Effect of Insulin and Oral Glutathione on Glutathione Levels and Superoxide Dismutase Activities in Organs of Rats With Streptozocin-Induced Diabetes

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SUMMARY

The effect of insulin or glutathione treatment on glutathione content of liver and jejunal mucosa and on superoxide dismutase (SOD) activity of liver, kidney, and erythrocytes was investigated in pair-fed animals with streptozocin (STZ)-induced diabetes. Diabetes lowered hepatic glutathione concentration, but glutathione concentration of the jejunal mucosa was not affected. Insulin, but not oral glutathione, restored hepatic glutathione concentration to normal levels. Diabetes depressed activity of the cytosolic form of SOD in liver, kidney, and erythrocyte. Treatment of diabetic rats with oral glutathione or intramuscular insulin increased cytosolic SOD activity of renal cortex and liver (but not erythrocytes) to control levels. These results suggest a link between glutathione metabolism and cytosolic SOD activity in diabetes. DIABETES 1986; 35:503–507.

Alterations in superoxide dismutase (SOD), peroxidase, and catalase activities and tissue glutathione concentrations have been reported in diabetes.[1] For example, in some tissues of untreated diabetic rats, the activity of the cytosolic (or Cu-Zn-containing) form of the enzyme superoxide dismutase (Cu-Zn SOD) decreases,[2–6] and the activity of the mitochondrial (or Mn-containing) form of superoxide dismutase (Mn SOD) increases.[5,6] Rats with streptozocin (STZ)- or alloxan-induced diabetes have increased liver peroxidase activity, decreased heart peroxidase activity, and increased catalase activity in heart, liver, and kidney.[2] Furthermore, total glutathione concentrations decline in livers of diabetic rats,[7] although hepatic glutathione reductase activity levels do not change.[8] Erythrocyte total glutathione levels of humans with untreated diabetes are lower than normal.[9]

The abnormal levels for both Cu-Zn SOD and Mn SOD activity in diabetes are restored to normal by insulin treatment in all tissues studied except erythrocytes.[5,6] We have previously proposed that the decreased Cu-Zn SOD activity observed in diabetes is the result of an inactivation of the enzyme due to an inability to metabolize hydrogen peroxide via glutathione peroxidase.[1,6] However, diabetes may affect other aspects of the glutathione-peroxide interaction. Glutathione reacts with peroxide to form glutathione disulfide in a reaction catalized by glutathione peroxidase. Glutathione is then regenerated by an NADPH-linked reduction of glutathione disulfide, a reaction catalyzed by glutathione reductase. NADPH is generated by glucose catabolism using the hexose monophosphate shunt. Because hexose monophosphate shunt activity is abnormal in diabetes,[1] NADPH availability might be reduced and the ability to recycle glutathione disulfide to glutathione altered in diabetes. If this hypothesis is correct, treatments that increase glutathione levels may also increase SOD activity in tissues of diabetic animals in the absence of insulin treatment. One such treatment might be administration of glutathione. Novi[10] reported that oral glutathione can cause the regression of aflatoxin-induced tumors. Orally administered glutathione is probably not transported intact into the enterocyte, but rather is hydrolyzed in the lumen to its component amino acids (glutamate, glycine, and cysteine). However, these amino acids are absorbed and can serve as precursors for synthesis of intracellular glutathione.[11,12]

In the present study, we administered glutathione to diabetic rats and determined the effect of such treatment on tissue glutathione levels and SOD activity. We also measured SOD activity in tissues of untreated diabetic animals and in diabetic animals treated with insulin.

MATERIALS AND METHODS

Male Sprague-Dawley rats (King Laboratories, Oregon, Wisconsin) weighing 160–180 g were housed in the animal care

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of Iowa. Animals were provided with Purina rat chow (number 5012) and water ad libitum and were weighed daily. One week after arrival, the animals were randomly divided into control and experimental groups and were entered into the experimental protocol.

On day 1 of this experimental period, control animals were injected intraperitoneally (i.p.) with 0.5 ml of citrate buffer (pH 4.5, prepared by adjusting 2.2% aqueous citric acid to pH 4.5 with NaOH) and animals in the experimental groups were injected with STZ dissolved in citrate buffer (80 mg/kg, provided by the Upjohn Company, Kalamazoo, Michigan). Diabetes was confirmed on the second day postinjection by polyuria, polydipsia, glucosuria (Tes-Tape, Eli Lilly and Company, Indianapolis, Indiana), and weight loss.

Animals were pair fed by the following protocol. The amount of food given to the control animals for the first 4 days after the injection of STZ was the amount of food consumed by the matched group of diabetic animals on the previous day. Beginning with the 4th day after STZ injection, when the diabetic animals began to exhibit hyperphagia, the amount of food given to diabetic animals was restricted to the amount consumed by the control animals on the previous day. All food given was consumed.

On the 5th day after STZ administration, the diabetic animals were randomly divided into three groups. One group received glutathione (100 mg dissolved in 2 ml of water) by gavage at 1600 h daily for 5 days. The second group received an equal volume of isotonic saline. The third group of diabetic animals was treated with 1 U insulin/50 g body wt intramuscularly (i.m.) at 1600 h (NPH, Iletin, Lilly) for 5 days. Insulin-treated diabetics were fed ad libitum. Control animals were divided into two groups of five animals each. One group received oral glutathione for 5 days as described above; the other group received an equal volume of isotonic saline.

Animals were killed on the 10th or 11th day after injection of STZ (5th or 6th day after glutathione or insulin treatment). Rats were anesthetized with an injection of sodium pentobarbital (Nembutal, 50 mg/kg i.p.), and a laparotomy was performed. Blood was drawn from the bifurcation of the aorta, collected in tubes containing 50 U panheparin (Abbott Laboratories, North Chicago, Illinois), mixed, and chilled. Plasma was separated by centrifugation, and the red cells were saved for SOD activity analysis. Plasma glucose concentration was analyzed by glucose analyzer (Beckman Instruments, Inc., Fullerton, California). The entire small intestine was removed and divided into three segments of approximately equal length. The segments were flushed with chilled saline and blotted dry on absorbent paper. Weights of the full-thickness segments were recorded and the segments kept cold under saline-soaked gauze. Mucosa was obtained by slitting open the segments lengthwise on a glass plate over ice and scraping the mucosa off with a microscope slide. Mucosa from the distal 12 cm of the proximal segment was placed in ice-cold sodium-phosphate buffer (50 mM, pH 7.8), homogenized with three 5-s bursts in a Brinkman polytron at maximum power, and set aside for SOD analysis. Mucosa from the midsegment was used as follows: a 1-g aliquot was homogenized by polytron for 3 min in 3 vol of 3% cold metaphosphoric acid, centrifuged to remove precipitated protein, and the supernatant solution frozen (−80°C) for later assay of glutathione content.

The liver was removed, rinsed, blotted, and weighed. Approximately 1 g was homogenized in 20 vol of 3% cold metaphosphoric acid, centrifuged, and the supernatant stored at −80°C for glutathione analysis. A second piece of tissue was removed, homogenized in potassium-phosphate buffer (50 mM, pH 7.8) with three 5-s bursts on the polytron at maximum power setting, and saved for SOD activity analysis. The kidneys were removed, rinsed, blotted, and weighed. The kidneys were slit open, and the renal cortex was dissected from the medulla. The cortex from both kidneys was pooled and homogenized in cold potassium-phosphate buffer (50 mM, pH 7.8) for analysis of SOD activity.

Glutathione content was determined by the procedure of Owens and Belcher.[13] This spectrophotometric procedure measures the change in absorbance (412 nm) occurring when glutathione reduces 5,5′-dithiobis(2-nitrobenzoic acid). Glutathione disulfide (so-called oxidized glutathione) concentrations were also measured in selected samples of liver with the method of Akerboom and Sies.[14] The following methods were used by T.T.D. for data presented as unpublished observations: cysteine content was measured by the procedure of Gaitonde[15] and glycine and glutamate concentrations were analyzed on a Beckman 121 M amino acid analyzer.

SOD activity was assayed with a previously described indirect inhibition assay, in which xanthine and xanthine oxidase serve as a superoxide generator, and nitroblue tetrazolium is used as a superoxide indicator.[5,16] Each sample was assayed twice, once in the absence of inhibitors to measure total (Cu-Zn plus Mn SOD) activity and once in the presence of 5 mM cyanide (inhibits Cu-Zn SOD activity) to measure Mn SOD activity. The Cu-Zn SOD activity was calculated as the difference between total and Mn SOD activity. The specificity of the SOD assay was tested by performing control experiments on one sample from each organ and treatment group. Measurement of endogenous xanthine oxidase activity by following urate formation at 290 nm when nitroblue tetrazolium was omitted from the assay indicated very low levels of endogenous xanthine oxidase activity in the samples. This low activity level did not vary with experimental treatment. Addition of exogenous xanthine oxidase in the absence of nitroblue tetrazolium caused a similar increase in urate formation in all treatment groups, indicating an absence of xanthine oxidase activity inhibitors. Reduction of nitroblue tetrazolium did not occur in the absence of added xanthine oxidase, indicating that reduction of nitroblue tetrazolium by other pathways did not occur. Finally, boiling the tissue homogenate abolished SOD activity, indicating that the activity measured was enzymatic.

Protein content was measured by the procedure of Lowry et al.[17] Data were analyzed by the unpaired *t*-test or by analysis of variance with Dunnett's test for multiple comparisons against one control,[18] with the rejection level of 0.05.

RESULTS

Before experiments with pair-fed animals, we performed an initial study with ad libitum-fed animals (6–9 per group). These studies demonstrated depressed Cu-Zn SOD activity in renal cortex, jejunal mucosa, liver, retina, and erythrocytes. Treatment with oral glutathione increased Cu-Zn SOD activity significantly in all tissues except erythrocytes. Because it was

TABLE 1
Glutathione (GSH) content of liver and jejunal mucosa (μmol/100 g wet wt, mean ± SEM for 5 animals)

Treatment	Liver	Mucosa
Control	671 ± 74	516 ± 44
Control + GSH	653 ± 62	456 ± 32
Diabetic	412 ± 55*	477 ± 38
Diabetic + GSH	508 ± 22†	514 ± 37
Diabetic + insulin	961 ± 72‡	524 ± 37

*Significantly different from control, $P < 0.05$.
†Significantly different from untreated diabetes, $P < 0.05$.
‡Significantly different from diabetes, $P < 0.05$.

possible that differences in food intake between control and diabetic animals accounted for these results, we extended our studies to pair-fed animals.

Initial body weights were similar in all groups (mean group weights ranged from 203 to 211 g). At the time of killing, mean (±SEM) body mass was significantly lower in diabetic animals than in controls (controls, 252 ± 5 g; diabetics, 175 ± 4 g). Liver mass of diabetics was also significantly lower than control (controls, 9.9 ± 0.5 g; diabetics, 6.8 ± 0.3 g). Oral glutathione treatment did not affect body or liver mass of either controls or diabetic animals. Insulin treatment of diabetic animals increased body mass (234 ± 7 g) and liver mass (12.8 ± 1.2 g). No significant change in kidney weight was seen in any experimental group. Untreated diabetic animals had increased plasma glucose levels (744 ± 47 mg/dl) as compared with controls (158 ± 9 mg/dl). Oral glutathione did not affect the plasma glucose concentrations for either controls or diabetic animals. However, insulin treatment significantly lowered the plasma glucose levels of diabetic animals (148 ± 39 mg/dl).

Diabetes decreased liver glutathione concentrations without affecting mucosal levels (Table 1). Glutathione treatment of control animals had no effect on either liver or mucosal glutathione concentrations. Glutathione treatment of diabetic animals increased liver glutathione levels above those of untreated diabetics but did not affect mucosal levels. Insulin treatment of pair-fed diabetic animals significantly increased liver, but not mucosal, glutathione levels relative to untreated diabetic animals.

The SOD activity levels in organs of animals are shown in Table 2. Cu-Zn SOD activity was decreased in renal cortex and liver of diabetic animals as compared with controls. Oral glutathione treatment increased Cu-Zn SOD activity of liver and kidney to control levels in diabetics but had no effect on controls. Insulin treatment restored Cu-Zn SOD activity of liver and kidney to control levels. Although diabetes did not decrease Cu-Zn SOD activity of erythrocytes significantly, mean Cu-Zn SOD activity was lower in diabetics than in controls or controls treated with glutathione. Cu-Zn SOD activity was significantly depressed in erythrocytes from diabetics treated with glutathione or insulin. Mn SOD activity of renal cortex or liver of diabetics was not significantly different from controls or controls treated with glutathione.

DISCUSSION

Our data show that hepatic glutathione levels are decreased by diabetics, confirming earlier work by Krahl.[7] Insulin treatment, but not oral glutathione, restored hepatic levels of glutathione to normal or greater than normal values. Administration of oral glutathione raised hepatic glutathione to a level intermediate between control and diabetic values. The reasons for the decreased hepatic glutathione levels in diabetes are not defined by our study. The decrease might be caused by STZ toxicity in producing diabetogenic action or could be the result of decreased synthesis or increased degradation of glutathione in diabetes. Cellular glutathione metabolism is a complex process involving both the degradation and resynthesis of glutathione as well as interconversion of glutathione and glutathione disulfide.[12] The interconversion of glutathione and glutathione disulfide is closely linked to the NADPH levels produced by the hexose monophosphate shunt, since glutathione reductase uses NADPH to reduce glutathione disulfide to glutathione.[19] Because activity of the hexose monophosphate shunt is altered in diabetes (reviewed in ref. 1), NADPH may be affected.

STZ can deplete erythrocyte levels of glutathione, causing hemolysis.[20] Because other agents deplete cellular glutathione by causing its oxidation,[19,21] STZ may lower the glutathione content of various organs by causing its oxidation to glutathione disulfide, thus increasing its efflux from the cell;[20] however, this seems unlikely. If glutathione synthesis occurs at normal rates, glutathione levels should return to normal values within several hours after STZ administration.[21] Furthermore, STZ is rapidly cleared from the body,[22,23] suggesting that effects noted at 10–11 days after administration of the compound probably are not directly due to the presence of STZ in the tissues.

The lowered level of liver glutathione noted in diabetic animals may also reflect a decreased rate of glutathione synthesis. Rat liver glutathione turns over rapidly.[24] Although the activity of enzymes involved in glutathione synthesis has not been measured in diabetes, a decreased rate of glycine incorporation into glutathione has been observed in liver and diaphragm of diabetic animals,[7] suggesting a decrease in the activity of γ-glutamylcysteine synthetase. Our data are

TABLE 2
Tissue SOD activity (U/mg protein, mean ± SEM, N = 5)

Tissue	SOD activity	
	Cu-Zn	Mn
Renal cortex		
Control	81 ± 10	68 ± 4
Control + GSH	92 ± 14	54 ± 8
Diabetes	47 ± 12*	77 ± 3
Diabetes + GSH	83 ± 10	69 ± 25
Diabetes + insulin	65 ± 11	61 ± 5
Liver		
Control	190 ± 27	67 ± 19
Control + GSH	222 ± 23	51 ± 7
Diabetes	98 ± 12*	47 ± 6
Diabetes + GSH	143 ± 40	87 ± 12
Diabetes + insulin	125 ± 23	86 ± 12
Erythrocyte		
Control	40 ± 3	NP†
Control + GSH	39 ± 2	NP
Diabetes	33 ± 2	NP
Diabetes + GSH	27 ± 3*	NP
Diabetes + insulin	26 ± 5*	NP

*Significantly different from control or control + GSH, $P < 0.05$.
†Mn SOD activity is not present in erythrocytes.

consistent with a block at this point, since both liver cysteine and glutamate levels are elevated in diabetic animals, suggesting a build up of precursor amino acids (T.T.D., unpublished observations). Glutathione normally inhibits γ-glutamylcysteine synthetase,[12] thus regulating total glutathione levels in the cell.[25] Because glutathione levels are depressed in the liver of diabetic animals, this inhibitory effect should no longer be rate limiting. However, tissue glutamate and cysteine content suggests that there may still be a block in the synthetic cycle.

It is also possible that glutathione synthesis is normal but that increased quantities are being exported to meet the glutathione or cysteine requirements of other tissues.[26-30] Endogenously synthesized glutathione is transported from the liver primarily in its sulfhydryl form.[21] However, increased oxidative stress increases the formation and efflux of glutathione disulfide.[19,21] Thus, it is possible that the decreased liver glutathione levels observed in diabetic animals reflected oxidative stress and conversion of glutathione to glutathione disulfide. If the rate of formation of glutathione disulfide exceeded its rate of efflux, tissue levels of glutathione disulfide might increase. However, glutathione disulfide levels did not differ from those of controls in the livers of ad libitum-fed diabetic animals studied (data not shown).

Alternatively, diabetes may increase the rate of glutathione degradation. One-third to one-half of hepatic glutathione is labile and is thought to serve as a cysteine reservoir.[29] Liver and blood glutathione levels decrease during starvation[27,31] or protein restriction,[31] and these changes are thought to reflect the utilization of glutathione to maintain the cysteine pool. If a similar situation occurs in diabetes, we might expect levels of glutathione component amino acids to be affected. In the present study, the decrease in liver glutathione levels noted during diabetes was accompanied by an increase in liver cysteine and glutamate levels, although glycine levels did not change (T.T.D., unpublished observations). Plasma glycine, cysteine, and glutamate levels are depressed in rats with STZ-induced diabetes (T.T.D., unpublished observations), suggesting an increased demand for these amino acids.

Although oral glutathione treatment had only a minimal effect on tissue glutathione levels, treatment raised Cu-Zn SOD activity to control levels. The failure of oral glutathione to affect tissue glutathione levels is not surprising. Oral glutathione is probably not transported intact from the intestinal lumen to the portal blood but is hydrolyzed to component amino acids, which are absorbed.[32,33] Oral glutathione treatment has been reported to have other activities. At the same doses we used, oral glutathione is reported to cause regression of aflatoxin B-induced liver tumors,[10,34] although others could not duplicate these findings.[35,36]

The mechanism by which diabetes affects SOD activity levels is also not clear. STZ can directly inhibit Cu-Zn SOD activity;[3,4] however, due to the short half-life of STZ,[22,23] it is doubtful that STZ directly caused the lowered SOD activity. Furthermore, insulin or glutathione treatment can restore Cu-Zn SOD activity to normal.

Intramuscular insulin or oral glutathione treatments restored Cu-Zn SOD activity in tissues where protein synthesis occurs but not in erythrocytes, which are unable to carry out protein synthesis. The cause of this restoration of Cu-Zn SOD activity is not clear. Activation of the enzyme is probably not involved, because erythrocyte Cu-Zn SOD activity was not increased in diabetic animals by insulin or glutathione treatment.

It is possible that orally administered glutathione transiently increased glutathione levels after dosing but that glutathione levels had dropped to the typical level for diabetes by the time the animals were killed. Yoshimura et al.[37] found the maximal rise in hepatic glutathione levels at 2 h after the administration of oral glutathione or cysteine. A transient rise in glutathione levels may have been adequate to metabolize enough cellular peroxide to protect the Cu-Zn SOD activity. Studies on glutathione levels and SOD activity at various times after the administration of insulin or glutathione are planned to test this hypothesis in diabetic animals.

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