Effect of Oxidative Stress on Glutathione Pathway in Red Blood Cells From Patients With Insulin-Dependent Diabetes Mellitus

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Recently, increased oxidative stress and impaired antioxidant defense have been suggested as a contributory factor for initiation and progression of complications in diabetes. Although glutathione (GSH) and the enzymes included by glutathione redox cycle have an important role for protection of cells against free radical-mediated damage, they may be susceptible to oxidation themselves. We examined the susceptibility of the GSH pathway to oxidation and inactivation in subjects with well-controlled and poorly controlled insulin-dependent diabetes mellitus (IDDM) versus controls and the effect of glycemic control on this susceptibility. Red blood cells (RBCs) were isolated, RBC level of GSH, activity of glutathione peroxidase (G-Px), and glutathione reductase (G-Red) were measured at the baseline and after a 2-hour incubation with hydrogen peroxide. Significant decreases were observed in the GSH level and in the activity of GSH peroxidase and GSH reductase in all the groups after the incubation with hydrogen peroxide. Maximum decrease was observed in the poorly controlled diabetic group for all parameters. This result indicates that the GSH pathway is susceptible to oxidation; and this susceptibility increases in poorly controlled diabetics. Therefore, insufficient antioxidant defense by the GSH pathway may be one of the factors responsible for development of complications in patients with IDDM.

IDDM OCCURS ONCE most pancreatic β cells have been destroyed and therefore have stopped secreting normal insulin levels. Pancreatic β-cell death may occur via immune or nonimmune mechanisms.[1] Pancreatic β-cell lysis by cytotoxic T lymphocytes and cytokine-mediated lysis by macrophages and helper T cells through reactive oxygen species (ROS)-mediated mechanisms are well documented.[2] Recently ROS have gained considerable interest as a contributory factor for the development of diabetic complications.[3-5]

ROS are inactivated by enzymic and nonenzymic antioxidants. Glutathione (GSH) and the enzymes included by glutathione redox cycle are physiologic constituents of the intracellular antioxidant defense system and are present in all mammalian cells.[6] Normal red blood cells (RBCs) are resistant to oxidative damage through their antioxidant system, particulary, GSH redox cycle, so GSH and enzymes that use it are very important in vivo.[7] Glutathione peroxidase (G-Px) removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced GSH. Oxidized glutathione (GSSG) is regenerated by the reaction catalyzed by glutathione reductase (G-Red). The nicotinamide adenine dinucleotide phosphate (NADPH) required is provided by pentose phosphate pathway. Chronic hyperglycemia leads to activation of NADPH-dependent aldose reductase (polyol pathway), which diminishes the NADPH available for G-Red.[7]

Cellular components, such as lipids, proteins, and DNA are targets of ROS-mediated oxidative damage. Increased lipid peroxidation,[5] protein,[8] and DNA[9] oxidation have been shown in diabetes. GSH, G-Per, and G-Red may be susceptible to oxidative damage in diabetic conditions in which oxidative stress is increased. On the other hand, GSH and these enzymes can also be damaged by glycation reactions in poorly controlled diabetics. Nonenzymatic glycation is a spontaneous chemical reaction between glucose and the amino groups of proteins in which reversible Shiff bases and more stable Amadori products are formed. Advanced glycation end produts (AGEs) are then formed through oxidative reactions and cause irriversible chemical modifications of proteins, which can lead the alterations in their structures and functions.[10]

The purpose of the present study was to examine oxidative inactivation of the GSH pathway in subjects with insulin-dependent diabetes mellitus (IDDM) and to disclose the probable contributory effect of elevated blood glucose on this impariment.

MATERIALS AND METHODS

We studied 37 patients with IDDM and 22 healthy volunteers. Patients with levels of glycosylated hemoglobin (HbA_{1c}) higher than 6.2 were considered poorly controlled diabetics. Eighteen of the patients had diabetic complications, such as retinopathy and nephropathy. The characteristics of all the subjects are given in Table 1. All subjects selected for investigation were nonsmokers, and none of them had been administered antioxidant vitamins and drugs. They showed body mass index (BMI) within the normal range. Decleration of Helsinki was adhered to in this study. Informed consent was obtained from each subject.

Blood Sampling

Blood samples were drawn into glass tubes containing ethylendiamine tetra acetic acid (EDTA). RBCs were separated from plasma by centrifugation at 700 $\times$ *g* at 4°C for 15 minutes and washed 3 times with 0.9% saline solution with removal of the buffy coat. Aliquots of the RBCs were taken for determination of GSH, G-Px, and G-Red activities and for incubation with H_2O_2.

GSH and Enzyme Assays

GSH level of the RBCs was measured by the method of Beutler et al[11] using metaphosphoric acid for protein precipitation and 5,5dithiobis 2-nitro benzoic acid (DTNB) for color development at 412 nm. GSH levels were determined using the molar absorption coefficient of

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Table 1. Main Characteristics of the Subjects

	Controls (n = 22)	Well-Controlled Diabetics (n = 17)	Poorly Controlled Diabetics (n = 20)
Age (yr)	29 ± 6	26 ± 8	31 ± 10
Female/male	12/10	9/8	10/10
Serum glucose (mg/dL)	86 ± 11	107 ± 45	207 ± 90
HbA_{1c} (%)	5.4 ± 0.4	5.5 ± 0.6	9.1 ± 2.0

the GSH at 412 nm (1.36×10^4 L · mol^{-1} · cm^{-1}). GSH levels were expressed as micromoles/gHb (μmol/gHb). The Hb concentration was measured by Drabkin's reagent.

G-Px and G-Red activities of the RBCs were measured by kits from Randox (Catolog no: RS504 and GR 2368, respectively) and expressed as U/gHb. One unit of enzyme activity was defined as 1 μmol NADPH oxidized per minute for both of them.

Incubation of Erythrocytes With H_2O_2

A total of 1 mL of a 0.5% RBC suspension in phosphate-buffered saline, 1.5 mmol/L H_2O_2 and 4mmol/L NaN_3 (to inhibit endogenous activity of catalase) was incubated at 37°C for 2 hours in a shaking water bath,[12] and the GSH level and activity of G-Px and G-Red were measured again.

The results are expressed as means ± SD. Statistical analysis was performed by 1-way analysis of variance (ANOVA). Bonferroni and Dunnett tests were used for paired comparisons. Significance level was considered as P less than .05.

RESULTS

RBC level of GSH, activity of G-Px, and G-Red at the baseline and after a 2-hour incubation with H_2O_2 are shown in Table 2. Treatment with H_2O_2 decreased the GSH level and activity of these enzymes in all groups. The magnitude of the decreased functional activity are shown as percentages in Fig 1. The magnitude of the decreased functional activity of GSH was not found to be significantly different from the control and well-controlled diabetic groups. However, it was found to be higher in the poorly controlled diabetic group than in the control group ($P < .001$). The magnitude of the decreased functional activity of G-Px and G-Red was found to be similar in the control and well-controlled diabetic groups; but they were found to be higher in the poorly controlled diabetic group than in both the control ($P < .02$ for G-Px and $P < .001$ for G-Red) and well-controlled diabetic ($P < .05$ for G-Px and $P < .001$ for G-Red) groups. The magnitude of the decreased functional activity was the greatest in the poorly controlled diabetic group.

DISCUSSION

The GSH system has a high capacity to deal with H_2O_2. However GSH, G-Px, and G-Red in RBCs may expose the oxidation via enhanced free radical generation in diabetic conditions. Mechanisms that contribute to increased oxidative stress in diabetes include autooxidative glycosylation, nonenzymatic glycation, metabolic stress resulting from changes in energy metabolism, changes in the level of inflammatory mediators, and the status of antioxidant defense systems.[13] In the present study, 2-hour incubation with H_2O_2 inactivated the GSH pathway in all groups. Previously Pigeolet et al[14] determined 50% inactivation of G-Px, which was purified from bovine erythrocytes, by H_2O_2 in vitro. There is no report indicating G-Red inactivation by H_2O_2 to the best of our knowledge. It has been known that molecules having an easily oxidizable thiol group, such as GSH, are targets of inactivation in vivo.[7] GSH interacts with free radicals by its protein thiol groups in the reduced state.[15] Oxidized glutathione is non functional unless regenerated by G-Red. G-Px and G-Red may be susceptible to ROS via their protein structure. On the other hand, when a protein is damaged in vivo by ROS, it is often marked for proteolytic degradation.[16] Therefore, inactivation of G-Px and G-Red by treatment with H_2O_2, determined in the present study, may be derived from oxidation of enzymes and/or increased degradation of enzymes via heavy oxidation.

It has been observed that the GSH pathway was more susceptible to in vitro oxidation in the RBCs from patients with IDDM than those in the healthy controls. On the other hand, the susceptibility of the GSH pathway to oxidation by H_2O_2 was higher in the subjects with poorly controlled IDDM than those in the subjects with well-controlled IDDM. When the baseline level of GSH and the baseline activity of these enzymes were considered 100%, it was observed that the level of GSH decreased 44% in the well-controlled diabetics and 56% in the poorly controlled diabetics, the activity of G-Px decreased 14% in the well-controlled diabetics and 32% in the poorly controlled diabetics, the activity of G-Red decreased 17% in the well-controlled diabetics and 42% in the poorly controlled diabetics after 2-hour incubation with H_2O_2. Muruganandam et al[17] reported decreased intraplatelet GSH level in the poorly controlled type 1 diabetic subjects as compared with controls. They showed approximately 4-fold higher apparent K_m of G-Px in the platelets from type 1 diabetics with high glycated-Hb in comparison to low glycated-Hb diabetics, and they also suggested that kinetic parameters of the platelet G-Red activity was independent from the glycation state of the type 1 diabetics. The results of the present study are in agreement with

Table 2. GSH Level, G-Px, and G-Red Activities in RBCs of the Subjects at Baseline and After a 2-Hour Incubation with H_2O_2

	Baseline			After Incubation		
	Controls (n = 22)	Well-Controlled Diabetics (n = 17)	Poorly Controlled Diabetics (n = 20)	Controls (n = 22)	Well-Controlled Diabetics (n = 17)	Poorly Controlled Diabetics (n = 20)
GSH (μmol/g Hb)	5.24 ± 0.81	3.69 ± 0.82	2.68 ± 0.50	3.53 ± 0.87*	2.01 ± 0.46‡	1.15 ± 0.38‖
G-Px (U/g Hb)	44.60 ± 9.20	38.50 ± 7.62	34.9 ± 9.41	39.30 ± 7.83†	33.07 ± 5.99	23.56 ± 6.21‖
G-Red (U/g Hb)	10.82 ± 0.81	9.96 ± 1.53	9.77 ± 1.33	9.07 ± 2.02*	8.20 ± 0.96§	5.61 ± 1.05‖

*$P < .001$ *v* controls at baseline, †$P < .01$ *v* controls at baseline, ‡$P < .01$ *v* well-controlled diabetics at baseline, §$P < .01$ *v* well-controlled diabetics at baseline. ‖$P < .001$ *v* poorly controlled diabetics at baseline.

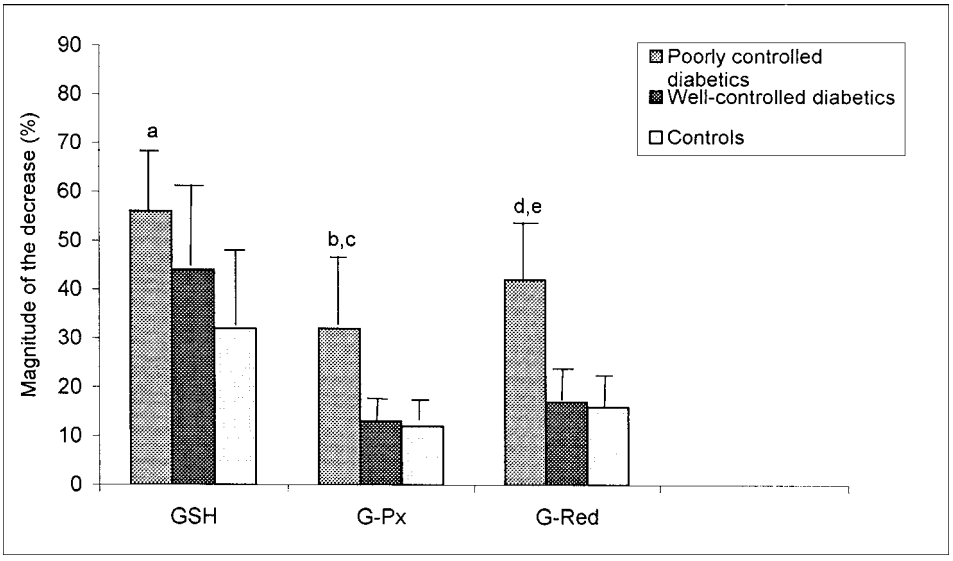

Fig 1. Magnitude of the decreased functional activity of GSH, G-Px, and G-Red after a 2-hour incubation with H_2O_2. [a]$P < .001$ *v* controls, [b]$P < .02$ *v* controls, [c]$P < .05$ *v* well-controlled diabetics, [d]$P < .001$ *v* controls, [e]$P < .001$ *v* well-controlled diabetics.

their data for GSH and G-Px: glycemic control is highly effective on oxidative inactivation of GSH and G-Px. Increased sorbitol synthesis in diabetic subjects causes NADPH depletion, which limits the reduction of oxidized GSH to reduced GSH, catalyzed by G-Red.[7] Furthermore, the activity of enzymes involved in the pentose phosphate pathway which generates NADPH decrease in diabetics.[3] The highly decreased GSH level in the RBCs from the patient with poorly controlled IDDM after a 2-hour incubation in vitro with H_2O_2, determined here, may be derived from NADPH depletion, increased membrane permeability for GSSG due to oxidative stress-induced membrane damage, and GSH consumption in the removal of peroxides.[18] Although our data are in agreement with data from Muruganandam et al[17] for GSH and G-Px, there is conflict for G-Red. Glycemic control is also effective on the magnitude of the inactivation of G-Red according to our data. As far as we know, there is no further report about the effect of glycemic control on the oxidative inactivation of G-Red.

Finally, the GSH pathway in RBCs is impaired by in vitro oxidation with H_2O_2, and poorly controlled diabetic conditions increase the susceptibility of the GSH pathway to oxidation in the subjects with poorly controlled IDDM. Data imply that impaired cellular antioxidant defense by the GSH pathway due to susceptibility of GSH-related antioxidant enzymes to oxidation may be a contributory factor for development of complications in patients with IDDM.

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