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Hepatic gene expression profile associated with non-alcoholic steatohepatitis protection by *S*-nitroso-*N*-acetylcysteine in ob/ob mice ☆,☆☆

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***Background/Aims*: To understand the molecular mechanisms underlying non-alcoholic steatohepatitis (NASH) prevention by *S*-nitroso-*N*-acetylcysteine (SNAC), an NO donor that inhibits lipid peroxidation, we examined hepatic differentially expressed genes between ob/ob mice receiving or not SNAC treatment concomitantly with a methionine–choline deficient (MCD) diet.**

***Methods*: Ob/ob mice were assigned to receive oral SNAC fed solution (MCD+SNAC group) or vehicle (MCD group) by gavage. After four weeks, histopathological analysis and microarray hybridizations were conducted in liver tissues from groups. GeneSifter® system was used to identify differentially expressed genes and pathways according to Gene Ontology.**

***Results*: NASH was absent in the MCD+SNAC group and no significant changes in food intake or body weight were observed in comparison to MCD group. After SNAC treatment, several genes belonging to oxidative phosphorylation, fatty acid biosynthesis, fatty acid metabolism and glutathione metabolism pathways were down-regulated in comparison to the MCD group.**

***Conclusions*: SNAC treatment promotes down regulation of several genes from FA metabolism related pathways, possibly through abrogation of the cytotoxic effects of reactive oxygen species and lipid peroxides with consequent prevention of mitochondrial overload. Further studies are required to investigate the clinical implications of these findings, in attempt to develop novel therapeutic strategies for NAFLD treatment.**

***Keywords*: Nitric Oxide; *S*-Nitroso-*N*-acetylcysteine; Hepatic gene expression; Non-alcoholic steatohepatitis**

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Abbreviations: NAFLD, Non-alcoholic fatty liver disease; NASH, steatohepatitis; FA, fatty acid; SNAC, *S*-nitroso-*N*-acetylcysteine; MCD, methionine–choline deficient diet.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses the whole spectrum of fatty liver, including non-alcoholic steatosis, steatohepatitis (NASH) and NASH-related cirrhosis [1]. Although major advances have been made in understanding the physiologic characteristics of NAFLD, the pathogenesis of this condition and its progression to fibrosis remains to be clearly defined [2–5]. Insulin resistance, mitochondrial dysfunction and oxidative stress seem to play important roles. Increased hepatic fatty acid (FA) synthesis secondary to elevated plasma glucose and/or insulin levels and increased hepatic uptake of FA resulting from peripheral lipolysis favors fat deposits in the presence of insulin resistance. Teleologically, in an attempt to limit additional lipid accumulation, a new steady state is achieved with a compensatory increment in hepatic removal of lipids through mitochondrial oxidation of FA [6], as evident in ob/ob mice [7] and in patients with NASH [8]. Increased oxidation of FA augments the formation of NADH and $FADH_2$, thus delivering more electrons to the respiratory chain which, concomitantly to a decreased activity of respiratory chain complexes [9,10], results in increased mitochondrial reactive oxygen species (ROS) generation and lipid peroxidation. As a result, a vicious cycle is triggered, which further increases mitochondrial ROS formation [6].

Nitric oxide (NO) can act as a potent inhibitor of the lipid peroxidation chain reaction by scavenging propagatory lipid peroxyl radicals, and by inhibiting many potential initiators of lipid peroxidation, such as peroxidase enzymes [11]. However, in the presence of superoxide $O_2^{\cdot -}$, NO forms peroxynitrite ($OONO^-$), a powerful oxidant, which is able to initiate lipid peroxidation [12]. Thus, the balance between NO and ($O_2^{\cdot -}$) may have important implications in NAFLD, where oxidative stress seems to have a pivotal role in the onset and/or progression of the disease [12,13]. *S*-nitroso-*N*-acetylcysteine (SNAC) is a *S*-nitrosothiol (RSNO) that is considered to be an endogenous NO carrier and donors in mammals [14]. A previous study has shown that treatment with SNAC, a potent inhibitor of lipid peroxidation, prevented the rise of hydroperoxides in liver homogenates and NAFLD induced by a choline deficient diet in rats [15].

In the present study, we demonstrate that steatohepatitis induced by a methionine–choline deficient diet in ob/ob mice can also be completely prevented by treatment with SNAC and we used microarray analysis of gene expression profiling to identify mechanisms potentially responsible for the observed effects.

2. Materials and methods

2.1. SNAC synthesis

SNAC was synthesized through the S-nitrosation of *N*-acetyl-L-cysteine (Sigma Chemical, St. Louis, MO, USA) in an acidified sodium nitrite solution [16]. Stock SNAC solutions were further diluted in PBS. Solutions were diluted to 2.4×10^{-4} mol/L in PBS (pH 7.4) before administration. All the experiments were carried out using analytical grade water from a Millipore Milli-Q Gradient filtration system.

2.2. Animals

Male ob/ob mice (Jackson Laboratories, Bar Harbor, ME, USA), eight-weeks-old, weighting 30–40 g, were housed in temperature and humidity controlled rooms, kept on a 12 h light/dark cycle and provided unrestricted amounts of food and water. All procedures for animal experimentation were in accordance to the Helsinki Declaration of 1975, (NIH Publication No. 85-23, revised 1996) and the Guidelines of Animal Experimentation from the University of São Paulo School of Medicine. Ob/ob mice [Control group ($n = 6$)] were submitted to a standard diet (Nuvilab® Nutrientes Ltda, Colombo, Brazil) *ad libitum*. NASH was induced in the ob/ob mice by methionine–choline deficient [MCD group ($n = 6$)] (produced in house: carbohydrate – 62,5% with starch and sucrose; protein – 17% with casein without methionine–choline; lipid – 7% with soybean oil; AIN-93M vitamin mix – 1%; AIN-93M mineral mix – 3.5%) 5 g/day for each ob/ob mice for four weeks. Concomitantly, ob/ob mice were randomly assigned to receive oral SNAC fed solution (1.4 μmol/kg) [MCD+SNAC group ($n = 6$)] or vehicle (physiologic Ringer's solution) in MCD group, both daily by gavage. After four weeks of treatment with SNAC or vehicle associated with MCD diet, ob/ob mice were sacrificed (12-weeks-old) and their livers were collected for mRNA isolation and histological examination.

2.3. Biochemical and histopathological analysis

Serum alanine amininotransferase (AST), aspartate aminotransferase (ALT), cholesterol and triglycerides levels were analyzed by standard methods using automated techniques.

Fragments of liver tissues were previously fixed by immersion in formaldehyde saline (4%) solution and processed for hematoxylin–eosin (HE) and Masson Trichrome stains for histological analysis. Histological variables were blindly semi-quantitated from 0 to 4+ with respect to: both macro and microvacuolar fatty change (0: <5% hepatocytes involved; 1: 6–25%; 2: 26–50%; 3: 51–75%; 4: 76–100%) and its zonal distribution, lobular inflammatory changes (0: absent; 1: minimal; 2: mild; 3: moderate; 4: severe); hepatocyte ballooning (0: absent; 1: minimal; 2: mild; 3: moderate; 4: severe); fibrosis (0: absent; 1: only perisinusoidal fibrosis, no septa; 2: some septa, no nodule; 3: frequent septa with incipient nodule formation; 4: cirrhosis).

2.4. Microarray assay

Gene expression profiles liver tissue from ob/ob mice from the Control group, MCD group and MCD+SNAC group were compared by microarray analysis using the CodeLink™ UniSet Mouse 20K I Bioarray (GE Healthcare Bio-Sciences, Chalfont St. Giles, UK). Two biological replicate were prepared for each experimental group. This platform consists of 19,801 probes as well as 300 negative controls, 108 positive control probes and 100 housekeeping genes on a single slide. Total RNA from liver tissues was prepared using TRIzol® reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to standard protocols provided by the manufacturer.

For each CodeLink™ Bioarray, double-stranded cDNA and subsequently cRNA was synthesized from 2 μg of total RNA using the CodeLink™ Expression Assay Kit (GE Healthcare Bio-Sciences) according to manufacturer's instructions. Briefly, cRNA was prepared by *in vitro* transcription using a single labeled nucleotide (biotin-11-UTP) in the IVT reaction. The labeled cRNA was then purified using