Glutathione peroxidase, superoxide dismutase and catalase activities in hepatic tissue from children with glycogen storage disease

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Abstract

Introduction: The glycogen storage diseases are caused by inherited deficiencies of enzymes that regulate the synthesis or degradation of glycogen. The most common forms of glycogen storage disease (GSD) are types I, II, III, and IV, which may account for more than 90% of all cases. The most common form is type I, or von Gierke's disease, which occurs in one out of every 100,000 births. Intracellular antioxidant defence is primarily provided by antioxidant enzymes, which catalyse decomposition of reactive oxygen species. To study the oxidative stress status in children with glycogen storage disease by determining activities of glutathione peroxidase, superoxide dismutase and catalase in liver tissue.
Material and methods: Nine children suffering from glycogen storage diseases types I and III were studied. They were selected from the Hepatology Clinic, Cairo University and compared with children who happened to have incidental normal liver biopsy. Glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) levels were measured in fresh liver tissue using ELISA.
Results: Glycogen storage disease patients showed significant increases in SOD and GPX, and there were significant correlations between SOD and both direct bilirubin and prothrombin concentration, and between GPX activity and both ALT and AST.
Conclusions: Oxidative stress could play a role in the pathogenesis of glycogen storage disease. These preliminary results are encouraging to conduct more extensive clinical studies using adjuvant antioxidant therapy.

Key words: glutathione peroxidase, superoxide dismutase, catalase, glycogen storage disease, oxidative stress.

Introduction

The glycogen storage diseases are caused by inherited deficiencies of enzymes that regulate the synthesis or degradation of glycogen [1].

There are 12 glycogen diseases [2]. Glycogen storage disease (GSD) that principally affect the liver include type I, type III, type IV, type VI (liver defect). Because carbohydrate metabolism in the liver is responsible for phosphorylase deficiency, phosphorylase kinase deficiency, glycogen synthetase deficiency, and glucose transporter-2 plasma glucose homeostasis, this group of disorders typically causes fasting hypoglycaemia and hepatomegaly [3].

The most common forms of GSD are types I, II, III, and IV, which may account for more than 90% of all cases. The most common form is type I, or von Gierke's disease, which occurs in one out of every 100,000 births. Other forms, such as types VI and IX, are so rare that reliable statistics are not available. The overall frequency of all forms of glycogen storage disease is approximately one in 20,000-25,000 live births [4].

Oxidative stress is an abnormal phenomenon occurring inside our cells or tissues when production of oxygen radicals exceeds their antioxidant capacity. Excess of free radicals damages essential macromolecules of the cell, leading to abnormal gene expression, disturbance in receptor activity, proliferation or cell death, immunity perturbation, mutagenesis, protein or lipofuscin deposition [5].

Intracellular antioxidant defence is primarily provided by antioxidant enzymes, which catalyse decomposition of reactive oxygen species. The three major antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), differ from each other in structure, tissue distribution, and cofactor requirement [6, 7].

Nguyen *et al*. (2008) showed that sera from GSD-Ia patients have increased total antioxidant capacity compared to controls and this increase correlates with elevated levels of uric acid, a powerful plasma antioxidant. Antioxidant capacity in the sera of GSD-Ia patients may contribute to protection against premature atherosclerosis [8].

To our knowledge, no studies to evaluate the oxidative status in paediatric liver tissue in children with GSD have been performed so far. Therefore, our aim was to study the oxidative status in children with GSD, using an indirect approach in which antioxidant enzymes, namely, glutathione peroxidase, superoxide dismutase and catalase, were determined in the liver tissue.

Material and methods

The present study included 9 children and adolescents (4 males and 5 females) suffering from type I and type III glycogen storage diseases. Patients were diagnosed and selected from the Hepatology Clinic, New Children's Hospital, Cairo University between May 2004 and November 2006. The mean age of these patients was 4.44 ±3.84 years.

They were compared with a group of seven children who happened to have incidental "normal" liver biopsy – they had persistent or intermittent high levels of liver enzymes for more than 6 months (as a control group). Informed consent was taken from the parents of children according to guidelines of the ethical committee of the National Research Centre, Dokki, Egypt.

Exclusion criteria

Patients with acute viral hepatitis, concurrent conditions in which free radical production is elevated such as other inflammatory processes outside the liver, or intake of antioxidant drugs affecting free radical scavenging such as vitamin C, A and E at time of biopsy.

All cases were subjected to:

I) full history taking including – personal history details, past and family histories; vaccination history,

II) thorough clinical examination including – anthropometric measurements including height and weight, vital signs, general and local abdominal examination,

III) routine laboratory investigations including – urine and stool analysis, complete blood count using Coulter counter, assessment of liver functions (total and direct bilirubin, ALT, AST, ALP and GGT; serum total proteins and albumin; prothrombin time and concentration),

IV) other laboratory investigations when needed, e.g. IGg,

V) serological markers of HBV and HCV (e.g. HBsAg, HBcAb, HCVAb, HCV PCR),

VI) abdominal ultrasonography,

VII) glutathione peroxidase, superoxide dismutase and catalase levels in fresh liver tissue (cell free homogenates) using ELISA.

Collection of samples

A liver tissue core was taken from each case using a modified Menghini needle (sure cut). Each sample was divided into 3 portions, one for estimation of each enzyme, and was stored at –80°C till assay.

Glutathione peroxidase assay

Liver biopsy was washed in phosphate buffer, pH 7.4. Then, the tissue was homogenized in 5 ml/gm cold buffer, which consisted of 50 mM tris-HCl, pH 7.5, 5 mM EDTA, 1 nM DTT. The homogenate was centrifuged at 10,000 xg for 15 min at 4°C. The supernatant was removed for assay of GPX.

Glutathione peroxidase activity was measured using the Glutathione Peroxidase Assay Kit provided by Cayman Chemical Company, USA. Glutathione peroxidase catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage. With the exception of phospholipid-hydroperoxide GPX, a monomer, all of the GPX enzymes are tetramers of four identical subunits. Each subunit contains a selenocysteine in the active site, which participates directly in the two-electron reduction of the peroxide substrate. The enzyme uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine [9].

The Cayman Chemical Glutathione Peroxidase Assay Kit measures GPX activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) is produced upon reduction of hydroperoxide by GPX, and is recycled to its reduced state by GR and NADPH.

The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPX activity is rate limiting, the rate of decrease in the A340 is directly proportional to the GPX activity in the sample [10].

Superoxide dismutase assay

Liver biopsy was washed with 0.9% NaCl to remove red blood cells. The tissue was then blotted dry and weighed followed by homogenization in 200 µl buffer (0.05 M potassium phosphate and 0.1 mM EDTA, pH 7.8) and centrifuged at 15,000 xg for 30 min at 4°C. The supernatant was used for determination of SOD.

Superoxide dismutase was measured using the Superoxide Dismutase assay kit provided by Oxis research, USA SOD-525™. The BIOXYTECH assay is based on the SOD-mediated increase in the rate of autoxidation of 5, 6, 6a, 11b-tetrahydro-3, 9, 10-trihydroxybenzo[c]fluorene R1 in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm [11].

Interference due to mercaptans (RSH) such as reduced glutathione is controlled by pre-treating samples with 1-methyl-2-vinylpyridinium R2, which directly eliminates mercaptans by means of a fast alkylation reaction. The kinetic measurement of the 525 nm absorbance change is performed after the addition of R1.

The SOD activity is determined from the ratio of the autoxidation rates in the presence (Vs) and in the absence (Vc) of SOD. The Vs/Vc ratio as a function of SOD activity is independent of the type of SOD (Cu/Zn-SOD, Mn-SOD, Fe-SOD) being measured [11]. One SOD-525 activity unit is defined as the activity that doubles the autoxidation rate of the control blank (Vs/Vc = 2).

Catalase assay

Biopsy was washed with phosphate buffer, pH 7.4, to remove red blood cells. The tissue was then blotted dry and weighed, followed by homogenization in 1.5 ml cold buffer (50 mM potassium phosphate and 1 mM EDTA pH 7) and centrifugation at 10,000 g for 15 min at 4°C was done. The supernatant was used for the assay.

Catalase assay kit provided by Cayman Chemical Company, USA. Catalase is a ubiquitous antioxidant enzyme that is present in most aerobic cells. Catalase (CAT) is involved in the detoxification of hydrogen peroxide (H_2O_2). This enzyme catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water (catalytic activity). Catalase also demonstrates peroxidatic activity, in which low molecular weight alcohols can serve as electron donors, while the aliphatic alcohols serve as specific substrates. In humans, the highest levels of catalase are found in the liver, kidney, and erythrocytes, where it is believed to account for the majority of hydrogen peroxide decomposition.

The Cayman Chemical Catalase Assay Kit utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-trizazole as the chromagen. The assay can be used to measure CAT activity in plasma, serum, erythrocyte lysates, tissue homogenates, and cell lysates [12].

Statistical analysis

SPSS for Windows, version 7.0 computer program was used for statistical analysis. A *p*-value of less than 0.05 was considered statistically significant. The *t*-test was used to compare 2 independent means. Pearson correlation coefficient (*r*) was used to measure the linear relationship between two quantitative variables. Data are represented as the mean ± standard deviation.

Results

Table I shows the descriptive data of the studied children with GSD and the control group. The majority of children with GSD had growth retardation. All patients had abdominal distension, divarication of recti and hepatomegaly. Only 33.3% had splenomegaly. Mild anaemia was positive in 77.7%. Liver biopsy of our patients did not show evidence of hepatic adenomas.

As regards the control group (they were not healthy children but they had normal liver biopsy) only 2 children had mild hepatomegaly, with no splenomegaly. Mild anaemia was positive in 42.86%.

Data in Table II show the values of liver function tests of GSD patients and the control group. In our study, we demonstrated that patients with type I and type III GSDs showed significantly increased SOD and GPX levels in hepatic tissue, reflecting increased antioxidative defence. Also, there were significant correlations between SOD and both direct bilirubin and prothrombin concentration, and between GPX and both ALT and AST levels. Mean levels of superoxide dismutase, glutathione peroxidase and catalase enzymes in the studied

group and controls are shown in Table III. Glycogen storage disease patients showed significant increases in SOD and GPX. In Table IV different correlations between the three enzymes and liver function tests are shown. There was a significant correlation between SOD levels and both DB and PC levels, whereas GPX activity has been found to be significantly correlated with both ALT and AST levels.

Discussion

Growth is generally impaired in patients with GSD I and GSD III, although growth can be improved with good dietary therapy in most patients. However, growth remains unimproved by treatment in some patients; the endocrine parameters of growth in this group are not measurably different from the larger number of patients [3, 13].

In our study, we demonstrated that patients with type I and type III GSDs were mildly anaemic. The cause of this mild anaemia could be nutritional. None of our GSD patients had severe anaemia. The cause of severe anaemia in the absence of renal function compromise in children with GSD I has remained unclear. Some have recently proposed that hepcidin production by hepatic adenomas plays a central role in patients with GSD I. Hepcidin is a peptide hormone that is also a key regulator of the egress of cellular iron; in excess, it may interfere with intestinal iron transport as well as iron release from macrophages [13]. Liver biopsy of our patients did not show evidence of hepatic adenomas.

Oxidative stress is a major pathogenetic event occurring in several liver disorders ranging from metabolic to proliferate ones. The main sources of ROI are mitochondria, cytochrome P450 enzymes in the hepatocytes, Kupffer cells and neutrophils [14].

In our study, we demonstrated that patients with type I and type III GSDs showed significantly increased SOD and GPX levels in hepatic tissue (Table III), reflecting increased antioxidative defence. Also, there were significant correlations between SOD and both direct bilirubin and prothrombin concentration, and between GPX and both ALT and AST levels (Table IV). This increase in the antioxidant activity was compatible with the results of Wittenstein and colleagues, who found that the total radical-trapping antioxidant parameter (TRAP) was elevated in GSD patients. Among single antioxidants analyzed, only elevated uric acid levels in GSD patients strongly correlated with TRAP levels [15].

Glycogen storage disease patients do not seem to develop premature atherosclerosis despite severe hyperlipidaemia present already in infancy. This was shown by different investigators: measurement

Table I. Descriptive data of the studied children with GSD and control group

Items	GSD mean ± SD	Control mean ± SD
Age [years]	7.52 ±4.19	3.62 ±3.17
Male, *n* (%)	4 (44.1%)	3 (42.9%)
Female, *n* (%)	5 (55.6%)	4 (57.1%)
Weight-for-age z score "WAZ"	–1.69 ±1.25	–1.08 ±1.24
Height-for-age z score "HAZ"	–2.75 ±0.88	–1.9 ±2.01
Body mass index "BMI" [kg/m^2]	18.73 ±2.01	17.37 ±2.67
Clinical manifestations	***n* (%)**	***n* (%)**
Abdominal distension	9 (100%)	0 (0%)
Divarication of recti	9 (100%)	0 (0%)
Hepatomegaly	9 (100%)	0 (0%)
Splenomegaly	3 (33.3%)	0 (0%)
Pallor	7 (77.7%)	4 (57.1%)
HB [gm/dl]	10.39 ±1.17	10.74 ±1.26
Platelets [× 1000/mm^3]	480.7 ±136.3	387.1 ±77.3
WBCs [× 1000/mm^3]	11.37 ±2.24	7.59 ±2.51

Table III. Comparison between mean levels (± SD) of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) enzymes in liver tissue of the GSD and control children

Variable	SOD [U/mg Ptn]	GPX [nmol/min/ml]	CAT [nmol/min/ml]
GSD (*n* = 9)	0.85 ±0.21*	78.66 ±31.33*	8.07 ±0.78
Control children	0.33 ±0.15 (*n* = 7)	10.62 ±6.68	7.24 ±1.74

** significant difference ($p < 0.05$) vs. controls*

Table II. Values of liver function tests of GSD patients and control group

Group studied	TB [mg/dl]	DB [mg/dl]	ALT [U/l]	AST [U/l]	AST : ALT ratio	GGT [U/l]	ALB [gm %]	PT Second	PC [%]
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
GSD (*n* = 9)	0.60 ±0.31	0.20 ±0.14	211.91 ±219.25	264.81 ±281.31	1.24 ±0.544	82.47 ±101.59	4.15 ±1.20	11.87 ±0.91	94.23 ±10.04
Control (*n* = 7)	1.04 ±1.22	0.40 ±0.50	115.10 ±136.46	82.22 ±63.37	1.46 ±0.69	46.21 ±51.71	4.55 ±0.49	12.22 ±0.88	94.78 ±7.68

TB – total bilirubin, DB – direct bilirubin, GGT – γ-glutamyl transferase, ALB – albumin, ALT – alanine aminotransferase, PT – prothrombin time, AST – aspartate aminotransferase

Table IV. Correlation between liver function tests and superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) in GSD

	SOD [U/mg Ptn]		GPX [nmol/min/ml]		CAT [nmol/min/ml]	
	r	*p*	*r*	*p*	*r*	*p*
TB	–0.686	> 0.05	–0.618	> 0.05	–0.450	> 0.05
DB	0.122	< 0.05	–0.155	> 0.05	0.111	> 0.05
ALT	–0.308	> 0.05	–0.844	< 0.05	–0.581	> 0.05
AST	–0.322	> 0.05	–0.783	< 0.05	–0.387	> 0.05
ALB	0.248	> 0.05	0.497	> 0.05	0.093	> 0.05
PT	–0.294	> 0.05	–0.394	> 0.05	–0.121	> 0.05
PC	–0.716	< 0.05	–0.616	> 0.05	–0.011	> 0.05
ALP	–0.115	> 0.05	–0.500	> 0.05	–0.207	> 0.05
GGT	–0.490	> 0.05	–0.489	> 0.05	0.025	> 0.05

TB – total bilirubin, DB – direct bilirubin, ALT – alanine transaminases, AST – aspartate transaminases, ALB – albumin, PT – prothrombin time, PC – prothrombin

of vascular endothelial function with high-resolution ultrasonography of the brachial artery in young adults with GSD revealed normal results. Examination of carotid and femoral artery intima/media thickness and aortic dispensability in adolescents with GSD gave no evidence of atherogenic vascular wall alterations [16]. In contrast, early impairment of vascular endothelial function was found in children and adolescents with a family history of coronary artery disease [17, 18]. It was suggested that this increased antioxidative defence in GSD patients might partially contribute to the low risk of atherosclerosis despite severe hyperlipidaemia in GSD [8, 15].

In conclusion, superoxide dismutase and glutathione peroxidase levels are significantly increased in hepatic tissue in children with glycogen storage disease. These findings suggest that oxidative stress is involved in glycogen storage disease, and that superoxide dismutase and glutathione peroxidase are key enzymes in protection of the liver against oxidative stress. Understanding redox regulation may have important clinical ramifications in understanding the pathogenesis of GSD and developing therapeutic approaches.

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