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

Hernán Gonzalo Villagarcía[a], María Cecilia Castro[a], Luisa González Arbelaez[b], Guillermo Schinella[c], María Laura Massa[a], Eduardo Spinedi[a], Flavio Francini[a,*]

[a] CENEXA (Centro de Endocrinología Experimental y Aplicada; UNLP-CONICET-FCM), CEAS-CICPBA, Argentina
[b] CIC (Centro de Investigaciones Cardiovasculares; UNLP-CONICET-FCM), Argentina
[c] Cátedra Farmacología Básica, Facultad de Ciencias Médicas UNLP and CICPBA, 1900 La Plata, Argentina

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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 prediabetic (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],

* Corresponding author at: CENEXA, (UNLP-CONICET-FCM), 1900 La Plata, Argentina.
E-mail address: f_francini@yahoo.com (F. Francini).

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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 $MgCl_2$, 0.2 mM dNTPs and 0.15 μL Platinum Taq DNA polymerase (6 U/μL) (Invitrogen).

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