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Proteomic approach to identify molecular signatures during experimental hepatic fibrosis and resveratrol supplementation☆,☆☆

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ABSTRACT

We document the changes in sera and liver proteome during experimental hepatic fibrosis (HF) and its protection by resveratrol (Rsvtrl) in rats. HF was induced by *N*′-Nitrosodimethylamine (NDMA) administration thrice a week for 21-days. Compared to the control group, significant ($P < 0.05$) elevations in ALT, AST, ALP, γGT and bilirubin occurred during fibrosis. H&E, IHC for α-SMA and reticulin stainings of liver demonstrated that collagen amassing and HF were concurrent. Rsvtrl-supplementation refurbished hepatic architecture and lessened collagen deposition. 2DE proteome analyses showed ~398 (pH, 3–10) and ~129 signatures (pH, 4–7) within 5–201 kDa range in liver and sera, respectively. Nearly, 45 spots in liver and 18 in sera were differentially expressed in fibrotic and Rsvtrl-supplemented animals. MALDI—TOF–MS/MS analysis revealed at least ten signatures as the potential biomarkers for HF, because of the significant changes (>2 fold) in their expression. Out of them, two signatures have not so far been characterized and we have accomplished it in this study. Our proteomics data provides new evidence on an overall involvement of carbohydrate, lipid, Ca^{2+} signaling and oxidative pathways during NDMA-induced HF. More importantly, the oxidative pathway appears to be the principal mediator in the NDMA-induced HF as well as the hepato-protection by Rsvtrl.

1. Introduction

Rodent model for liver fibrosis, usually generated by bile duct ligation (BDL) or administration of environmental toxicants like *N*′-Nitrosodimethylamine (NDMA), *N*′-Nitrosodiethylamine (NDEA), carbon tetrachloride (CCl_4) or thioacetamide (TAA) are among the most commonly studied fibrosis models [1–5]. Besides, numerous other causes such as viral/protozoal insult, alcohol abuse, autoimmune diseases *etc.* [6] may also contribute to the generation of fibrosis. During the present study, NDMA was used to induce liver fibrosis in rats within a short span of 21 days in the animals exposed to its repeated lower doses, with a histological appearance that perfectly imitates liver fibrosis in human beings. The metabolism of NDMA proceeds through the intermediate radical [$CH_3(CH_2•)NBN{=}O$] generation by the action of cytochrome CYP2E1-dependent oxidases, either *via* α-hydroxylation or denitrosation of the nitrosamine [7,8]. During α-hydroxylation pathway, the hydroxymethylnitrosamine ($HOCH_2CH_3HBN{=}O$) decomposes to formaldehyde and monomethylnitrosamine ($CH_3NHN{=}O$) which undergo rearrangement and form methyldiazonium ion ($CH_3N^+{\equiv}N$). Methyldiazonium alkylates biological macromolecules like DNA, RNA and proteins. On the other hand, metabolic translation of the intermediate radical *via* denitrosation leads to the formation of methylamine (CH_3NH_2) and formaldehyde. α-Hydroxylation pathway is believed to form the active metabolites responsible for the genotoxic and carcinogenic potential of NDMA [7]. Moreover, NDMA biotransformation stimulates generation of free radicals and reactive oxygen species (ROS) by hepatocyte, Kupffer cells and neutrophils which further enhance the oxidative damage to contribute hepatic fibrosis [9,10]. The net consequence of these changes is transdifferentiation of quiescent hepatic stellate cells (HSCs) into myofibroblast-like activated HSCs. The highly proliferative activated HSCs are characterized by the loss of vitamin-A droplets and the release of proinflammatory, profibrogenic and promitogenic cytokines such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), endothelin-1 (ET-1) and interferon-γ (INF-γ) [11]. The characteristic features of liver fibrosis

Abbreviations: NDMA, *N*′-Nitrosodimethylamine; Rsvtrl, resveratrol; LFT, liver function test; H&E, hematoxylin and eosin; IHC, immunohistochemistry; ALP, alkaline phosphatase; AST, aspartate aminotransferases; ALT, alanine aminotransferases; TBil, total bilirubin; α-SMA, α-smooth muscle actin; 2DE, 2 dimensional gel electrophoresis; MALDI—TOF–MS/MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; HSCs, hepatic stellate cells; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; ET-1, endothelin-1; INF-γ, interferon-γ; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; PMF, Peptide Mass Fingerprint; kDa, kilodalton; GST, glutathione-*S*-transferase; CA-III, carbonic anhydrase-III; RGN, Regucalcin; IMPase-1, inositol monophosphatase-1.

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are irregular proliferation and deposition of types-I, -III and -IV collagen-rich tough fibrous connective tissue, which create an imbalance between synthesis and degradation of extracellular matrix (ECM) within liver [1,12–14]. The details of underlying biochemical and molecular mechanisms and integrated pathway of NDMA-induced hepatic fibrosis are yet to be elucidated.

Owing to the significant role of ROS in liver diseases, increased levels of intracellular antioxidants should be one of the options in any strategy to avert liver damage. In the present study, we have examined the hepatoprotective potential of resveratrol which reportedly possesses anti-inflammatory, anti-cancerous/pro-apoptotic, anti-microbial and anti-oxidative properties [15–20]. Literature also suggests that the hydrogen electron donation from hydroxyl group of Rsvtrl counters several types of oxidative damage [21]. The *in vivo* use of Rsvtrl as a restorative medicine has always been a subject of research against different ailments including hepatic disorders [22]. A number of reports are available indicating remedial role of Rsvtrl in non-alcoholic fatty liver diseases (NAFLD), heart diseases, neurological disorders, diabetes and obesity [23,24]. While a definite progress has been made in researching friendly alternatives to treat liver fibrosis, the molecular mechanism of action of many of them, in general, and resveratrol in particular, is yet to be explained in aggressive fibrotic process.

Broadly, the purpose of present study was to measure and identify changes in the serum and liver proteomic profiles during fulminant stages of liver fibrosis caused by NDMA and subsequent recovery due to resveratrol treatment. In addition to biochemical markers which reflect changes in metabolism, we also compared differentially expressed proteins in the liver and sera proteome in NDMA-induced fibrotic rats with those which receive Rsvtrl supplement. For identifying differentially expressed proteomic markers, two-dimensional electrophoresis (2-DE) coupled with mass spectrometric analysis (MS), has gained wide recognition [25]. It has quality attributes of precision and rapid analysis when integrated with data obtained by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) [26]. Considering the scarcity of literature, it is realized that further studies shall be carried out to provide a better understanding of underlying mechanism of hepatic fibrosis by way of identifying differentially expressed protein candidates for predicting hepatic fibrosis [27]. Identifying changes in a larger perspective of protein expression of serum and liver will strengthen our understanding of NDMA-induced hepatic fibrosis and ameliorative potential of resveratrol in animal models with possible relevance to human exposure.

2. Materials and methods

2.1. Chemicals and reagents

N′-Nitrosodimethylamine (NDMA), resveratrol, acrylamide, bis-acrylamide, ammonium persulphate (APS) and TEMED were purchased from Sigma-Aldrich. Hematoxylin and eosin stains were obtained from SRL, India. α-Smooth muscle actin (α-SMA) antibodies and goat anti-mouse IgG-HRP conjugated antibodies were procured from Trends Bio-product Pvt. India and CALTAG laboratories, Bangkok respectively. All the other chemicals, reagents and salts used were of analytical grade.

2.2. Animals

Adult male albino rats (*Rattus norvegicus*) of Wistar strain between 6 and 8 weeks (160 ± 10 g), were utilized in the present study. The experimental animals were housed in sterilized polycarbonate cages under suitable hygienic conditions with 1:1 light:dark exposure. The animals were divided into different groups and fed regularly with sterilized diet and water *ad libitum*. Animal care and experiments were done as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The study synopsis was approved by the Board of Studies of the Department of Zoology (BOSDOZ) and finally recommended by the Institutional Committee for Advanced Studies and Research (CASR) of the University (No. 09-phd-L73).

2.3. Treatment design

After acclimatization for a week, the animals were finally categorized in three different groups of ten each (n = 10). Animals belonging to Group-I marked as saline control and received sterile normal saline (10 mL kg^{-1} body weight i.p.); rats belonging to Group-II served as resveratrol (Rsvtrl) control and these animals received 10 mg kg^{-1} body weight; experimental animals of Group-III were administered NDMA (10 μL diluted to 1 mL with 0.15 M sterile NaCl) in doses of 10 mg kg^{-1} body weight (i.p.), while the Group-IV rats were given resveratrol (10 mg kg^{-1} body weight) after 2 h lag of administering NDMA. The dose of resveratrol was narrowed down on the basis of previously published reports [18,28,29] and some preliminary experiments. The 2 h interval of resveratrol administration was based on few preliminary experiments and the published report [5]. The rats were given three injections of NDMA on three successive days/week for 21 days to generate hepatic fibrosis, whereas NDMA was replaced with saline (saline control) or Rsvtrl (Rsvtrl control and Rsvtrl-supplemented) in other groups, as described earlier [2,12,15]. Animals belonging to different groups were anaesthetized and sacrificed on day-21 from the start of treatment.

2.4. Sera collection and preparation

It was carried out according to the protocol described earlier [15]. Briefly, from the anesthetized rats belonging to different groups blood was collected in sterilized glass tubes on weekly basis. To obtain clear sera, blood was allowed to clot at room temperature for about an hour and centrifuged at 300 rpm under cooling for 10 min. As the pale yellow colored supernatant, sera was collected (20 μL aliquotes) in fresh microfuge tubes and analyzed freshly or stored at −20 °C for further biochemical investigations.

2.5. Clinical pathology

The activities of marker enzymes of liver function (LFT) were determined in the sera of treated and control group of animals. Parameters of blood biochemistry like sera alkaline phosphatase (SALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and bilirubin were quantitatively analyzed using commercial kits (Erba Diagnostics Mannheim Gmbh, Mumbai, India and AutoZyme, Accurex Biomedical Pvt. Ltd., Mumbai, India).

Hydroxyproline (HP) levels were estimated in sera of control and treated group of rats essentially by the protocol of Woessner [30]. Amount of glycogen in the liver samples of all the treated categories was estimated following the procedure of Vies [31]. In brief, 2 mL of liver extract was mixed with potassium hydroxide (10 N) and incubated in boiling water bath for an hour. Upon cooling, 1 mL of acetic acid was added to neutralize the alkalinity in the contents. Color was developed by adding ~4 mL of freshly prepared anthrone reagent (0.2% w/v in 95% H_2SO_4) in 2 mL neutralized solution following incubation in boiling water bath for 10–15 min. Optical densities were read at 650 nm and the glycogen levels were extrapolated from the known glucose standards.

2.6. Histopathology and immunohistochemical localization of α-smooth muscle actin (α-SMA)

The standard protocol of Ahmad et al. [2] was followed for histopathology. The degree of liver fibrosis was monitored by routinely performed hematoxylin and eosin (H&E), reticulin stainings and immunohistochemical staining (IHC) of α-SMA. For all types of