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Hepatocyte or serum albumin protein carbonylation by oxidized fructose metabolites: Glyceraldehyde or glycolaldehyde as endogenous toxins?

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ABSTRACT

Excessive sugar intake in animal models may cause tissue damage associated with oxidative and carbonyl stress cytotoxicity as well as inflammation. Fructose became a 100-fold more cytotoxic if hepatocytes were exposed to a non-toxic infusion of H_2O_2 so as to simulate H_2O_2 released by Kupffer cells or infiltrating immune cells. In order to determine the molecular mechanisms involved, protein carbonylation of fructose and its metabolites were determined using the 2,4-dinitrophenylhydrazine method. In a cell-free system, fructose was found to carbonylate bovine serum albumin (BSA) only if low concentrations of FeII/H_2O_2 were added. Protein carbonylation by the fructose metabolites glyceraldehyde or glycolaldehyde was also markedly increased by FeII/H_2O_2. The protein carbonylation may be attributed to glyoxal formation by hydroxyl radicals as the glyoxal trapping agent aminoguanidine or hydroxyl radical scavengers prevented protein carbonylation. Glyoxal was also much more effective than other carbonyls at causing protein carbonylation. When BSA was replaced by isolated rat hepatocytes, fructose metabolite glyceraldehyde in the presence of non-toxic 2 μM FeII:8-hydroxyquinoline (HQ) and a H_2O_2 generating system (glucose/glucose oxidase) markedly increased cytotoxicity, protein carbonylation and reactive oxygen species (ROS)/H_2O_2 formation. Furthermore this was prevented by hydroxyl radical scavengers or aminoguanidine, a glyoxal scavenger. CuII: 8-hydroxyquinoline increased H_2O_2 induced hepatocyte protein carbonylation less but was prevented by aminoguanidine. However, cytotoxicity and protein carbonylation induced by glyceraldehyde/CuII:HQ/H_2O_2 were not affected by hydroxyl radical scavengers. Although fatty liver induced by an excessive sugar diet in animal models has been proposed as the first hit for non-alcoholic steatohepatitis (NASH) we propose that oxidative stress induced by the oxidation of fructose or fructose metabolites catalysed by Fenton FeII/H_2O_2 could be a 'second hit'. A perpetual cycle of oxidative stress in hepatocytes could lead to cytotoxicity and contribute to NASH development.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is associated with obesity and sometimes with increased liver enzyme activity in the plasma (NASH). Whether dietary fructose enriched corn syrup increases NAFLD in the clinic is not known [1]. However mice fed high-fructose corn syrup developed non-alcoholic steatohepatitis (NASH), a necroinflammatory disease that can progress to cirrhosis with an increased risk of hepatocellular carcinoma [2]. Pigs fed a high-fructose atherogenic diet also developed an abnormal liver histology resembling human NASH and severe metabolic syndrome [3]. Fructose enriched diets fed to rats were also found to be much more effective at inducing NASH than rats fed fat-enriched diets. Lobular inflammation was also predominant over portal inflammation which is consistent with human NASH [4].

The molecular mechanisms of fructose induced NASH formation were not known but NASH was associated with an approximately 40% sustained increase in serum advanced glycation end product (AGE)-2 levels associated with glyceraldehyde formation. Four other carbonyl fructose metabolites may also contribute [5,6]. A two hit hypothesis has also been proposed for fructose associated hepatic steatosis progressing to the more serious non-alcoholic steatosis (NASH), with the first hit being hepatic steatosis, and the second hit being inflammation and associated oxidative stress caused by ROS formation [7]. However, the validity of this hypothesis has recently been questioned [8,9].

We have recently developed a hepatocyte inflammation model in which hepatocytes were exposed to a non-toxic H_2O_2 infusion to mimic H_2O_2 released by activated inflammatory cells. Isoniazid is an anti-tuberculosis drug that causes hepatotoxicity in about 20% of patients that is usually associated with an inflammatory response. Hydrazine, the major isoniazid metabolite, became much more toxic to hepatocytes when exposed to non-toxic H_2O_2 [10]. Hydralazine, used for long-term treatment of hypertensive dis-

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orders is associated with some patients developing hepatitis and lupus erythematous, an autoimmune syndrome. Hydralazine was also much more toxic in the hepatocytes inflammation model [11]. Fructose was not toxic towards isolated hepatocytes even at 1.5 M whereas only 12 mM fructose caused 50% cytotoxicity in 2 h when the hepatocytes were exposed to non-toxic low doses of H_2O_2, to model that released by activated neutrophils or macrophages [12,13]. It is not clear whether the marked increase in hepatocyte cytotoxicity is caused by fructose or a fructose oxidation product or a fructose carbonyl metabolite or a metabolite radical oxidation product.

The objective of our research was to determine the molecular basis of fructose cytotoxicity associated with inflammation. In the present study, FeII/H_2O_2 (FeII/EDTA/H_2O_2) was used as an iron mediated Fenton reaction model to form Fenton oxidants including hydroxyl radicals. CuI/H_2O_2 (CuII/ascorbate/H_2O_2) was also used as a copper mediated Fenton reaction model to form these oxidants [14]. It was found that protein carbonylation and cytotoxicity induced by the fructose metabolite was markedly increased in both Fenton models. The fructose metabolite glyceraldehyde also became much more cytotoxic in the presence of non-toxic amounts of FeII/H_2O_2 or CuI/H_2O_2. However hydroxyl radical scavengers inhibited FeII/H_2O_2 but not CuI/H_2O_2 catalysed glyceraldehyde cytotoxicity.

2. Materials and methods

2.1. Chemicals

Fructose was purchased from Alfa Aesar Co. Type II collagenase was purchased from Worthington (Lakewood, NJ). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from Boehringer–Mannheim (Montreal, Canada). BSA (pH 7, ≥98%, A7906), glyceraldehyde, glycolaldehyde, EDTA (ethylenediaminetetraacetic acid) and all other chemicals were of the highest quality commercially available and were purchased from Sigma–Aldrich Canada (Oakville, Ontario).

2.2. Animal treatment and hepatocyte preparation

Sprague–Dawley rats weighing 275–300 g (Charles River Laboratories) were used for experimental purposes. All animal care and experimental procedures were carried out according to the guidelines of the Canadian Council on Animal Care [15]. Rats were housed in ventilated plastic cages over PWI 8–16 hardwood bedding, with 12 air changes per hour, and a 12 h light photoperiod (lights on at 0800 h) and an environmental temperature of 21–23 °C with a 50–60% relative humidity. The animals were fed with a normal standard chow diet and water ad libitum. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described [16]. Isolated hepatocytes (10^6 cells/ml) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in 50 ml round-bottomed flasks continually rotating in a 37 °C water bath, under an atmosphere of 95% O_2 and 5% CO_2. Hepatocytes were allowed to acclimatize for 30 min before the addition of chemicals.

2.3. Hepatocyte inflammation model

A marked increase in hepatotoxin susceptibility was found if the hepatocytes were exposed to the products of activated immune cells such as H_2O_2 generated by their NADPH oxidase activity [10,11]. However, following its addition to hepatocytes, a bolus of H_2O_2 is completely metabolized by catalase within a minute [17]. To avoid rapid metabolism by catalase, a H_2O_2 generating system was used that involved adding glucose 10 mM to the hepatocyte suspension followed by glucose oxidase (0.5 units/ml). This system continuously supplied H_2O_2 over a 3 h period without affecting hepatocyte viability or GSH levels [10].

2.4. Cell viability assay

Hepatocyte viability was assessed microscopically by plasma membrane disruption using the Trypan blue (0.1%, w/v) exclusion test [16]. Hepatocyte viability was determined every 30 min during a 3 h incubation period. Hepatocytes used were 80–90% viable before use. 2 μM ferric chloride ($FeCl_3$) complexed with 8-hydroxyquinoline (HQ) was used to load hepatocytes for 5 min with iron. Whilst 2 μM copper sulphate ($CuSO_4$) complexed with HQ was used to load hepatocytes with copper. The hepatocyte reductases reduced the FeIII moiety to FeII or reduced CuII to CuI [18].

2.5. Determination of BSA and hepatocyte protein carbonylation

In a cell-free assay, FeII/H_2O_2 (FeII/EDTA 200 μM + H_2O_2 1 mM) was used as an iron mediated Fenton system whereas CuI/H_2O_2 (CuII 50 μM + H_2O_2 1 mM + ascorbate 1 mM) was used as a copper mediated Fenton model. The total protein bound carbonyl content of BSA was determined by derivatizing the protein carbonyl adducts with 2,4-dinitrophenylhydrazine (DNPH). BSA (2 mg/ml) was dissolved in 100 mM phosphate buffer (pH 7.4). BSA (500 μl) was incubated for 1 h at room temperature with 500 μl of DNPH (0.1%, w/v) in 2N HCl. 1 ml of TCA (20%, w/v) was added to the suspension to stop the reaction. The sample was centrifuged at 10,000 rpm to obtain the cellular pellet, and the supernatant was removed. DNPH was removed by extracting the pellet three times using 500 μl of ethyl acetate:ethanol (1:1) solution. After the extraction, the pellet was dried under a gentle stream of nitrogen and dissolved in 1 ml of Tris-buffered 8 M guanidine–HCl (pH 7.2). The solubilized hydrazones were measured spectrophotometrically by a SpectraMax plus 384 spectrophotometer using an extinction coefficient of 22,000 M^{-1} cm^{-1} at 374 nm [19]. Hepatocyte protein carbonyl content was measured using the same method described for determining BSA carbonyl content. Briefly, 500 μl sample of hepatocyte suspension (0.5×10^6 cells) was taken at 60, 120 and 180 min and added to 500 μl of DNPH (0.1%, w/v) in 2N HCl. The concentration of 2,4-DNPH derivatized protein carbonyls was determined at 374 nm after incubating hepatocytes for 1 h with DNPH [20].

2.6. H_2O_2 measurement

H_2O_2 formation by hepatocytes were measured by taking samples at 90, 120, and 180 min and adding FOX 1 reagent (ferrous oxidation of xylenol orange). The FOX 1 reagent consisted of 25 mM sulfuric acid, 250 μM ferrous ammonium sulfate, 100 μM xylenol orange and 100 mM sorbitol. At the above time points 50 μl of hepatocytes suspension (1.0×10^6 cells/ml) were added to 950 μl FOX 1 reagent and incubated for 30 min at room temperature. Samples were spectrophotometrically analyzed at 560 nm. The extinction coefficient 2.35×10^5 M^{-1} cm^{-1} was used to quantify the molar concentration of H_2O_2 through Beer–Lambert law [17].

2.7. ROS formation

Hepatocyte ROS generation was determined using dihydrodichlorofluorescein diacetate which was taken up by cells rapidly and cleaved by unspecific esterases to form a non fluorescent alcohol dihydrodichlorofluorescein H_2DCF. This was oxidised by ROS to the green fluorochrome dichlorofluorescein followed by lysosomal iron relocation [21]. Aliquots (1 ml) were taken from the hepatocytes suspension at 120 min after incubating with