RESEARCH ARTICLES

High dietary salt decreases antioxidant defenses in the liver of fructose-fed insulin-resistant rats

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Abstract

In this study we investigated the hypothesis that a high-salt diet to hyperinsulinemic rats might impair antioxidant defense owing to its involvement in the activation of sodium reabsorption to lead to higher oxidative stress. Rats were fed a standard (CON), a high-salt (HS), or a high-fructose (HF) diet for 10 weeks after which, 50% of the animals belonging to the HF group were switched to a regimen of high-fructose and high-salt diet (HFS) for 10 more weeks, while the other groups were fed with their respective diets. Animals were then euthanized and their blood and liver were examined. Fasting plasma glucose was found to be significantly higher (approximately 50%) in fructose-fed rats than in the control and HS rats, whereas fat liver also differed in these animals, producing steatosis. Feeding fructose-fed rats with the high-salt diet triggered hyperinsulinemia and lowered insulin sensitivity, which led to increased levels of serum sodium compared to the HS group. This resulted in membrane perturbation, which in the presence of steatosis potentially enhanced hepatic lipid peroxidation, thereby decreasing the level of antioxidant defenses, as shown by GSH/GSSG ratio (HFS rats, 7.098±2.1 versus CON rats, 13.2±6.1) and superoxide dismutase (HFS rats, 2.1±0.05 versus CON rats, 2.3±0.1%), and catalase (HFS rats, 526.6±88.6 versus CON rats, 745.8±228.7 U/mg ptn) activities. Our results indicate that consumption of a salt-rich diet by insulin-resistant rats may lead to regulation of sodium reabsorption, worsening hepatic lipid peroxidation associated with impaired antioxidant defenses.

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1. Introduction

Increasing evidence suggests the involvement of oxidative stress in insulin resistance [1] and has generated high interest in the role of free radicals in the maintenance of adequate levels of antioxidant defenses [2,3]. In type 2 diabetes, a significant inverse correlation exists between hepatic fat load and the antioxidant defense system [4], which may prevent generation of an adequate compensatory response for restoration of cellular redox balance [5]. In particular, changes have been demonstrated in some components of the free radical defense system in different models [6−8].

We observed that the expression of genes encoding the antioxidant enzymes glutathione peroxidase (GPx), gamma-glutamylcysteine synthetase and superoxide dismutase (SOD) decreased in the liver tissue of streptozotocin-induced diabetic rats because of increased oxidative stress [9] associated with overproduction of reactive oxygen species (ROS)[10]. Under normal conditions, almost all of the produced superoxide anions (\(O_2^-\)) are converted to hydrogen peroxide (\(H_2O_2\)) by the action of SOD, which is further detoxified to water by catalase (CAT) or GPx [11]. However hyperglycemia induce the overproduction of \(O_2^-\) [12] and dramatic change in the oxidant/antioxidant balance has been postulated to play a role in the pathogenesis of diabetes.

Increasing the sugar intake has been reported to result in dyslipidemia, as indicated by elevated levels of serum triglycerides, cholesterol, and low-density lipoproteins [13,14].These underlying metabolic disturbances appear to induce insulin resistance commonly observed in high-fructose fed human and animal models [15] when fructose consumption causes progressive liver disease stimulated...
lipogenesis [16]. Furthermore, despite recent advances in elucidating the pathogenesis of related conditions, studies have shown that presence of insulin resistance and compensatory hyperinsulinemia would lead to sodium retention [17].

Therefore in the present study we examined whether a high-salt diet could impair antioxidant defenses in the liver of fructose-fed rats due activation of enhanced renal sodium reabsorption potentiating oxidative stress.

2. Materials and methods

2.1. Animal and diets

Forty-five male 12-week-old Fischer rats, weighing approximately 300 g, were individually housed in a temperature- and humidity-controlled room under a 12 h light/dark regimen. Initially, the rats were randomly assigned to three experimental groups (n = 10–12) as follows: the control group (CON), fed with the AIN93M diet [18] and water; the high-salt group (HS), fed with the AIN93M diet plus 8% w/w NaCl and water; and the high-fructose group (HF), fed with the AIN93M diet and a 20% w/v fructose solution as drinking water. After 10 weeks of treatment, the animals belonging to the HF group were further divided into 2 groups: rats that continued to be fed on the fructose solution (HF) and rats that were switched to a high-fructose + high-salt regimen (HFS) for 10 more weeks. Details of the experimental diets are given in Table 1. Food and water were provided ad libitum and their intake was measured. At the end of the experimental period, the rats were fasted for 12 hours, anesthetized with isoﬂurane and euthanized by total blood collection from the brachial plexus. The blood was centrifuged at 1500g for 15 min. One liver lobule from each animal was separated for histological analysis and the rest was frozen at –80°C until further analysis. All the procedures were approved by the Ethical Committee for Animal Care and Use of the Federal University of Ouro Preto.

2.2. Biochemical determinations

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities and plasma glucose concentration were determined using commercial kits from Labtest Diagnostica SA (Lagosa Santa, MG, Brazil) # 108, 109 and 84, respectively, by following the manufacturer’s instructions. ELISA was utilized to quantify plasma insulin and leptin levels using commercial kits from Ultra Sensitive Rat Insulin ELISA, Crystal Chem Downers Grove, IL, USA, and Rat Leptin ELISA Kit, Linco Research, USA, (Catalog #90060 and #90040, respectively). The homestasis model assessment (HOMA), described by Matthews et al. [19] as a measure of insulin resistance, was calculated using the formula (insulin [μmol/ml] × glucose [mM/L])/22.5. Hepatic fat was extracted using a chloroform-methanol mixture (2:1, v/v) according to the method of Folch et al. [20] and the total lipids were quantified gravimetrically by evaporating the solvents in the extract. Sodium concentrations were measured by flame photometry (Olidef model C-71 apparatus; São Paulo, Brazil).

2.3. Antioxidant defenses and oxidative stress

Liver SOD activity was measured by the method of Marklund and Marklund [21]. One unit of SOD activity was defined as the amount of enzyme that inhibited the rate of autoxidation of pyrogallol by 50%, which was determined at 570 nm. Catalase activity was measured according to Aebi [22] and was expressed in units per milligram of protein using the extinction coefficient of 0.0394 L/mmol/L/cm. The rate of H2O2 decomposition was followed by monitoring absorption at 240 nm in 50 mM phosphate buffer, pH 7.0, containing 5 mM H2O2. Tissue protein content was determined according to the method developed by Lowry et al. [23] using bovine serum albumin as the standard. The total glutathione (GSH + GSSG) was measured after precipitation of proteins with an equal volume of 4% sulfosalicyclic acid using the enzymatic method previously described [24]. Oxidized glutathione (GSSG) was determined after derivatization of total GSH with 2-vinyllpyridine. Oxidative stress index was calculated from the GSH/GSSG ratio and by lipid peroxidation status through of levels of thiobarbituric acid reactive substances (TBARS) as described by Buege and Aust [25].

2.4. Liver histology

After removal from each animal, the livers were immediately fixed in 10% buffered formaldehyde, embedded in paraffin, cut (4-μm thickness) and mounted on glass slides. The sections were deparaffinized in xylene, stained with hematoxylin and eosin (H&E) using the standard technique and then examined. Histological examination of the slides was performed by using concentrated light microscope equipped with photographic digital camera (DM5000; Leica) with software Qwin Plus. Scoring of the slides was performed using a semi-quantitative method reported by Brunet et al. [26]. Fat degeneration was graded according to the percentage of fat-containing hepatocytes. Grade of vesicular steatosis according to the original system involved 10 grades, whereas in this system, steatosis was graded from 0–4 based on the percentage of hepatocytes involved in the biopsy (0, none; 1, 10%; 2, 10–33%; 3, 33–66% and 4, ≥66%).

2.5. Statistical analysis

Normality of the sample distribution for each continuous parameter was tested with the Kolmogorov–Smirnov test. The significance of any differences in proportions of medians was tested with Kruskal–Wallis test and in means by one-way analysis of variance (ANOVA), followed by Dunns and Tukey tests, respectively. Correlation analysis was used to measure the degree to which 2 variables were related. Significance for all measures was defined when P < 0.05. GraphPad Prism version 5.00 for Windows (San Diego, CA, USA) was used for statistical analyses.

3. Results

Notably, the mean food intake was significantly different amongst different dietary groups. In relation to the control group, high dietary NaCl resulted in a lower caloric value leading to higher food intake in HS rats (P < 0.01). On the other hand, fructose supplementation led to lower food intake (P < 0.001) due to its energy content (Fig. 1A). Mean values for liquid intake were significantly higher in the high-salt groups (P < 0.001) than in the CON and HF groups (Fig. 1B). Higher fructose intake lowered the energetic demand for solid food (P < 0.001) in HFS rats than in other groups (Fig. 1C). The high-salt diet led to increased liquid intake, thus compelling HS and HFS rats to drink approximately 4.0 and 3.0 times the liquid volumes consumed by the CON animals, respectively, resulting in the corresponding differences in the liquid calorie intake of HFS rats drinking the fructose solution (P < 0.001) in relation to HF rats (Fig. 1D). There were no significant differences in the total energy intake (Fig. 1E). Moreover, plasma leptin concentrations in HF rats was higher (P < 0.01) compared to that in other groups (Fig. 1F).

The average final body weight was lower in HFS rats than in HF and CON rats (P < 0.05). Relative liver weights were higher (P < 0.05) in fructose-fed rats (HF and HFS) and a corresponding increase was observed in the liver lipid content and plasma glucose in fructose-fed rats in relation to CON (P < 0.05) and HS (P < 0.001). The highest insulin concentration was found in HFS rats (P < 0.05) and HOMA analysis revealed that this group had significantly higher values than the controls animals (P < 0.01), thereby indicating that the combination of dietary fructose with NaCl in HFS rats impaired insulin response. For determination of whether the dietary treatment induced liver injury, serum AST and ALT activities were examined, and ALT but not AST in the HF group were found to be significantly higher than that of the control group (P < 0.05). Augmented natriuretic response to a high-salt diet significantly decreased serum sodium in HS rats compared to that in other groups (P < 0.05) and particularly HFS showed a substantial increase in relation to the HS group (Table 2).

Photomicrographs of hepatic specimens stained with H&E are shown in Fig. 2A–D, and the scores of histological variables are presented as medians in Fig. 2E. Mild or no hepatic steatosis occurred in CON rats (Fig. 2A). HS rats did not show predominant occurrence of steatosis, but hyperemic vessels were observed in the parenchyma
cells (Fig. 2B). As expected, the high fructose treatment caused hepatic lipid accumulation, which was evident in both HF and HFS rats (Fig. 2C and 2D) with no signs of necroinflammation. Fat deposition in the HF group was classified as macrovesicular, while livers of HFS rats showed mainly a microvesicular pattern with lesser grade of lipid accumulation in relation to HF rats. A statistically significant higher steatosis score ($P<0.05$) was seen in livers from HF compared to CON animals (Fig. 2E).

Compared with the control group, the hepatic levels of total glutathione and GSH were significantly lower in the HFS group ($P<0.01$), although no significant difference was observed in the GSSG levels (Fig. 3). The GSH/GSSG ratio was calculated to determine whether oxidative stress had been augmented and was found to be lower in HFS rats than in control rats ($P<0.01$). Assessment of lipid peroxidation showed damage in hepatocytes, as verified through TBARS content of HFS in relation to CON animals ($P<0.05$). Furthermore, a progressive functional deficiency in antioxidant defenses was also evidenced in the HFS group, with a significant decrease in SOD and CAT activities, ($P<0.05$; $P<0.001$, respectively) in relation to the CON group. Additionally, a negative correlation was found between TBARS and GSH/GSSG ratio ($r=-0.40, P<0.01$), SOD ($r=-0.56, P<0.005$) and CAT activities ($r=-0.50, P<0.002$) (Table 3).

### 4. Discussion

The present study suggested that the osmotic load caused by augmenting dietary NaCl increased plasma osmolality, stimulated thirst, and enhanced liquid intake, as observed by Manesh et al. [27]. Studies in both rats [28] and humans [29] have reported that chronic fructose ingestion is associated with increase in plasma leptin levels and leptin resistance, which alters the information that is relayed to the central nervous system on energy intake and body fat stores for regulation of food intake and energy homeostasis [30]. Roglans et al. [31] reported that this increase precedes obesity, suggesting that the liver is a key organ in the development of metabolic derangements induced by fructose consumption. Nevertheless, in our study this increase of leptin levels only in HF rats occurred in the absence of augmented body weight: HF and control rats had the same body weight, although leptin potently activates cellular fuel consumption by stimulating fatty acid oxidation and reducing lipogenesis [32].

On the other hand, while fructose is more soluble, sweeter, and less glucogenic than glucose or sucrose and has been recommended as a replacement for these sugars in the diets of diabetic and obese people, it is lipogenic and usually causes greater elevation in triglyceride levels [3]. Significant differences due to fructose diet-induced development of fatty liver were observed in our experiments, as well as in other studies [33,34]. Fructose treatment-induced increased fatty liver and consequent hepatomegaly was found in HF and HFS animals. Fructose-fed rats served as a model for diet-induced insulin resistance, suggesting that pathophysiological mechanisms and lipid retention in hepatocytes (hepatic steatosis) was an important early sign in the development of a metabolic abnormalities spectrum. Our model was thus proved appropriate because it reproduced historically detectable steatosis resulting primarily from the deposition of fat in hepatocytes of fructose-fed rats. However, our results demonstrated that HFS rats consumed more fructose than HF rats did, but displayed lower degree of steatosis.
development. This may indicate that the high-salt diet model, as in other studies, attenuated gain in body weight [35,36], which can lead to lower accumulation of fat in hepatocytes, as supported by our data. Determination of liver function parameters also revealed liver dysfunction due to fructose-feeding and hepatic damage in fructose-fed rats. Fructose feeding was found to significantly enhance serum ALT activity, indicating considerable hepatocellular injury in HF rats. ALT has been routinely measured and is considered a surrogate marker of liver fat accumulation [37]. Injury to the hepatocyte leads to disruption of the plasma membrane and leakage of the enzyme to the extracellular fluid. Thus it can be detected at abnormal levels in the serum and this condition may be a cause of hepatocyte death.

Rats are an excellent animal model to study the effects of fructose intake because their fructose metabolism closely resembles that of humans [38] and studies have shown that fructose induces hyperglycemia and hyperinsulinemia [39,40]. The results demonstrated that fructose administration produces insulin resistance in HFS rats consistently with previous studies carried out using different techniques to assess insulin resistance [40,41]. Insulin resistance in fructose-fed rats has been attributed to a low level of insulin-stimulated glucose oxidation due to modifications in the post-receptor cascade of insulin action [42]. Thus elevated plasma insulin concentrations enhance the synthesis of very-low-density lipoprotein, and this may induce increase in fatty liver as observed by us in HF and HFS rats with decreased in response of HFS rats to glucose utilization, featuring a lower insulin action as indicated by higher HOMA values. In contrast, Nishimoto et al. [43] used fructose-fed insulin-resistant rats with a low or high-sodium diet and found no significant differences in plasma glucose or

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**Fig. 2.** (A-D) Representative photomicrographs H&E staining of liver sections of experimental rats. Hepatocytes abnormalities were not observed in the CON group (A); note hyperemic vessels (arrowhead) and normal parenchyma appearance in the HS group (B); aspect of macrovesicular steatosis in the HF group (C). Note hepatocytes with a large negative image in the cytoplasm with nucleus displaced into the periphery of the cell, large fat globule in most cases (white arrow); aspect of microvesicular steatosis in the HFS group (D). Note cells with small cytoplasmic vacuoles without displaced nucleus (black arrow) magnification ×440. Grade of liver steatosis of rats in experimental groups (E). Values (median, n=8–11) (*P<0.05 vs. control rats).
insulin among their groups, although they used a lower dosage for both salt and fructose than in our study. This discrepancy among studies may be better explained by the fact that diet-induced modifications in metabolic and hormonal profile are probably dependent on the duration of diet treatment, on the amount of carbohydrate and salt in the diet besides interactions with other nutrients [44–46].

Our model was based on the administration of a high-fructose diet inducing insulin resistance that resembles the so-called ‘fast food’ that is highly popular nowadays. This type of diet constitutes an important, typically westernized lifestyle, which includes consumption of processed foods that are high in salt and sugar. Fructose has been broadly used in metabolic studies, although there is no data concerning liver abnormalities associated with ‘high-salt’ regimens.

In this study, the high-salt diet led to greater urinary excretion of sodium, but hyperinsulinemia showed in HFS rats has influence on sodium retention as demonstrated by higher serum sodium concentrations in HFS when compared with the HS group. This antinatriuretic effect may be opposed by a concomitant decrease in proximal tubular sodium reabsorption [47] or an increase in glomerular filtration rate (GFR) [48]. Chronic hyperinsulinaemia increases GFR in normal dogs [48], but not in obese insulin-resistant dogs [49] which suggest that insulin could increase GFR, and thus the filtered sodium load, only in insulin-sensitive subjects. Insulin has been known to enhance sodium reabsorption in the proximal tubule and stimulates not only sodium but also volume absorption in the rabbit proximal convoluted tubule. Thus, from these stimulatory effects, it is clear that insulin acts on proximal tubules to reabsorb sodium filtered from the glomeruli and yet, important regulatory mechanisms exist subsequently in the Henle’s loop, distal tubule and connecting tubule [17].

Besides, Vasdev et al. [50] showed that intracellular sodium levels increase cytosolic free calcium, which can increase oxidative stress and this condition changes the membrane components compromising its integrity [51] because increased ROS generation has been shown to induce cell membrane lipid peroxidation [52]. Therefore, it has been suggested that lipid accumulation in the liver makes hepatocytes more sensitive to oxidative stress [53], which in this study, potentially activated lipid peroxidation, as demonstrated by increased TBARS in HFS rats. The observed steatosis could have affected lipid composition and fluidity of mitochondrial membranes, which increased oxidative stress in the liver. In addition, changes in liver glutathione redox status were monitored in this work by recording the GSH/GSSG ratio, because severe oxidative stress may deplete cellular GSH, and glutathione play an important function in detoxification of free radicals [54]. Glutathione is the major intracellular non-protein antioxidant, and GPx converts H$_2$O$_2$ to H$_2$O by oxidizing glutathione to glutathione disulfide [2]. We observed a decrease in liver GSH levels in HFS rats as well as in

![Fig. 3. Antioxidant defenses and oxidative stress in the liver of experimental rats. Different letters indicate significant differences at $P<.05$ by one-way ANOVA followed by Tukey’s test.](image-url)
the GSH/GSSG ratio, which could be a consequence of adaptation of the fatty liver, as suggested by the negative correlation of TBARS with GSH/GSSG ratio. It may represent a consequence of the higher pro-oxidant status developed in HFS rats, which is likely responsible for the high consumption of cellular and circulating antioxidants. Moreover, decreased SOD and CAT activities were observed in HFS rats that could participate in hepatic vulnerability to oxidative stress. Decreased feedback regulatory mechanisms involving these antioxidant enzymes, prevents the restoration to normal enzyme level. Thus, the susceptibility of the tissue to oxidative stress was dependent on the alteration in lipid composition and tissue damage. SOD play a key role in cell protection against the deleterious effects of O$_2$ and catalase prevents damage by rapidly converting H$_2$O$_2$ to water [55]. There are possible pathways by which cellular metabolism in this model may be altered, which in turn may accelerate oxidative stress. The increased oxidative stress could be due to production of oxygen free radicals [3] and H$_2$O$_2$ resulting from SOD activity, which can generate hydroxyl radicals through the Fenton reaction, and thus, ROS can themselves reduce the activity of antioxidant enzymes such as CAT and GPX [56]. Consequently, a lowering of these activities is suggestive of reduced scavenging potential in the insulin-resistant rats on high-salt diet. Another possibility is that accumulation of advanced glycation products may be mediated, in part, by visceral osmoreceptors. Am J Physiol Regul Integr Comp Physiol 2006;290:F625–9.

In conclusion, the high-salt diet reduced hepatic antioxidant defenses in the fructose-fed rats, providing evidence to support the idea that increased oxidative stress is involved in membrane defenses, worsening insulin sensibility in the early stage of experimental diabetes in rats; however, further research is needed to define the interdependencies/interactions among fructose, salt, oxidative stress more clearly.

In the authors’ model, the role of fructose in increasing oxidative stress and hepatic steatosis in diet-induced obese mice. Exp Mol Pathol 2011;91:419–28.

References


