

DNA Oxidative Damage is Correlated with JNK Activation in Hepatocytes from Rats with Experimental Insulin Resistance

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Abstract

Oxidative stress was improved as the main complication for a variety of pathological conditions. The present study was aimed to investigate the effect of insulin resistance development on c-Jun-N-terminal kinases (JNK) activity and DNA/RNA oxidative damage in rat's liver under fructose rich diet. Total JNK, phosphorylated JNK-1 and JNK-2 ([pThr183/Tyr185] c-Jun N-terminal protein kinase (pJNK1/2), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) amounts were measured in hepatocyte lysate. Glucose, insulin, free fatty acids, and triacylglycerols concentration and thiobarbituric acid reactive substances concentrations were measured in blood plasma. Catalase and superoxide dismutase activity, and thiobarbituric acid reactive substances (TBARS) were determined in liver homogenate. Fructose rich diet in rats provoked the insulin resistance that is accompanied by deep metabolic abnormalities detected in our study. These metabolic abnormalities induced by fructose overload are associated with an enhanced oxidative stress which appears to deregulate the JNK pathway. Consequently, accumulation of DNA/RNA oxidative damage stress marker – 8-OHdG– in hepatocytes positively correlates with JNK activity in the cells.

Keywords: Oxidative damage; Insulin resistance; c-Jun N-terminal kinases; DNA oxidative damage; High fructose diet

Introduction

At the present time, oxidative stress was improved as the main complication for a variety of pathological conditions. Especially dangerous component of oxidative stress generation is the formation of Reactive Oxygen Species (ROS). Their formation can cause damage to many cellular components such as lipids, DNA and proteins as a result of their oxidation [1,2]. The level of ROS, which far exceeds the possibilities of protecting cells, can lead to serious cell disorders (e.g., mutation, carcinogenesis, degenerative and other diseases, inflammation, aging, apoptosis etc.) [3]. Peroxidation products content and antioxidant enzymes activity used to be the accepted markers of oxidative stress in a numerous studies [4].

One of the consequences of oxidative stress is the formation of DNA lesions, which can result in genomic instability and lead to cell death [5]. Over 100 different types of oxidative DNA modifications have already been identified in the mammalian genome [6]. High levels of oxidative damage of DNA and RNA may significantly affect its function and cause detrimental effects to cells and organisms [7]. There are a lot of mechanisms to control the DNA/RNA oxidation levels in cells, suggesting that their damage is indeed a significant issue [8]. Guanine is the base that is most susceptible to oxidation, due to its low redox potential, and 8-oxoguanosine is the most widespread lesion. Therefore, this product is the most common and best-characterized lesion created by ROS. These characteristics and the strong relation between ROS production and 8-oxoguanosine make it derivatives the good cellular biomarkers to indicate the extent of oxidative stress [9,10].

Speaking nowadays about investigation of the oxidative stress provocation, development and consequences it is impossible not to mentioned JNK-signaling pathways. During the last years great interest has been directed toward the c-Jun N-terminal kinases (JNK) - group of mitogen-activated protein kinases (MAPKs) that are activated by oxidative stress, pro-inflammatory cytokines, mitogenic stimuli, hyperglycemia etc. Ten JNK isoforms were described from three genes known as: JNK1 (four isoforms), JNK2 (four isoforms) and JNK3 (two isoforms) [11-13]. JNKs play a key role in MAPK pathways provides the interconnection between increased ROS levels and development of disorders such as insulin resistance [14,15]. The JNK pathway is important in modulating cellular responses to stress. Increased oxidative stress was implicated in different animal models of diabetes, insulin resistance, metabolic syndrome etc. through activation of the JNK. JNK regulates the activities of many transcription factors and that the JNK pathway is required for the regulation of inflammatory responses, cell proliferation, and apoptosis [16-19].

Activation of the JNK signaling pathway can mediate many of the effects of stress on insulin resistance through inhibitory phosphorylation of insulin receptor substrate. JNK1 activity is believed to be implicated in obesity and insulin resistance [20]. Several studies have reported that JNK activity is increased in response to diabetes, and elevated JNK activity interferes with insulin action both in cell culture and in animal models [14,17]. The ingestion of a single, sucrose-enriched meal or elevation of portal vein fructose concentrations via fructose infusion in rats *in vivo* also increased hepatic JNK activity and phosphorylation of insulin receptor substrate-1 (IRS-1) on serine, a downstream target of JNK [21].

Both DNA oxidation and JNK activation could induce the apoptosis in different tissues. But whether the oxidative damage or signaling pathways play the leading role in this process remains unclear.

One aspect of the multifaceted problem of oxidative stress is the study of tissue specific mechanisms of its course. There are a lot of data testifying that both JNK activity changes in different tissues and the DNA oxidative damage accompanied oxidative stress [22-25] but reports involving the investigations of both this indices in liver tissue are partial and not numerous.

So the present study was aimed to investigate the effect of insulin resistance development on JNK activity and DNA/RNA oxidative damage in rat's liver under fructose rich diet.

Materials and Methods

The experimental protocol and design were approved by the Institutional Animal Care and Use Committee at National Pharmaceutical University, and the animals were maintained in accordance with the guidelines for animal care and use of laboratory animals.

Healthy male Wistar rats (12 months old) weighting between 490-530 g were selected for the study. 40 animals were divided into two groups: the study group (IR, n=20) received fructose-rich diet (FRD, containing 18.3% protein, 60.3% fructose and 5.2% fat) while the control group received regular rat chow (C, n=20) for 6 weeks [26].

Body weight (g) was recorded after 3 and 6 weeks of experiment.

On the 22nd and on the 43rd day of experiment (after 12 hours fasting period) animals were killed by decapitation (n=10). Blood samples were collected for plasma preparation.

Liver was immediately removed from the animals and the sample for total JNK, phosphorylated JNK-1 and JNK-2 ([pThr183/Tyr185] c-Jun N-terminal protein kinase (pJNK1/2), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) determination was quickly frozen in liquid nitrogen and stored at -80°C until use. Tissue samples were prepared according to kit instructions (*commercial Human/Mouse/Rat Total JNK Pan Specific DuoSet IC ELISA; [pThr¹⁸³/Tyr¹⁸⁵] JNK1/2 EIA kit; K-ASSAY 8-OHdG ELISA*), respectively.

Other samples of hepatic tissues were used to prepare tissue homogenate (10% w/v) in ice-cold buffer of pH 7.4 (in 0.25 M sucrose, 0.02 M triethanolamine hydrochloride containing 0.12 M dithiothreitol). The homogenate was then centrifuged at 5000g for 60 min to remove debris. The supernatant was again centrifuged at X12,000g for 40 min to obtain cytosolic fraction. All the procedures were carried out at 4°C. Clear upper supernatant fluid was taken for Thio Barbituric Acid-Reactive Substance (TBARS) level, Catalase (C) and Superoxide Dismutase (SOD) activity determination. Protein assays were made by the method of Lowry [27].

Plasma glucose concentration was determined by a glucose oxidase method (commercially available kit SIGMA, USA), and plasma insulin concentration was measured in plasma with the use of sandwich immunoassay on MSD platform (*Meso Scale Discovery Multy-Array Assay System Mouse/Rat Insulin Kit*).

TBARS were measured spectrophotometrically at 532 nm against blank and the results are expressed as Malonic Dialdehyde (MDA) formed micromol/L (blood) and nmol/mg protein (liver) [28].

Plasma free fatty acids (FFA) levels were determined by enzymatic method with a commercially available kit (*Free Fatty Acid Quantitation Kit, Sigma-Aldrich, USA*).

Plasma Triacylglycerols (TAG) content were determined by enzymatic method with a commercially available kit (*Triglyceride Colorimetric Assay Kit, Sigma, USA*).

Catalase (CAT) activity in liver was determined based on Aebi's [29].

The activity of superoxide dismutase (SOD) in liver was measured by using commercially available kit (*Superoxide Dismutase (SOD) Activity Assay Kit, BioVision Incorporated, USA*).

Total JNK in liver was determined with the help of commercial *Human/Mouse/Rat Total JNK Pan Specific DuoSet IC ELISA (R&D Systems, Inc., USA)*.

Phospho JNK1/2 in liver was determined with the help of commercial *[pThr¹⁸³/Tyr¹⁸⁵] JNK1/2 EIA kit (Enzo Life Sciences)*.

Content of 8-OHdG in liver was determined by the commercial kit *K-ASSAY 8-OHdG ELISA (KAMIYA BIOMEDICAL COMPANY, USA)*. For quantifying DNA in cell lysate was used the commercial DNA quantification kit (*KAMIYA BIOMEDICAL COMPANY, USA*).

All values were calculated as mean ± SD. The ANOVA test (XLSTAT) was employed for statistical comparison between control and various groups. Significance was considered at P<0.05.

Results

IR animals exhibited a significant increase in body weight (Table 1) when compared with intact rats (C) after 6 weeks of experiment. After 3 weeks of FRD diet, body weights were similar in both control and IR rats. But levels of plasma glucose, TAG and FFA in IR animals were significantly different than those of the control animals in both measurement terms.

Tested parameters	initial		after 3 weeks		after 6 weeks	
	C	IR	C	IR	C	IR
body weight(g)	510 ± 5	510 ± 5	512 ± 6	520 ± 8	552 ± 7	635 ± 8 ^a
glucose (mmol/L)	4.7 ± 0.12	4.8 ± 0.11	4.9 ± 0.14	6.9 ± 0.12 ^a	5.1 ± 0.09	14.24 ± 0.23 ^{a b}
insulin (pg/ml)	1290 ± 34	1310 ± 43	1340 ± 29	2110 ± 32 ^a	1320 ± 28	2920 ± 41 ^{a b}
TAGmmol/L	0.84 ± 0.06	0.86 ± 0.07	0.85 ± 0.09	1.48 ± 0.09 ^a	0.87 ± 0.08	2.59 ± 0.11 ^{a b}

FFA mmol/L	0.40 ± 0.05	0.38 ± 0.03	0.39 ± 0.03	0.51 ± 0.02 ^a	0.41 ± 0.03	0.68 ± 0.04 ^{a,b}
TBARS mkmol/L	2.57 ± 0.18	2.61 ± 0.15	2.49 ± 0.22	3.29 ± 0.56 ^a	2.51 ± 0.21	3.37 ± 0.24 ^a

Values represent the mean ± SD of 10 experimental animals each group.
^ap<0.05 (Compared to control).
^bp<0.05 (Compared to IR 3 weeks).

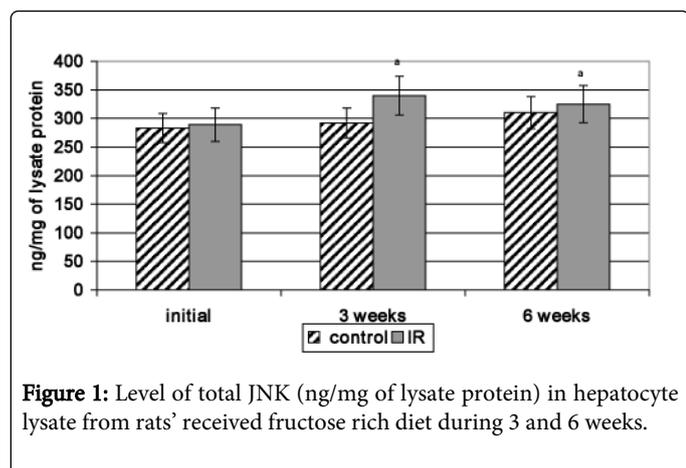
Table 1: Effect of fructose rich diet on body weight, plasma levels of insulin, glucose, triacylglycerols, free fatty acids, and TBA active products after 3 weeks and 6 weeks of diet.

Tested parameters	initial		after 3 weeks		after 6 weeks	
	C	IR	C	IR	C	IR
SOD activity U/ mg protein	28.5 ± 1.2	26.3 ± 1.8	27.9 ± 1.5	28.8 ± 1.2	30.1 ± 1.8	15.4 ± 1.1 ^{a,b}
CAT activity (U/mg protein)	205.2 ± 10.5	210.5 ± 9.8	201.6 ± 11.1	225.4 ± 8.5 ^a	210.1 ± 10.1	101.9 ± 9.4 ^{a,b}
TBARS nmol MDA/mg protein	0.72 ± 0.11	0.69 ± 0.09	0.74 ± 0.12	1.45 ± 0.11 ^a	0.71 ± 0.08	2.01 ± 0.14 ^{a,b}

Values represent the mean ± SD of 10 experimental animals each group.
^ap<0.05 (Compared to control).
^bp<0.05 (Compared to IR 3 weeks).

Table 2: Effect of fructose rich diet on superoxide dismutase and catalase activity, and peroxidation level in liver homogenate after 3 weeks and 6 weeks of diet.

Although fasting glycemia was significantly changed by 6 weeks of fructose overload, insulinemia was significantly increased in FRD compared to control rats (Table 1) indicating insulin resistance development in these animals.

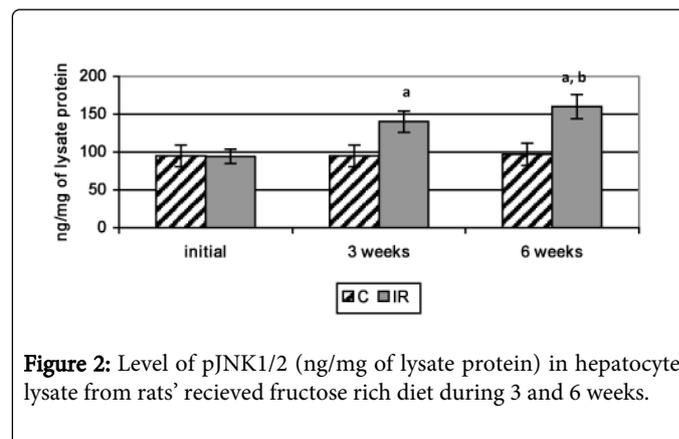


^ap<0.05 (Compared to control).

^bp<0.05 (Compared to IR 3 weeks).

Total JNK and pJNK1/2 levels were measured in liver tissue lysates. The observed changes after 3 and 6 weeks of experiment are displayed in Figures 1 and 2, respectively. Total JNK amount increased after 3 weeks of a diet reaching 341.3 ng/mg of lysate protein compared to control 292.7 ng/mg of lysate protein but to sixth week the total

amount decreased to 325.6 ng/mg of lysate protein compared to control 312.4 ng/mg of lysate.



^ap<0.05 (Compared to control).

^bp<0.05 (Compared to IR 3 weeks).

The different dynamics was observed as for pJNK1/2 amount. It levels in tissue lysates increased significantly to the third week of experiment (142.34 ng/mg of lysate protein) and continued to increase reaching to the end of sixth week 164.21 ng/mg of lysate protein.

Accumulation of 8-OHdG in hepatocytes began to be registered in early terms of experiment (Figure 3). And at a later period the 8-OHdG formation measured in experimental groups showed the significant increasing in it content.

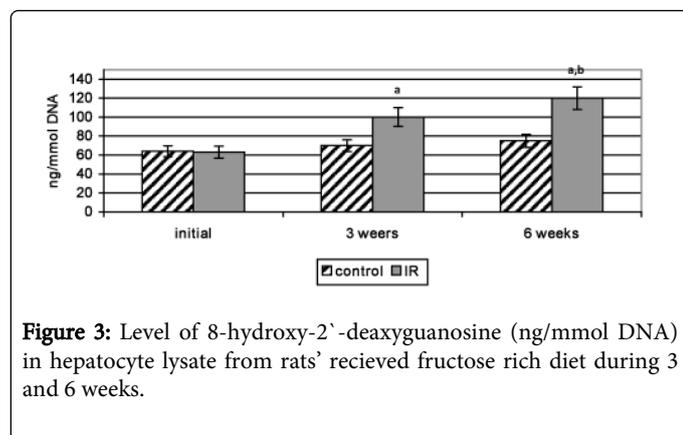


Figure 3: Level of 8-hydroxy-2'-deoxyguanosine (ng/mmol DNA) in hepatocyte lysate from rats' received fructose rich diet during 3 and 6 weeks.

^ap<0.05 (Compared to control).

^bp<0.05 (Compared to IR 3 weeks).

Discussion

In the present study, investigation of insulin resistant effects confirms our assumption about the correlation between JNK1/2 activation and formation of 8-OHdG in rat liver. Feeding of rats with fructose rich diet for 3 and 6 weeks induced hyperinsulinemia, hyperglycemia, hypertriglyceridemia increasing of FFA and TBARS level in blood. Insulin levels and high triglyceridemia are known to enhance free radical production [30-32]. These results suggest that in our experiment conditions FRD led to metabolic disturbances accompanied by oxidative stress development and proatherogenic state. Also hypercaloric diet provoked the deep shift in hepatocytes metabolism that led to oxidative stress development. Oxidative stress in hepatocytes was evaluated by the measure of the validated biomarkers: catalase and SOD activities and TBARS accumulation.

But all this parameters was significantly increased only to the sixth week of experiment. A number of other studies using the FRD have also reported increased fasting plasma insulin, glucose and FFA [30] as well as a consistent hypertriglyceridemia in accordance with our results [31]. These metabolic parameters indicated the insulin resistance development [33] and could result in type 2 diabetes mellitus (DM2). Representative metabolic changes in IR rats included body weight gain, hyperglycaemia, hyperinsulinemia, increased TAG, FFA and TBARS level in blood.

In the same time, metabolic links between insulin resistance, oxidative stress and other consequences of DM2 still uncertain. The role of signaling pathways disorders in this process could explain these interconnections.

Our data showed that in early terms occurred the increase of total JNK and its phosphorylated isoforms 1/2 accumulation in liver. This fact pointed that the JNK overexpressed at the beginning of insulin resistance development (hyperglycemia and hyperinsulinemia). One of JNK overexpression manifestations could be the cell oxidative damage.

The DNA oxidation resulting in accumulation 8-OHdG in tissues was observed in more later early terms compared with JNA activation. These results suggest that the signaling disturbances may play the initial role in metabolic complications of hypercaloric diet and insulin resistance.

At the same time, increasing of DNA oxidation damage testifies that the oxidative stress under insulin resistance development goes far and plays the additional role of an independent factor of metabolism disturbance.

Thus, fructose rich diet in rats provoked the insulin resistance that is accompanied by deep metabolic abnormalities detected in our study. These complications induced by fructose overload are associated with an enhanced oxidative stress which appears to deregulate the JNK pathway.

Consequently, accumulation of DNA/RNA oxidative damage stress marker-8-OHdG- in hepatocytes strong positively correlates with JNK activity in the cells.

To conclude the present work gives additional insights about the potential mechanisms linking insulin resistance, oxidative stress development and their consequences.

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