.

These changes correlate with enhanced overall inflammation [4,8],

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namely at hepatic level [17], and accompanied by a liver metabolism displaced to increased lipid production (higher glucose flux throughout increased glucokinase activity and enhanced lipogenic genes expression) [17], a characteristic that, combined with a chronic glucocorticoid-rich milieu [7,8], resembles those appearing in the human Cushing's and Metabolic Syndromes phenotypes.

It is recognized that a high OS level (a cell imbalance between free radical generation and free radical scavenger activity) has been implicated as a major pathogenic cause in several illnesses, from cancer [18] up to metabolic diseases [19–21]. We previously demonstrated in a model of unhealthy diet intake that inflammation, insulin resistance (IR) and OS constitute a pathological triad that could be effectively reversed by mitigating endogenous OS [22].

N-Acetyl-L-Cysteine (a highly active antioxidant form; NAC) is a well-known compound that, by acting through its GSH enhancing effect [23], effectively counteract, at least in part, the development of several OS-related dysfunctions, including peripheral IR in rodents [24–26] and humans [27,28], dysfunctional liver [29–31] and obesity [32,33]. However, no studies have been focused on the application of NAC treatment in order to prevent the development of pre-diabetes, overall dys-metabolism and liver malfunction in the neonatally-damaged adult MSG male rat [17].

In this regard, we presently examined whether pre-diabetes, dysmetabolism, inflammation and liver dysfunction developed in adult MSG male rats could be prevented by arresting the high OS endogenous environment. With this aim, adult male MSG rats were supplemented, since weaning up to adult age, with an oral low dose of NAC (25 mg/rat/day, in the drinking solution) as antioxidant. Several features (e.g. circulating levels of endocrine-metabolic biomarkers, peripheral and liver OS status, IR indexes and inflamed-dysmetabolic liver) were compared with NAC-untreated MSG litter-mate rats.

2. Materials and methods

2.1. Chemicals and drugs

Reagents of the purest available grade including MSG and NAC were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies anti-P-AKT (reacting with Ser473) and anti-AKT were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA; catalog number 6040S and 9272 respectively), anti-COX-2 from CAYMAN Laboratories (MI, USA catalog number 160106), anti-iNOS and anti-eNOS were obtained from Sigma (catalog number N7782), anti-P-eNOS (Ser 1177) was obtained from Cell Signaling Laboratory (Danvers, MA, USA; catalog number N3893) and anti-GAPDH from Millipore (CA, USA; catalog number 92590). Finally, a secondary antibody anti-rabbit IgG Peroxidase (developed in goats) was obtained from Sigma (catalog number A9169).

2.2. Experimental animals

Animal model preparation has been largely and previously reported by one of present authors [16,34–38]. Briefly, adult male and female Wistar rats were allowed to mate in colony cages, in a light- (lights on: 07:00–19:00 h) and temperature (20–22 °C)-controlled room. Rat chow and water were available ad libitum. Pregnant rats were transferred into individual cages. Beginning on day 2 after parturition, newborn male pups were i.p. injected with either 4 mg/g BW MSG dissolved in a small volume (25–150 μL) of sterile 0.9% (w/v) NaCl; due to MSG solution hypertonicity, a similar volume of 10% (w/v) NaCl was i.p. injected to litter-mate controls (C). Injections were performed day after day up to day 10 of age [15,34–38]. Rats were weaned at 21 days of age and housed (3 rats per cage) in a controlled environment (20–22 °C and lights on between 07:00–19:00 h). On the morning of the weaning day, male rats were divided into four groups: two of them, Control-litter mates (C) and MSG-treated rats (MSG), received Purina rat chow and water ad libitum until the experimental day. The other two groups, C and MSG rats, received Purina rat chow and either water or NAC solution ad libitum (C-NAC and MSG-NAC groups). NAC solution (varying 2.5–0.8 mg/mL; adapted from Dhouib IB et al. 2014) intake ranged between 11 ± 2 (21 day-old rats) and 34 ± 4 (150 day-old rats) mL/day/rat, thus rendering an average of NAC-intake of 25 mg/rat/day [39]. Each group included a total of 8–10 rats. Individual daily body weight (BW) and fluid/food intake were recorded until the experimental day (150 days of age). On the morning (between 08:00–10:00 h) of the experimental day, non-fasting animals were weighed, rapidly euthanized and trunk blood was collected (into EDTA-coated tubes). The brain was immediately dissected-out in order to check effectiveness of MSG treatment by macroscopic observation of degenerated optic nerves (inclusion criteria). Thereafter, liver was dissected and weighed; finally, hepatic medial lobes were excised for biochemical assays. Animals were killed by decapitation according to protocols for animal care and use (NIH Guidelines for care and use of experimental animals). All experimental procedures were approved by our Institutional Animal Care Committee (FCM-CICUAL N: T01-01-2014).

2.3. Circulating metabolites and insulin sensitivity indexes

Glucose-oxidase GOD-PAP method (Roche Diagnostics, Mannheim, Germany) was utilized to measure glycemia. Plasma levels of triglycerides, uric acid, and transaminases, aspartate aminotransferase (GOT) and alanine aminotransferase (GPT), were assayed by commercial (enzymatic-colorimetric) kits (Wiener Lab., Argentina). Circulating immunoreactive insulin was determined by a previously described specific radioimmunoassay [9], with intra- and inter-assay coefficient of variation ranging 2–4% and 6–9%, respectively. TBARS (thiobarbituric acid-reactive substances) as an index of malondialdehyde production was measured as a circulating OS marker. The amount of TBARS formed was expressed as pmol/mg of plasma protein quantified by the Bio-Rad Protein Assay kit [42].

Glycemia and insulin values were used to estimate peripheral IR by homeostasis model assessment-insulin resistance (HOMA-IR) (insulin × glycemia / 22.5) and β-cell function by HOMA-β [(20 × insulin / glycemia) − 3.5]. Liver insulin sensitivity index (LISI) was calculated by the following formula: k/(fasting plasma insulin) × fasting glycemia, where k = 22.5 × 18 (insulin/glycemia) [40]. In all three indexes insulin was expressed in μIU/mL and glycemia in mM.

2.4. Liver Protein carbonyl groups and reduced glutathione (GSH)

Hepatic OS markers (protein carbonyl and GSH levels) were determined as described elsewhere [41]. Both components were spectrophotometrically measured at 366 and 414 nm for protein carbonyl groups and GSH, respectively. Results were expressed in nmol of carbonyl residues per mg of protein and GSH content expressed in μmol of GSH per g of tissue.

2.5. Total liver RNA isolation and mRNA expression levels (qPCR)

A 100 mg liver-piece was used for total RNA isolation using the TRIzol Reagent (Gibco-BRL, Rockville, MD, USA) as described in a previous report [42]. Integrity and quality of RNA isolated was checked by agarose-formaldehyde gel electrophoresis and by measuring the 260/280 nm absorbance ratio. DNA contamination was avoided by using DNase I digestion reagent (Gibco-BRL). cDNA was obtained by reverse transcription-PCR using SuperScript III (Gibco-BRL) and total RNA (50 ng) as a template. qPCR was performed with a Mini Opticon Real-Time PCR Detector Separate MJR (BioRad), using SYBR Green I as fluorescent dye. Details of this procedure were reported elsewhere [17]. Briefly 10 ng of cDNA was amplified in a qPCR reaction mixture containing 0.36 μM of each specific primer, 3 mM MgCl2, 0.2 mM dNTPs and 0.15 μL Platinum Taq DNA polymerase (6 U/μL) (Invitrogen).

Table 1
Rat specific primers employed for real-time PCR analyses.

		GBAN	bp
b-actin	F, 5′-AGAGGGAAATCGTGCGTGAC-3′ R, 5′-CGATAGTGATGACCTGACCGT-3′	NM_031144	138
Fas	F, 5′-GTCTGCAGCTACCCACCCGTG-3′ R, 5′-CTTCTCCAGGGTGGGGACCAG-3′	NM_017332.1	214
Gpat	F, 5′-GACGAAGCCTTCCGAAGGA-3′ R, 5′-GACTTGCTGGCGGTGAAGAG-3′	AF_021348	68
Srebp1c	F, 5′-TTTCTTCGTGGATGGGGACT-3′ R, 5′-CTGTAGATATCCAAGAGCATC-3′	XM_213329.5	208
Il1b	F, 5′-ACAAGGAGAGACAAGCAACGAC-3′ R, 5′-TCTTCTTTGGGTATTGTTTGGG-3′	NM_031512.2	140
Pai1	F, 5′-CCACGGTGAAGCAGGTGGACT-3′ R, 5′-TGCTGGCCTCTAAGAAGGGG-3′	NM_012620.1	195
Tnfa	F, 5′-GGCATGGATCTCAAAGACAACC-3′ R, 5′-CAAATCGGCTGACGGTGTG-3′	NM_012675.3	**130**

(Abbreviations: F: forward primer; R: reverse primer; GBAN: GenBank Accession Number; amplicon length, in bp).

Specific oligonucleotide primers (obtained from Invitrogen) are shown in Table 1. Amplicons were designed in a size range of 90 to 250 bp, with *b-actin* used as housekeeping gene. Results are shown as relative to *b-actin* gene expression using Qgene96 and LineRegPCR software.

2.6. Western blot analysis

Immunodetection of TNFα, COX-2, iNOS, eNOS/P-eNOS, AKT/P-AKT and GAPDH proteins was performed in liver homogenates from each experimental group. Protein concentration was quantified by the Bio-Rad Protein Assay kit. Thereafter, dithiothreitol and bromophenol blue were added (final concentrations 100 mM and 0.1% w/v, respectively). Aliquots of 50–100 μg of whole protein were placed in reducing 10% (w/v) SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. GAPDH density was used to normalize protein content: the relative content of target protein was divided by the relative GAPDH protein level in each group. Non-specific binding sites of membranes were blocked by overnight incubation with non-fat dry milk at 4 °C. Enzyme identification and quantification were performed with specific primary antibodies against COX-2 iNOS, eNOS, P-eNOS (Ser1177), AKT, P-AKT and GAPDH. All these primary antibodies were overnight incubated at a final dilution of 1:1000. After the respective incubation period, membranes were rinsed with TBS and further incubated (1 h) with the corresponding secondary antibody at room temperature using Anti-Rabbit IgG Peroxidase, an antibody produced in goat (1:5000). ECL western blotting substrate was used for development. Bands were quantified by densitometry using Gel-Pro Analyser software.

2.7. Glucokinase (GCK) activity

Freshly removed hepatic pieces were homogenized in hand-held homogenizers (20 times) containing ice cold phosphate saline buffer, with 0.1 mM PMSF, 0.1 mM benzamidin, 2 mM DTT, 4 μg/mL aprotinin and 0.3 M sucrose (pH 7.5). Then, homogenates were centrifuged (600 xg) to separate and discard the nuclear fraction. Supernatants were centrifuged (100,000 xg, at 4 °C), collected and identified as cytosolic fractions (where GCK is active). Phosphorylation in cytosolic fraction was measured at 37 °C, pH 7.4, by recording at 340 nm increasing absorbance in a well-established enzyme-coupled photometric assay containing glucose-6-phosphate dehydrogenase, ATP, and NADP. GCK activity was then calculated by subtracting activity measured at 1 mM glucose (hexokinase) from that measured at 100 mM glucose, and expressed in mU per mg of protein. One unit of enzyme activity was defined as 1 μmol glucose-6-phosphate formed from glucose and ATP, per minute at 37 °C [17].

2.8. Fructokinase (KHK) activity

Pieces of liver were homogenized in buffer containing 25 mM HEPES (pH 7.1), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, spun at 10,000 ×*g* at 4 °C for 20 min, and KHK activity was measured by a coupled enzymatic assay [43]. Briefly, 20 μL of clear supernatant were added to 200 μL of the reaction mixture [25 mM HEPES (pH 7.1), 6 mM MgCl2, 25 mM KCl, 10 mM NaF, 5 mM D-fructose, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 40 U/mL pyruvate kinase, 40 U/mL lactate dehydrogenase, and 50 mM *N*-acetyl-D-glucosamine to inhibit hexokinase activity]. This reaction was started by adding 10 μL of ATP (5 mM final concentration) and quantitatively measured by recording changes in optical density at 340 nm (30 min).

2.9. Statistical analysis

Data were analyzed by ANOVA, followed by Tukey's multiple comparisons test using the Prism analysis program (GraphPad). Brown–Forsythe test (sensitive to departures from normality) and Bartlett's test were used to assess normality distribution and variance homogeneity. Results were expressed as means (± SEM) of the indicated number of observations; differences were considered significant when P values were < 0.05 [42].

3. Results

3.1. Effect of chronic NAC therapy on rat phenotype, circulating metabolites and insulin sensitivity

As expected, between weaning and the experimental day, MSG rats were hypophagic and hyperadipose (namely their visceral pads) compared to C rats, regardless of whether rats received NAC therapy or not (not shown). On the experimental day, all MSG rats displayed optic nerves degeneration (by macroscopic observation), one-third reduction in hypothalamic NPY mRNA levels (data not shown) and significantly ($P < 0.05$ vs. C rats) lower body weight (Table 2); thus confirming key features of the MSG phenotype [4]. As it can be seen, both C and MSG rats treated with NAC (C-NAC and MSG-NAC) showed only a trend to decrease their body weights when compared with those from NAC-untreated rats, respectively (Table 2). Nevertheless, C and MSG rats having received NAC maintained significant ($P < 0.05$) differences in their BWs, in a similar fashion as both groups of untreated rats did (Table 2). Similar qualitative results were noticed after analyzing wet liver weight values among all experimental groups (Table 2).

While rats from all four groups displayed a similar glycemia (approximately 1 g/L in all groups) (data not shown), significantly ($P < 0.05$ vs. C rats) higher plasma insulin levels characterized MSG rats (Fig. 1, panel A). NAC treatment in MSG animals was fully able to significantly ($P < 0.05$ vs. MSG values) decrease peripheral insulin

Table 2
Body weight (BW; in grams), wet liver weight (wLW; in grams per 100 g BW) and insulin sensitivity indexes in normal (C) and hypothalamic obese (MSG) rats treated (C-NAC and MSG-NAC) and untreated (C and MSG) with NAC.

	C	C-NAC	MSG	MSG-NAC
BW	406.2 ± 8.9	397.8 ± 7.1	341.2 ± 6.8^{ab}	327.3 ± 11.1^{ab}
wLW	3.32 ± 0.08	3.37 ± 0.05	2.89 ± 0.05^{ab}	3.02 ± 0.05^{b}
HOMA-IR	5.35 ± 0.46	3.49 ± 0.29^{a}	11.35 ± 0.98^{ab}	4.31 ± 0.79^{ac}
HOMA-β	60.01 ± 4.21	40.7 ± 3.3^{a}	129.3 ± 29.2^{ab}	56.8 ± 9.4^{c}
LISI	3.24 ± 0.31	5.33 ± 0.39^{a}	1.99 ± 0.19^{ab}	4.71 ± 0.59^{ac}

Values are means ± SEM ($n = 8$ rats per group). a, $P < 0.05$ vs. C values; b, $P < 0.05$ vs. C-NAC values; c, $P < 0.05$ vs. MSG values. Data were analyzed by ANOVA, followed by Tukey's multiple comparisons test Brown–Forsythe test, and Bartlett's test was used to assess normality distribution and variance homogeneity. LISI: Liver Index of Insulin Sensitivity.

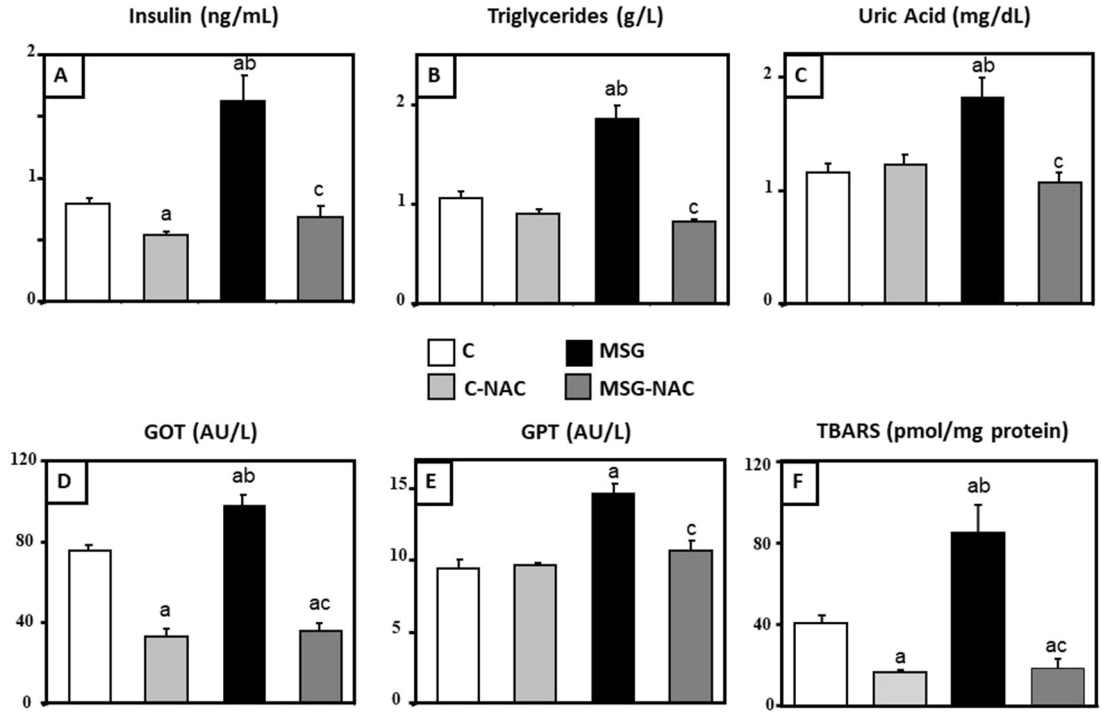

Fig. 1. Circulating concentrations of metabolites in adult normal (Control) and hypothalamic obese (MSG) rats. Control (white bars), C-NAC (pale gray bars), MSG (black bars) and MSG-NAC (dark gray bars) rats. Values are means ± SEM (n = 8 rats per group).a, $P < 0.05$ vs. C values; b, $P < 0.05$ vs. C-NAC values; c, $P < 0.05$ vs. MSG values. Data were analyzed by ANOVA, followed by Tukey's multiple comparisons test Brown–Forsythe test and Bartlett's test were used to assess normality distribution and variance homogeneity.

concentrations, thus reaching values similar to those displayed by C rats (Fig. 1, panel A). Interestingly, NAC administration was also efficient to significantly ($P < 0.05$) reduce plasma insulin concentrations in C rats to values even lower than those displayed by NAC-untreated C rats (Fig. 1, panel A). Consequently, the high HOMA-IR and HOMA-β values and the reduced LISI values ($P < 0.05$ vs. C rats) displayed by MSG rats (Table 2) were all fully abrogated when treating MSG rats with NAC (Table 2). Moreover, NAC treatment in C rats significantly ($P < 0.05$ vs. C) improved all three insulin indexes (see also Table 2).

The peripheral triglyceride (Fig. 1, panel B) and UA (Fig. 1, panel C) levels in MSG animals were significantly ($P < 0.05$ vs. C values) higher than those found in C rats. Interestingly, NAC treatment in MSG rats fully ($P < 0.05$ vs. MSG rats) prevented the above mentioned increments (Fig. 1, panels B and C, respectively). Similarly, the circulating levels of GOT (Fig. 1, panel D) and GPT (Fig. 1, panel E) were significantly ($P < 0.05$ vs. C) higher in MSG animals, and NAC treatment in MSG rats abolished ($P < 0.05$ vs. MSG rats) such increases (Fig. 1, panels D and E, respectively). Moreover, NAC administration in C rats even significantly ($P < 0.05$ vs. C) reduced circulating GOT concentrations (Fig. 1, panel D).

Finally, the elevated circulating TBARS levels, a key marker of peripheral OS, were blunted by NAC treatment in both MSG ($P < 0.05$ vs. MSG rats) and C ($P < 0.05$ vs. C rats) rats (Fig. 1, panel F).

The partial analysis of the liver insulin-signaling pathway, as indicated by P-AKT:AKT and P-eNOS:eNOS proteins ratios, indicated that the first one was significantly ($P < 0.05$ vs. C) reduced in MSG rats (Fig. 2, panel A). Conversely, the second one was similar in both groups (C and MSG) of NAC-untreated rats (Fig. 2, panel B). Interestingly, chronic NAC treatment was highly effective to significantly ($P < 0.05$ vs. C and MSG, respectively) enhance both proteins ratios, regardless of the experimental group examined (Fig. 2, panels A and B, respectively).

3.2. Preventive efficacy of NAC treatment on the development of liver oxidative stress, inflammation and dysmetabolism in hypothalamic obese rats

Concordant with the enhanced peripheral OS found in MSG rats, liver content of protein carbonyl groups was also significantly ($P < 0.05$ vs. C) higher in these rats (Fig. 3, Panel A). Whereas GSH, a peptide highly protective against OS development, content was significantly ($P < 0.05$ vs. C) lower in MSG rats (Fig. 3, panel B). Also, similarly to that accounted for hepatic carbonyl groups, liver protein content of iNOS was significantly ($P < 0.05$ vs. C values) higher in MSG animals (Fig. 3, panel C). Once again, chronic NAC administration in MSG rats fully prevented such derangements, indeed antioxidant treatment was able to significantly ($P < 0.05$ vs. MSG) reduce in liver both carbonyl groups and iNOS protein content (Fig. 3, panels A and C, respectively), while enhancing local tissue GSH content (Fig. 3, panel B).

Neonatal MSG treatment resulted in rats with deeply inflamed livers if compared with C tissues. In fact, MSG animals displayed a significant ($P < 0.05$ vs. C values) increase in the local tissue mRNA levels of *Il1b*, and *PAI1* (Fig. 4, panels A and B, respectively), with only just a trend in same direction for those of *TNFa* (Fig. 4, panel C). Of relevance, treating MSG rats with NAC those increases were fully abrogated ($P < 0.05$ vs. MSG values). Similarly, liver protein content of TNFα and COX-2 were significantly ($P < 0.05$ vs. C values) increased in MSG animals (Fig. 4, panels D and E, respectively), and the enhancement in both parameters was efficiently prevented ($P < 0.05$ vs. MSG values) by chronic oral NAC therapy (Fig. 4, panels D and E, respectively).

Regarding liver carbohydrate metabolism, GCK activity (the hepatic glucose sensor) was significantly ($P < 0.05$ vs. C) higher in MSG tissues and such an increase resulted fully ($P < 0.05$ vs. MSG values) prevented by chronic NAC therapy (Fig. 5, panel A). Conversely, hypertriglyceridemic MSG rats have developed a drastic inhibition of KHK activity ($P < 0.05$ vs. C) and NAC treatment was also effective to counteract ($P < 0.05$ vs. MSG values) such a low value in these rats (Fig. 5, panel B). As for liver lipid metabolism, lipogenesis-related genes

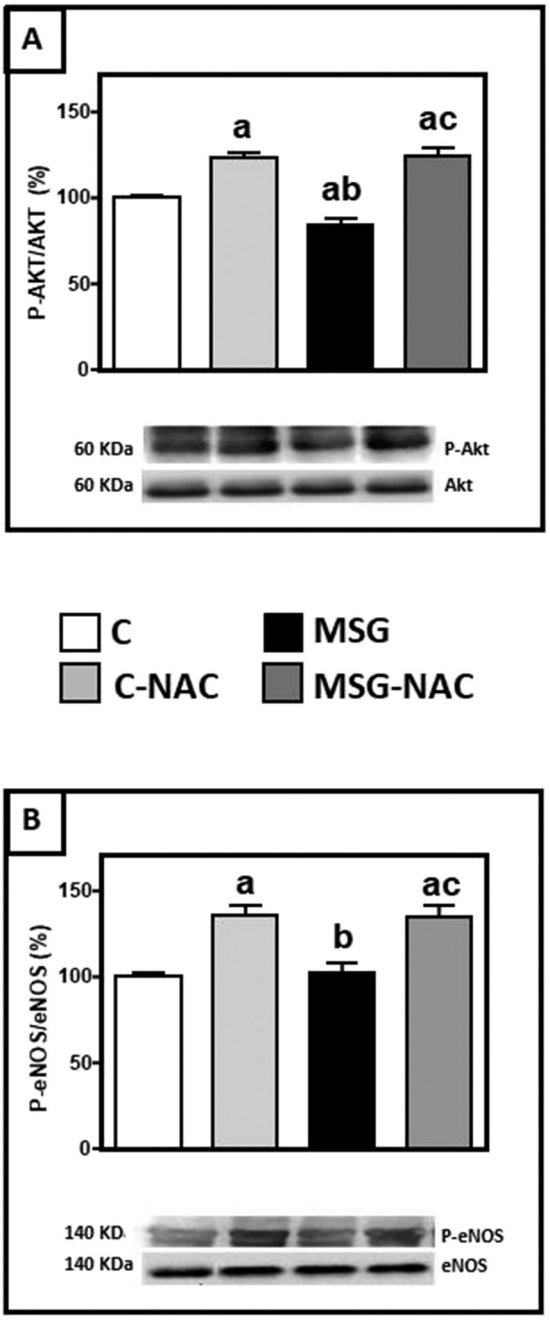

Fig. 2. Liver P-eNOS:eNOS and P-AKT:AKT protein content ratios (panels A and B, respectively) in Control (white bars), C-NAC (pale gray bars), MSG (black bars) and MSG-NAC (dark gray bars) rats. Inserts are respective representative Western blots. Results are means ± SEM (n = 8 rats per group). a, $P < 0.05$ vs. C values; b, $P < 0.05$ vs. C-NAC values; c, $P < 0.05$ vs. MSG values. Data were analyzed by ANOVA, followed by Tukey's multiple comparisons test Brown–Forsythe test and Bartlett's test were used to assess normality distribution and variance homogeneity.

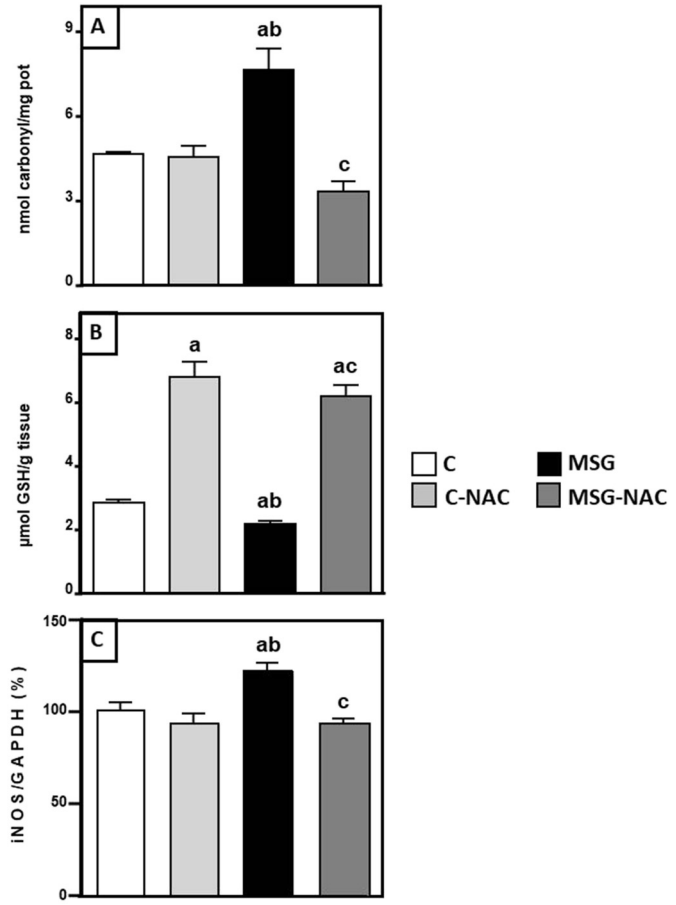

Fig. 3. Liver oxidative stress markers: carbonyl groups (panel A), reduced glutation (GSH) (panel B) and protein iNOS (panel C) in C (white bars), C-NAC (pale gray bars), MSG (black bars) and MSG-NAC (dark gray bars) rats. Values are means ± SEM (n = 8 rats per group). a, $P < 0.05$ vs. C values; b, $P < 0.05$ vs. C-NAC values; c, $P < 0.05$ vs. MSG values. Data were analyzed by ANOVA, followed by Tukey's multiple comparisons test Brown–Forsythe test and Bartlett's test were used to assess normality distribution and variance homogeneity.

expressions were also increased in MSG rats. Indeed, hepatic mRNA expression levels of *Srebp1c*, *Fas* and *Gpat* were significantly ($P < 0.05$ vs. C) higher (approximately 1.5, 2 and 5 fold, respectively) in MSG tissues (Fig. 5, panels C, D and E, respectively). Being NAC oral administration fully ($P < 0.05$ vs. respective MSG values) efficient to prevent all above mentioned distortions (Fig. 5, panels C-E).

4. Discussion

Adult male, neonatally ARC-damaged, rats are hypophagic, display low BW and endocrine-metabolic dysfunctions, being highly prone to develop hypertrophic hyperadiposity and chronic hyperleptinemia [14], peripheral and tissue inflammation [8,17], enhanced OS [17,44] and hyperuricemia, indicating an enhanced protein catabolism. All these changes are similar to those occurring in the human Metabolic and Cushing's Syndromes [45,46]. We now have addressed that several endocrine-metabolic disturbances and hepatic dysfunction developed by MSG rats can be fully prevented by chronic treatment with a daily-oral low antioxidant (NAC) dose suggesting that these dysfunctions are clearly dependent on an increased OS endogenous environment.

It is known that enhanced endogenous OS is a key factor for inducing IR [22,42]. High HOMAs scores (and low LISI score), like those recorded in our study, are closely related to enhanced pancreatic B cell function and, as a consequence, compensatory hyperinsulinemia is installed. In addition, we currently report in the MSG liver two distorted main steps downstream the insulin-signaling pathway (the P-AKT:AKT and P-eNOS:eNOS proteins ratios), being both crucial to physiologically avoid inflammation (Fig. 6). Our experiments shown that these altered pathways were effectively corrected by the early starting (weaning age) with antioxidant (NAC) therapy, thus strongly suggesting that poor MSG insulin signaling function seems to be dependent on a high OS endogenous environment. These results are in line with those studies showing that high fat [47] or carbohydrate [22,42] diet intake by rodents results in an increase in OS and IR, a dysfunction effectively corrected by the co-administration with other antioxidants, such as

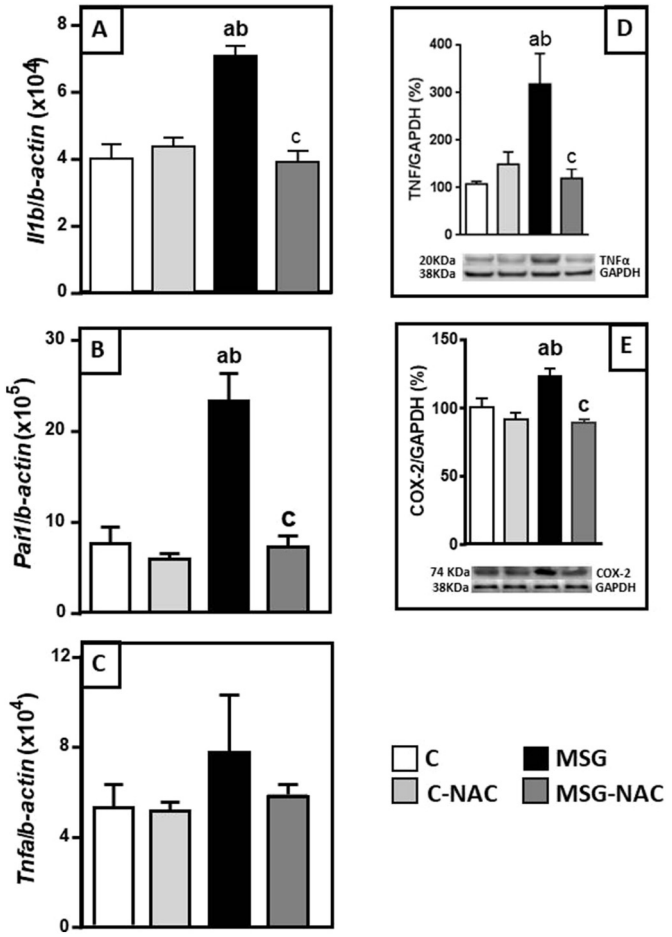

Fig. 4. Liver mRNA levels of *Il1b*, *Pai-1 and Tnfa* (panels A, B and C, respectively) and, TNF*a* and COX-2 protein content (panels D and E, respectively) in C (white bars), C-NAC (pale gray bars), MSG (black bars) and MSG-NAC (dark gray bars) rats. Inserts are respective representative Western blots. Values are means ± SEM (n = 8 rats per group). a, $P < 0.05$ vs. C values; b, $P < 0.05$ vs. C-NAC values; c, $P < 0.05$ vs. MSG values. Data were analyzed by ANOVA, followed by Tukey's multiple comparisons test Brown–Forsythe test and Bartlett's test were used to assess normality distribution and variance homogeneity.

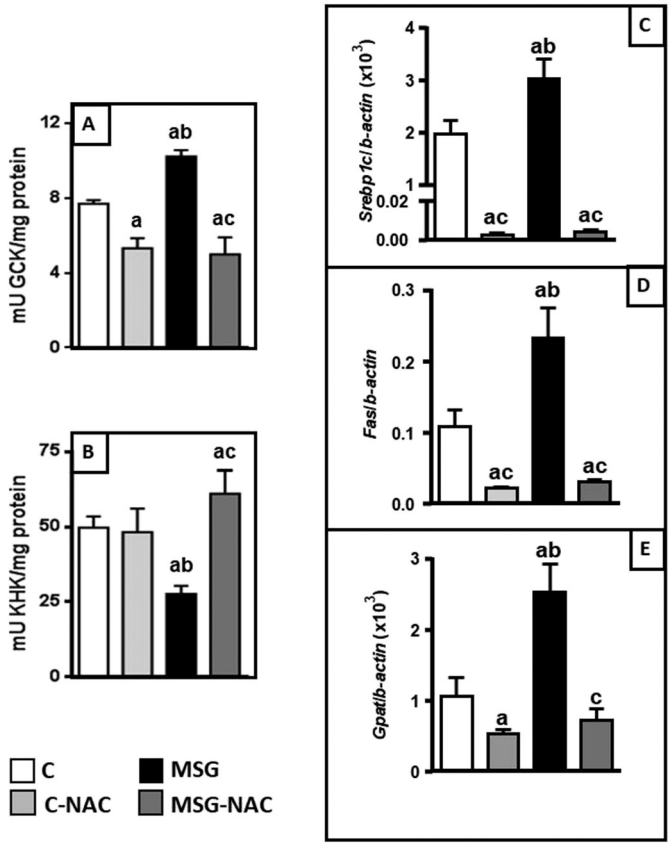

Fig. 5. Hepatic activity levels of GCK (panel A) and KHK (panel B) and, *Srebp1c* (panel C), *Fas* (panel D) and *Gpat* (panel E) gene expression levels in livers from C (white bars), C-NAC (pale gray bars), MSG (black bars) and MSG-NAC (dark gray bars) rats. Values are means ± SEM (n = 8 rats per group). a, $P < 0.05$ vs. C values; b, $P < 0.05$ vs. C-NAC values; c, $P < 0.05$ vs. MSG values. Data were analyzed by ANOVA, followed by Tukey's multiple comparisons test Brown–Forsythe test and Bartlett's test were used to assess normality distribution and variance homogeneity.

lipoic acid [22] or apocynin [42]. Reciprocally, enhanced eNOS activity has been claimed to prevent IR development [48]. Indeed, Akt-dependent phosphorylation at eNOS Ser residue is important for enhancing NO production [49,50] (see Fig. 6). High-fat diet intake-induced IR mice display diminished Erk1/2 and Akt phosphorylations, whereas eNOS phosphorylation is abolished [50]. Furthermore, defective PI3K/Akt-dependent eNOS phosphorylation surely contributes to impair NO-mediated vasodilatation and anti-inflammatory activity in pathological conditions, such as IR [39,51,52]. We currently demonstrated that NAC-induced normalization of defective eNOS phosphorylation, associated to an impaired insulin-signaling pathway function, assured an enhancement in P-eNOS levels to counteract tissue inflammation (Fig. 6). In this regard, previous reports [44,53,54] indicate that OS is a favoring key factor for the establishment and maintenance of an inflammatory state as observed in the periphery and liver of MSG rats. One distorted key marker found in our MSG rats was an enhanced liver COX2 protein content, a key enzyme involved in prostaglandin production and closely related to both OS- and inflammation-related mechanisms [55]. Additionally, other liver OS markers, such as high carbonyl protein groups and iNOS mRNA, as well as low OS-protecting GSH content, characterized MSG rats; thus once again indicating that a local (liver) enhanced OS has been installed in the MSG rat phenotype. It is known that enhanced iNOS production is a key mediator of liver OS development, due to its ability to mediate lipid free radical overproduction and IR [56]. Complementary, reduction in liver iNOS and, in turn, in local OS and hyperlipidemia, restores insulin sensitivity [57]. We currently addressed that NAC treatment efficiently reduced liver iNOS overproduction in hypothalamic obese rats, which in turn could be related to the above mentioned improvement in insulin sensitivity. In addition, the gene expression pattern of other liver pro-inflammatory markers (e.g. increased levels of *TNFα*, *Il1b and Pai-1* mRNAs) is also distorted in MSG animals, thus indicating an evident marker-correlation between local OS and inflamed liver. Nevertheless, as it occurred with peripheral TBARS levels, we were able to demonstrate that the overall disrupted liver OS pattern and its inflammation can be fully abrogated by NAC co-administration to MSG rats. Moreover, NAC is recognized as an enhancer of liver GSH production [58], a fact clearly noticed in our study; indeed, NAC therapy increased 2-fold (approximately) MSG liver GSH content.

Regarding liver carbohydrate metabolism, increased GCK activity, a key liver glucose sensor, could be dependent on local IR. As a consequence, impairment in insulin-dependent liver glucose influx should be expected, although this effect still remains fully compensated (pre-diabetic state). The restore of GCK activity to normal levels once NAC treatment was introduced could be suggesting, although some speculative, a novel OS sensor role of the enzyme. Additionally to GCK-dependent carbons supply, KHK activity independently brings carbons for triglyceride synthesis [59,60]. Having in mind that MSG rats are hypertriglyceridemic, a reduced liver KHK activity could cooperate against liver steatosis, thus resulting in ectopic lipid deposition at a

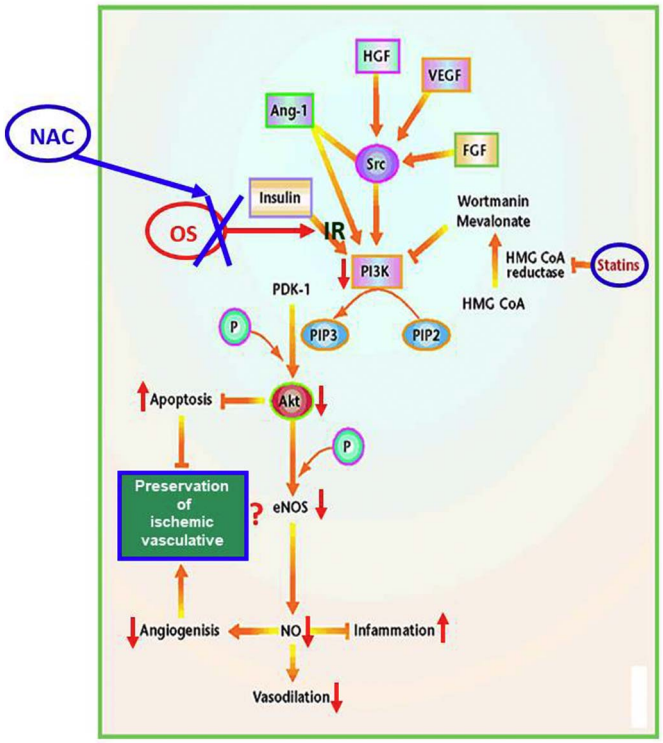

Fig. 6. Diagram displaying main steps downstream intra-target cell insulin signaling and consequent dysfunctions in an oxidative stress (OS)-reach endogenous environment inducing the development of insulin resistance (IR) (consequences are indicated in red arrows). Abbreviations PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B; PDK-1: phosphoinositide-dependent kinase-1; PIP2: phosphatidylinositol diphosphate; PIP3: phosphatidylinositol triphosphate; eNOS: constituve nitric oxide synthase; NO: nitric oxide. Orange arrows indicate stimulation, orange truncated-lines indicate inhibition, statins facilitate NO production. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

level different from the liver (e.g. adipose tissue). Interestingly, we have demonstrated without any doubt that NAC treatment fully normalized liver KHK activity in MSG rats.

As presently found, elevated MSG liver gene expression of *Srebp1c*, a master regulator of the lipogenic process, and those of its target genes, *Fas* and *Gpat* [61], also support a liver lipid metabolism clearly displaced to enhanced lipogenesis, thus resulting in a high peripheral concentration of triglycerides. Interestingly, NAC treatment in MSG animals resulted in the restoration of normal triglyceridemia and liver expression of the above mentioned gene-markers, and thus it is plausible to expect that lipid dysmetabolism could also be dependent on OS-induced IR in hypothalamic obese rats.

Other authors have demonstrated that NAC therapy effectively modulates adipogenesis [32,62–64] and inhibits adipocyte lipid accumulation [65]. Thus previous observations and present data strongly support for an overall beneficial NAC effect on liver lipogenesis. Thus, NAC seems to be efficiently acting not only on lipogenic genes but also, although through a different mechanism, in preventing GCK/KHK activity-derangement and dyslipidemia development.

5. Conclusions

We currently demonstrated that reducing an enhanced endogenous OS environment in MSG rats by antioxidant (NAC) therapy, the development of pre-diabetes, IR, dyslipidemia and inflamed-dysfunctional liver can be fully overridden. The pathogenic integrative mechanism displayed by MSG obese rats seems to involve an enhancement in endogenous OS that, in turn, impairs downstream insulin signaling pathway, by reducing Akt production and thus lowering eNOS activity. Low NO could then induce the distortion of various relevant physiological processes, among them: a) enhanced cell apoptosis (dependent on diminished Akt signaling), b) increased inflammation, and c) reduced vasodilatation and angiogenesis. The final result is, in combination with other pro-inflammatory adipocytokines released by MSG hypertrophic white adipocytes [66], an overall enhanced risk for the development of several co-morbidities, such as a compromising prothrombotic-inflammatory state and, consequently cardiovascular disease. Of relevance, antioxidant treatment (e.g. NAC) could be applied as adjuvant therapy to that of statin administration [13,67] (Fig. 6) in order to highly prevent/improve co-morbidities developed by individuals diagnosed with Metabolic Syndrome or Cushing's Syndrome.

Conflict of interests

The authors declare that there are no conflicts of interest.

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