Original Paper

Increased Oxidative Stress and Mitochondrial Dysfunction in Zucker Diabetic Rat Liver and Brain

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Key Words

Zucker diabetic rats • Liver • Brain • Oxidative stress • Mitochondrial dysfunction

Abstract

Background/Aims: The Zucker diabetic fatty (ZDF, FA/FA) rat is a genetic model of type 2 diabetes, characterized by insulin resistance with progressive metabolic syndrome. We have previously demonstrated mitochondrial dysfunction and oxidative stress in the heart, kidneys and pancreas of ZDF rats. However, the precise molecular mechanism of disease progression is not clear. Our aim in the present study was to investigate oxidative stress and mitochondrial dysfunction in the liver and brain of ZDF rats. **Methods:** In this study, we have measured mitochondrial oxidative stress, bioenergetics and redox homeostasis in the liver and brain of ZDF rats. **Results:** Our results showed increased reactive oxygen species (ROS) production in the ZDF rat brain compared to the liver, while nitric oxide (NO) production was markedly increased both in the brain and liver. High levels of lipid and protein peroxidation were also observed in these tissues. Glutathione metabolism and mitochondrial respiratory functions were adversely affected in ZDF rats when compared to Zucker lean (ZL, +/FA) control rats. Reduced ATP synthesis was also observed in the liver and brain of ZDF rats. Western blot analysis confirmed altered expression of cytochrome P450 2E1, iNOS, p-JNK, and IκB-α confirming an increase in oxidative and metabolic stress in ZDF rat tissues. **Conclusions:** Our data shows that, like other tissues, ZDF rat liver and brain develop complications associated with redox homeostasis and mitochondrial dysfunction. These results, thus, might have implications in understanding the etiology and pathophysiology of diabetes which in turn, would help in managing the disease associated complications.

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Introduction

Diabetes and obesity (diabesity) are among the most challenging global health problems. Metabolic syndromes and the complications associated with diabesity are related to compromised energy metabolism, hyperglycemia, hyperlipidemia, insulin resistance and cardiovascular abnormalities. However, the precise molecular mechanisms of disease progression and complications in various tissues are still unclear. The Zucker diabetic fatty (ZDF, FA/FA) rat is a genetic experimental model of type 2 diabetes characterized by inherited insulin-resistance with progressive metabolic complications, hyperglycemia and hyperlipidemia [1, 2]. While male rats develop spontaneous diabetes and metabolic complications, female rats become diabetic only after intake of high fat diet [2]. The ZDF rats exhibit progressive metabolic complications associated with increased insulin resistance, oxidative and nitrosative stress [3-6]. Studies have shown that obesity, inflammatory cytokines, excessive food intake are risk factors for development of hyperglycemia, insulin resistance and pancreatic β -cell dysfunction in ZDF rats when compared to Zucker lean controls (ZL,FA/+) [7]. The precise molecular mechanism that links progressive metabolic syndrome and insulin resistance in different tissues of ZDF rats is not completely understood.

Our previous studies, using young and elderly ZDF rats [8, 9] reported altered cardiovascular responses to progressive hyperglycemia and metabolic complications in comparison to lean controls. With age, the complications of diabetes worsen and ZDF rats may become more reliant on the use of lipid reserves to meet metabolic and energy requirements. A recent study in ZDF rats [10] suggests that diabetes per se is not a critical factor in the induction of clinically significant complications and hence some other factors related to obesity might have greater impact on cardiovascular abnormalities. Studies have also suggested that prolonged exposure to insulin suppresses mitochondrial biogenesis and function and this may lead to impairment of insulin sensitivity [11, 12]. Our recent studies [13, 14] using cardiomyocytes from the left ventricle and cellular fractions from the kidney and pancreas of ZDF rats have shown increased oxidative stress and mitochondrial dysfunction. We, therefore, have extended our study to other metabolic tissues, such as liver and brain, to elucidate the mechanisms of metabolic and oxidative stress in ZDF rats. We have demonstrated that the metabolic complications in ZDF rat liver and brain are also associated with increased oxidative stress, altered redox metabolism and mitochondrial dysfunctions, which are accompanied by altered cell signaling and compromised energy metabolism. These results have provided better understanding of metabolic and oxidative stress and the associated complications in ZDF type 2 diabetic rat tissues, which might have implications in the management of diabesity.

Materials and Methods

Chemicals and reagents

Cytochrome c, reduced glutathione (GSH), oxidized glutathione (GSSG), 5,5'-dithio-bis(2-nitrobenzoic acid), 1-chloro 2,4-dinitrobenzene (CDNB), cumene hydroperoxide, dimethylnitrosamine (DMNA), 7-ethoxyresorufin, resorufin, dinitrophenylhydrazine (DNPH), glutathione reductase, thiobarbituric acid, NADH, NADPH, coenzyme Q2, antimycin A, dodecyl maltoside, sodium succinate, apocynin and ATP Bioluminescent cell assay kits were purchased from Sigma-Aldrich Fine Chemicals (St Louis, MO, USA). 2', 7'-Dichlorofluorescein diacetate (DCFDA) was procured from Molecular Probes (Eugene, OR, USA). Kits for the measurement of SOD and NO were purchased from R&D System, MN, USA. Polyclonal antibodies against CYP450 2E1, p-JNK, I κ B- α , iNOS and β -actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Reagents for SDS-PAGE and Western blot analyses were purchased from Gibco BRL (Grand Island, NY, USA) and Bio Rad Laboratories (Richmond, CA, USA).

Animal and tissue preparation and sub-cellular fractionation

Studies were performed in elderly (30-34 weeks old) Zucker diabetic fatty (ZDF; FA/FA) rats (n=4, average body weight = 485g; average blood glucose = 478 mg/dL) and age-matched Zucker lean (ZL;

+/FA) controls (n=5, average body weight = 400 g; average blood glucose = 108 mg/dL) (Charles River Laboratories, UK). Approval for this project was obtained from the Animal Ethics Research Committee, College of Medicine & Health Sciences, United Arab Emirates University and all the animals were used according to the safe practice for animals in research guidelines as stipulated by NIH, USA.

Liver and brain tissues were dissected from male ZDF and age-matched ZL control rats and rinsed with ice-cold saline. Isolated liver and brain tissues were homogenized (10% w/v) in isotonic 100mM potassium phosphate buffer (pH7.4) containing 1mM EDTA and 0.1mM phenylmethylsulfonylfluoride (PMSF, a protease inhibitor). The homogenate was centrifuged at 1000xg for 10 min and the supernatant was used for further purification of mitochondrial and microsomal fractions as previously described [13,14]. Protein concentration in tissue homogenate and the isolated sub-cellular fractions was measured using BioRad reagent.

Measurement of ROS and NO

Production of ROS in ZDF and ZL rat tissues was measured using the DCFDA fluorescence method as previously described [13, 14]. Both, plasma membrane bound apocynin-sensitive NADPH oxidase (NOX)-dependent as well as mitochondrial-dependent ROS production were measured.

Nitric oxide production was determined by measuring the concentration of total nitrite using Griess reagent as described in the vendor's protocol (R & D Systems Inc).

Protein carbonylation, lipid peroxidation (LPO) and catalase/SOD activities

Protein peroxidation as a marker of increased oxidative stress was measured in ZDF and ZL rat tissue fractions by DNPH conjugation method as previously described [13-15]. NADPH-dependent membrane lipid peroxidation was measured as malondialdehyde formed using the standard thiobarbituric acid method [13-15]. Catalase activity was measured by the method of Beers and Sizer [16] in which the disappearance of peroxide was followed spectrophotometrically at 240 nm. One unit decomposes one micromole of hydrogen peroxide per minute at 25°C and pH 7.0 under the specified conditions. SOD activity was measured as percent conversion of NBT to NBT-diformazan according to the vendor's protocol (R & D System, MN, USA). The percent reduction in formazan formation was used as a measure of SOD activity.

Measurement of GSH pool and GSH-redox metabolism

The concentrations of cytosolic and mitochondrial GSH in the liver and brain were measured by NADPH-dependent GSSG-reductase catalyzed conversion of GSSG to GSH. Glutathione S-transferase (GST) activity using CDNB, glutathione peroxidase (GSH-Px) activity using cumene hydroperoxide and glutathione-reductase activity using GSSG/ NADPH as the respective substrates were measured by standard protocols as described before [13-15].

Measurement of CYP 2E1 and 1A1 activities

CYP 2E1-dependent N-demethylase activity was measured in the microsomal fraction from the ZDF and ZL rat liver and brain using dimethylnitrosamine (DMNA) substrate in the presence of NADPH in the appropriate buffer (pH 7.4) as previously described [17].

CYP 1A1 activity in the microsomal fraction from the ZDF and ZL rat liver and brain was measured spectrofluorometrically using 7-ethoxyresorufin as substrate as previously described [18].

Measurement of mitochondrial respiratory enzyme complexes

Freshly isolated liver and brain mitochondrial fractions (5µg protein) were suspended in 1.0 ml of 20 mM KPi buffer, pH 7.4, in the presence of the detergent, lauryl maltoside (0.2%). NADH-ubiquinone oxidoreductase (Complex I), Succinate-cytochrome reductase (Complex II/III) and cytochrome c oxidase (Complex IV) activities were measured using the substrates coenzyme Q2, succinate and reduced cytochrome c, respectively, according to the method of Birch-Machin and Turnbull, [19] as previously described [13-15].

Measurement of ATP level

ATP content in control ZL and ZDF rat liver and brain mitochondria was determined using ATP Bioluminescent cell assay kit (Sigma, St Louis, MO) according to the manufacturer's suggestion and samples were read using the TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

SDS-PAGE and Western blot analysis

Homogenates (50µg protein) from ZDF and ZL rat liver and brain were electrophoretically separated by 12% SDS-PAGE [20] and transferred on to nitrocellulose paper [21]. The expression of specific oxidative stress marker proteins (iNOS and CYP2E1) and cell signaling transcription regulatory proteins (p-JNK and IκB-α) was assessed by immunoreaction with their specific antibodies by Western blot analysis as previously described [13-15]. β-actin was used as the loading control. Densitometric analysis of the protein bands was performed using a gel documentation system (Vilber Lourmat, France) and expressed as relative intensity compared to the protein expression of control ZL which was arbitrarily taken as 100%.

Data analysis

Values were calculated as mean ± S.E.M of at least three determinations. Statistical significance of the data was assessed using the unpaired 't' test for differences between groups and p values ≤0.05 were considered significant.

Results

Alterations in sub-cellular ROS and NO production

A significant decrease (20%-30%) in total ROS production (Fig. 1A), apocynin-sensitive membrane bound NADPH oxidase-dependent ROS formation (Fig. 1B) as well as mitochondrial ROS formation (Fig. 1C) in the liver from ZDF compared to ZL control rats was observed. ROS production in brain was significantly increased (35-60%). NO production was significantly increased (~ 40%) in liver and brain of ZDF rats when compared to ZL controls (Fig. 1D).

Alterations in lipid peroxidation (LPO) and protein carbonylation

Microsomal and mitochondrial LPO were significantly increased (30-40%) in the brain of ZDF rat (Fig.s 2A and B) while in the liver, a significant increase (60%) in mitochondrial LPO was observed. Protein peroxidative carbonylation, as measured by DNPH-coupling in the liver and brain, was also significantly increased (70-80%) in the liver and brain of ZDF rats compared to ZL controls (Fig. 2C).

Fig. 1. ROS and NO production in ZDF rat liver and brain. ROS production (A,B,C) in the liver and brain of ZDF and ZL rats was measured using DCFDA as a probe as described in the Materials and Methods.

Total homogenates from the tissues of ZDF and ZL rats (n=5) were analyzed for total NO level (D) using Greiss reagent. Results are expressed as mean ± S.E.M. from three independent experiments and asterisks indicate significant difference (*p<0.05 and **p<0.01.)

Fig. 2. Lipid peroxidation and protein carbonylation in ZDF rat liver and brain. NADPH-dependent LPO (A,B) was measured in the liver and brain of ZDF and ZL rats (n=5) as malonedialdehyde formed using the standard thiobarbituric acid method as described before [13-15]. DNPH-coupled protein carbonylation (C) in the liver and brain total homogenates of ZDF and ZL rats (n=5) was measured as described in the Materials and Methods. Results are expressed as mean $\pm$ S.E.M.

from three independent experiments and asterisks indicate significant difference (*p<0.05 and **p<0.01).

Fig. 3. SOD and catalase activities in ZDF rat liver and brain. Tissue homogenate from ZDF and ZL rat liver and brain (n=5) was analysed for SOD (A) and catalase (B) activities as described in the Materials and Methods. Results are expressed as mean $\pm$ S.E.M. from three independent experiments and asterisks indicate significant difference (*p<0.05 and **p<0.01).

Alterations in SOD and catalase activity

SOD activity in the liver of ZDF rats was moderately increased (Fig. 3A) whereas it was moderately reduced in the brain. Catalase activity in the liver of ZDF rats was slightly reduced whereas that in the brain was significantly reduced (40%) compared to controls (Fig. 3B). These results show the differential response in the clearance of ROS in the liver and brain of ZDF rats with the progression of metabolic complications.

Alterations in GSH-dependent redox metabolism

A significant decrease (30-40%) in the cytosolic and mitochondrial protein-free GSH concentration was observed in the liver of ZDF rats (Fig. 4A and 4B). However, brain GSH was significantly increased (~40%) both in the cytosolic as well as mitochondrial fractions.

Fig. 4. GSH concentration and GSH-dependent enzyme activities in ZDF rat liver and brain. GSH concentration in the liver and brain of ZDF and ZL rats (n=5) was measured in the cytosol (A) and mitochondria (B) by the enzymatic recycling method of Griffith as described in the Materials and Methods. GST activity, using CDNB as substrate, in the liver and brain of ZDF and ZL rats was measured in the cytosol (C) and mitochondria (D) as described in the Materials and Methods. GSH-Px activity in the liver and brain of ZDF and ZL rats (n=5) was

measured in the cytosol (E) and mitochondria (F) using cumene hydroperoxide as substrate as described in the Materials and Methods. GSH-reductase activity in the liver and brain of ZDF and ZL rats (n=5) was measured in the cytosol (G) and mitochondria (H) as described in the Materials and Methods. Results are expressed as mean $\pm$ S.E.M. from three independent experiments and asterisks indicate significant difference (*p<0.05 and **p<0.01).

GSH-CDNB conjugating activity by GST enzyme in the liver from ZDF rats was significantly decreased (20%-30%) in the cytosolic and mitochondrial fractions (Fig. 4C and 4D). However, GST activity in the subcellular fractions of the brain from ZDF rats decreased significantly only in the mitochondrial fraction and not in the cytosolic fraction. These results again suggest the differential responses in maintenance of antioxidant GSH pools and its conjugating activity in the liver and brain tissues of ZDF rats.

Fig. 5. CYP activities in liver and brain of ZDF rats. CYP 2E1 (A) and CYP 1A1 (B) activities in the liver and brain microsomal fractions of ZDF and ZL rats (n=5) were measured using standard substrates as described in the Materials and Methods. Results are expressed as mean $\pm$ S.E.M. from three independent experiments and asterisks indicate significant difference (* p <0.05).

Fig. 6. Mitochondrial bioenergetics in ZDF rat liver and brain. Mitochondrial respiratory enzyme Complexes I, II/III and IV (A,B,C) activities were measured in freshly prepared mitochondrial fractions from ZDF and ZL rat tissues (n=5) using reduced enzyme specific substrates as described in the Materials and Methods. The ATP content (D) in control ZL and ZDF rat tissues was determined using ATP Bioluminescent cell assay kit (Sigma, St Louis, MO). Results are expressed as mean $\pm$ S.E.M. from three independent experiments and asterisks indicate significant difference (* p <0.05 and ** p <0.01).

GSH-Px activity, showed a significant decrease (20%-40%) in the mitochondrial compartment of the liver and brain tissues in ZDF rats (Fig. 4E and 4F) whereas cytosolic enzyme showed a mild decrease. Similarly, GSH-reductase activity in the mitochondria was significantly reduced (30%-50%) in the liver and brain of ZDF rats compared to the cytosolic fraction (Fig. 4G and 4H).

Fig. 7. Expression of redox regulatory and cell signaling marker enzymes / proteins in ZDF rat liver and brain. Homogenates (50 μ g protein) from ZDF and ZL rat kidney and pancreas were subjected to 12% SDS-PAGE and Western blot analyses to visualize immunoreactivity of specific marker proteins: iNOS, CYP2E1, p-JNK and I κ B- α . Beta-actin was used as loading control. The histograms in Fig. 7 B show densitometric analysis of the relative expression of respective proteins in Fig. 7 A with the statistical analysis. Asterisks indicate significant difference (* p <0.05 and ** p <0.01). The Figures shown are representative of 3-4 independent analyses. Molecular weights shown are in kDa.

Alterations in CYP activities

There was a significant increase (30%-40%) in CYP 2E1 and CYP 1A1 activities in the liver (Fig. 5A and 5B). However, these activities were not significantly altered in the brain of ZDF rats compared to controls.

Mitochondrial bioenergetics

Complex I, II/III and IV activities and mitochondrial ATP content were significantly decreased in the liver and brain tissue of ZDF rats compared to controls (Fig. 6A, 6B, 6C and 6D).

Alterations in the expression of Redox-sensitive marker proteins

Fig. 7 shows an increased expression of oxidative stress and redox-sensitive marker proteins, iNOS and CYP2E1 in ZDF rat liver and brain. On the other hand, the expression of p-JNK, I κ B- α were reduced in ZDF rats compared to ZL controls.

Discussion

ZDF rats exhibit progressive metabolic complications and insulin resistance. Elderly ZDF rats have multiple energy metabolism-associated complications [2, 3, 22, 23]. It has been shown that prolonged exposure to insulin suppresses mitochondrial function which may lead to insulin resistance [11, 12, 24]. Increased insulin resistance has been associated with increased oxidative stress associated cellular damage [25-28]. In the present study, we have provided evidence of increased oxidative and nitrosative stress in tissues from ZDF rats, particularly in the brain. A significant increase in ROS production was observed in the brain while liver exhibited a decrease in the production in ROS suggesting differential ROS

homeostasis in these tissues. An increase in the activity of liver SOD, which metabolizes superoxide radical to H_2O_2 , and a decrease in the catalase activity, which clears H_2O_2 , may have contributed to the differential ROS production and clearance in the liver and brain. ROS metabolizing enzyme CYP2E1, was increased in the liver of ZDF rats but no significant change in the brain was observed. Similarly, CYP1A1, which metabolizes xenobiotic and is under redox regulation, was only increased in the liver but not in the brain. NO production, on the other hand, was increased both in the liver and brain. An increase in lipid peroxidation and protein carbonylation also suggest increased oxidative stress in the brain and liver of ZDF rats. This observation supports our previous studies [13-14] confirming increased oxidative stress and associated complications in ZDF rat tissues. Reduced expression of p-JNK and I κ B- α protein also suggest increased NF- κ B -dependent redox responses in the ZDF rat tissues. Previous studies have also shown that increased NF- κ B activation is associated with inhibition of I κ B phosphorylation which negatively regulates the activation of JNK in ZDF rat tissues [14, 29].

Reduction in the cytosolic and mitochondrial GSH pools, GSH-conjugating activity by GSTs and regeneration of GSH from oxidized GSSG have been observed in the liver. GSH concentration on the other hand, was increased in the brain without any significant changes in the cytosolic GSH metabolism. Mitochondrial GSH metabolism in the liver and brain, however, was significantly decreased suggesting differential responses in ROS clearance and redox homeostasis in the liver and brain of ZDF rats. These results may have implications in affecting the metabolism of endogenous and exogenous substrates and developing insulin resistance. Increase in LPO products such as 4-hydroxynonenal (4-HNE) can enhance protein carbonylation and damage pancreatic β -cells and impair tissue response to insulin, and thus plays a key role in developing metabolic syndrome [30]. The GST activity in ZDF rat tissues was found to be decreased. There are reports suggesting that obese animals have low levels of GST activity for endogenous hydroperoxides; like 4-HNE, produced under oxidative stress conditions [31].

In type 2 diabetes, mitochondrial dysfunction in the tissues, particularly in the pancreas, due to proinflammatory responses and disturbance in redox homeostasis signaling have also been reported which may lead to increased oxidative stress [7, 32, 33]. Our previous studies on type 1 and type 2 diabetic rat models have also suggested that increase in oxidative stress is associated with increased mitochondrial dysfunction [13, 14, 15, 17]. Our present results also show a reduction in the activities of the respiratory complexes and reduced ATP synthesis in ZDF rat tissues, particularly in the brain. Mitochondrial oxygen consumption in tissues is coupled with the production of ROS. ROS production is mainly associated with the activities of Complex I and Complex III and the inhibitors of respiratory enzyme complexes in mitochondria have been reported to increase the production of ROS due to backflow and increased leakage of electrons [34, 35]. Our previous studies on the tissues from diabetic and non-diabetic rats and other species have also demonstrated that the inhibition of respiratory complexes activities are associated with increased ROS production and oxidative stress [13-15, 17, 36].

In conclusion, our experiments in ZDF rats have shown differential responses on the regulation of redox-homeostasis and metabolic stress in the liver and brain. Our results may help in better understanding the management of disease progression and associated complications in diabetes and obesity.

Abbreviations

CYP2E1 (cytochrome P450 2E1); CYP 1A1 (cytochrome P450 1A1); Cyt c ox (cytochrome c oxidase); DCFDA (2',7'-dichlorofluorescein diacetate); DMNA (dimethylnitrosamine); GSH (reduced glutathione); GST (glutathione S-transferase); GSH-Px (glutathione peroxidase); GSH-reductase (glutathione reductase); LPO (lipid peroxidation); NO (nitric oxide); iNOS (inducible nitric oxide synthase); ROS (reactive oxygen species); SOD (superoxide

dismutase); SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis); ZDF (Zucker diabetic fatty); ZL (Zucker lean).

Acknowledgements

We acknowledge the financial research supports from Sheikh Hamdan Bin Rashid Al-Maktoum Award for Medical Sciences, Terry Fox Cancer Research Fund and a grant from Research Committee, College of Medicine and Health Sciences Research Committee, UAE University.

Disclosure Statement

None

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