Changes in Hepatic Glutathione Metabolism in Diabetes

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Glutathione is important in the regulation of the redox state, and a decline in its tissue level has often been considered to be indicative of increased oxidative stress in diabetes. In this study of diabetic rats, the level of hepatic glutathione was normal unless food intake was restricted. Thus, the previous report of a reduction in hepatic glutathione in diabetes is likely to be the result of food deprivation rather than diabetes alone. In contrast to changes characteristic of oxidative stress, the efflux of glutathione in bile from diabetic animals was significantly decreased, whereas hepatic mixed disulfides were unchanged, and the hepatic γ-glutamyltransferase activity was considerably increased. These changes were not reproduced by food deprivation. The decrease in biliary excretion of glutathione in diabetes may reflect an attempt to conserve glutathione by activation of the hepatic γ-glutamyl cycle. We conclude that the disturbances of glutathione metabolism in diabetes are not typical of those seen in oxidative stress or food restriction. *Diabetes* **40:344–48, 1991**

The tripeptide glutathione is present in all cell types and is important in the regulation of the redox state and the protection of cells from oxidative damage (1,2). Its importance is exemplified by the findings that fibroblasts and hepatocytes depleted of glutathione die prematurely, a phenomenon prevented by the presence of an antioxidant (3,4).

There has been considerable interest in glutathione metabolism in diabetes because abnormalities in the generation and disposal of free radicals have been postulated to play a pathogenetic role in the chronic complications of diabetes. Several studies have examined the tissue level of glutathione in diabetes with varying results (5–9). The concentrations of glutathione in erythrocytes obtained from diabetic patients and the lens of diabetic rats were lower than normal, whereas the levels in peripheral nerves were normal (5,6). Hepatic glutathione concentration has been reported to be either normal or slightly decreased in diabetes (7–9).

Examination of the pattern and magnitude of glutathione abnormalities may clarify whether there is a generalized increase in oxidative stress in diabetes and whether oxidative stress plays any role in determining why some organs are more susceptible to the development of diabetic complications. Several factors need to be considered in interpreting the results of glutathione metabolism in diabetes. For example, change in food intake has been shown to affect hepatic glutathione levels (10). Diabetic animals consume more food, although weight loss still occurs. Moreover, tissue glutathione is only one component of a complex metabolic cycle. Apart from changes to the rate of its synthesis, glutathione levels can also be affected by its disposal via the formation of mixed disulfides, the inter- and intraorgan transfer via the γ-glutamyl cycle, and, in the case of liver, by its efflux via the biliary tract. In this study, we attempted to better define hepatic glutathione metabolism in diabetes by examining its metabolic disposal pathways and its tissue level. In addition, the relative contribution of diabetes and nutritional factors to changes in glutathione metabolism was investigated. To put into further perspective the possible role of oxidative stress in diabetes, we also studied superoxide dismutase activity and malondialdehyde levels in the liver of normal and diabetic rats.

RESEARCH DESIGN AND METHODS

Streptozocin was obtained from Calbiochem (La Jolla, CA). Reduced and oxidized glutathione, NADPH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), L-γ-glutamyl-*p*-nitroanilide, glutathione reductase, xanthine, xanthine oxidase, superoxide dismutase, cytochrome c (horse heart type VI), and 1,1,3,3,-

tetraethoxypropane were purchased from Sigma (St. Louis, MO). Insulin was obtained from CSL-Novo (Melbourne, Australia).

Several batches of female Wistar rats weighing 150–200 g were used for these experiments. Each batch of 20–30 animals was subdivided into diabetic and control groups. Diabetes was induced by intravenous injection of streptozocin (65 mg/kg), and only animals with a blood glucose level >18 mM 3 days after injection were regarded as diabetic. This resulted in 6–9 normal and 5–8 diabetic animals in each experiment, except for the study on starvation of normal animals when there were 3 animals in each group. All animals had access to water and were fed ad libitum, except where indicated. All experiments were approved by the animal ethics review committee.

The effects of diabetes on hepatic glutathione level and biliary glutathione excretion were studied after 1, 4, and 24 wk of diabetes. The hepatic γ-glutamyltransferase (GGT) activity was measured at 24 wk.

In another experiment, half of the diabetic animals were treated with insulin subcutaneously twice daily (2–4 U Actrapid in the morning and 4–6 U Monotard in the evening) from the onset of diabetes. Blood glucose levels were measured 3 times/day and maintained at <10 mM by adjustment of insulin dose. After 3 wk of diabetes, the rats were anesthetized and studied.

To study the effects of food restriction on glutathione metabolism, animals were divided into normal, diabetic, and food-restricted diabetic groups from the onset of diabetes. Diabetic animals were polyphagic and normally consumed 15–18 g chow/day. Intake of the food-restricted diabetic animals was limited to that consumed by normal animals (10 g/day). This regime was maintained for 24 days before the rats were studied.

In another experiment, the food intake of normal animals was withheld for 72 h before study of glutathione metabolism. Normal animals fed ad libitum were used as controls.

Cannulation of bile duct and preparation of tissue were performed as follows. At the specified times, the animals were anesthetized with an injection of ketamine (Ketalar, 5 mg/kg i.p., Parke-Davis, Detroit, MI), and the body temperature was maintained by placing the animals under a lamp. The bile duct was cannulated with polyethylene tubing with an internal diameter of 0.40 mm (Dural Plastics and Engineering, Dural, Australia), and bile was collected for a total of 20 min. To prevent in vitro oxidation of reduced glutathione, the bile was collected in four aliquots at 5-min intervals, and each aliquot was immediately acidified. Half of the samples were used for measurement of total glutathione (total GSH) and the other half for oxidized glutathione (GSSG). Results are expressed as nanomoles per minute per gram of liver. The animals were then killed by exsanguination by cardiac puncture. The liver was rapidly excised, weighed, and immediately processed for the measurement of total GSH, GSSG, and protein-bound mixed disulfides (XS-SX). A portion of the liver was frozen in liquid N_2 and then stored at −80°C for measurement of GGT activity.

For the determination of total GSH, 0.5 g liver was homogenized in 4 vol ice-cold 5% sulfosalicylic acid and then centrifuged at 3000 rpm (Beckman TJ-6, Fullerton, CA) for

10 min. A 20-μl aliquot of the supernatant was used for measurement of total GSH. Another 0.5-g piece of liver was homogenized in 4 vol 5% sulfosalicylic acid containing N-ethylmaleimide (20 mM) and centrifuged as before. A 400-μl aliquot of the supernatant was neutralized with 400 μl of dipotassium hydrogen phosphate (1.3 M). N-ethylmaleimide was removed by extracting five times with 1.6 ml of a saturated ethyl acetate solution. After excess ethyl acetate was evaporated under N_2, the sample was frozen at −80°C for 24 h. An aliquot of 40–100 μl was used for the measurement of GSSG.

Both the precipitate and the acid-soluble fraction obtained during the preparation of the total GSH sample were processed separately for the measurement of XS-SX. The precipitate was washed three times with 4 ml perchloric acid (0.1 M) and then resuspended in 1.75 ml Tris-HCL buffer (0.1 M, pH 8). The acid-soluble fraction was neutralized with an equal volume of dipotassium hydrogen phosphate (1.3 M). Both fractions were then reduced with sodium borohydride (100 mg). Excess borohydride was removed by the addition of 1 ml ice-cold 50% metaphosphoric acid, and the samples were centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was then neutralized with potassium hydroxide (10 N) and frozen at −80°C for 24 h before assay for XS-SX.

Total GSH, GSSG, and XS-SX (in both the protein precipitate and the acid-soluble form) were measured enzymatically by a method based on the catalytic action of glutathione on the reduction of DTNB by a mixture of NADPH and glutathione reductase (11). Results are expressed as micromoles per gram of liver. GGT was measured with L-γ-glutamyl-p-nitroanilide as a substrate, and the change in absorbance at 410 nm was monitored (12). Results are expressed as nanomoles per minute per milligram of protein. Protein concentration was measured with the Bio-Rad method (Richmond, CA).

The liver samples were prepared for the measurement of superoxide dismutase activity by homogenization in 5 vol potassium phosphate buffer (50 mM, pH 7.8). Triton X-100 (5%, 100 μl) was then added to a 2.5-ml aliquot, which was sonicated and kept on ice for 30 min. Cell membrane fragments were removed by centrifuging at 5000 rpm for 10 min (Sorval SS34 rotor); the supernatant fraction was then further purified by centrifugation at 42,000 rpm for 75 min (Beckman ultracentrifuge Ti 45 rotor). The clear supernatant was then applied to a PD10 column (Pharmacia, Uppsala, Sweden) preequilibrated with 25 ml phosphate buffer (50 mM, pH 7.8). The protein fraction was eluted with 3.5 ml of the same buffer, and the protein concentration was determined with the Bio-Rad method. The total superoxide dismutase activity was measured by the method of Fridovich (13). The activity of the Mn-dependent form of superoxide dismutase was determined after the addition of potassium cyanide (20 mM). The activity of the Cu-Zn form was calculated by subtracting activity of the Mn form from the total. Results are expressed as units per milligram of protein.

Hepatic malondialdehyde levels were determined according to the method of Ohkawa et al. (14) with 1,1,3,3,-tetraethoxypropane as a standard. Results are expressed as nanomoles per gram wet weight of liver.

Results are expressed as means ± SD and analyzed by

TABLE 1
Hepatic glutathione and mixed disulfide levels in normal and diabetic rats

Wk	Total glutathione		Oxidized glutathione (μmol/g liver)		Protein-bound mixed disulfides	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
1	5.73 ± 1.37	5.54 ± 0.44	1.85 ± 0.44	1.86 ± 0.84	0.21 ± 0.02	0.17 ± 0.05
4	6.17 ± 1.10	5.99 ± 0.99	0.95 ± 0.01	1.19 ± 0.28	0.41 ± 0.06	0.21 ± 0.12
24	5.29 ± 0.43	4.27 ± 0.50	0.31 ± 0.08	0.19 ± 0.04	0.19 ± 0.05	0.16 ± 0.03

Values are means ± SD.

analysis of variance with subsequent comparison with Duncan's multiple-range test. $P < 0.05$ was significant.

RESULTS

Diabetic animals gradually lost weight despite unlimited access to food, averaging 20% less than control animals after 24 wk of diabetes. The total GSH and GSSG in the liver and their ratio were not different from normal controls (Table 1). Overall, the XS-SX level in the protein precipitate was not different between normal and diabetic animals. (Although the diabetic XS-SX level was lower at 4 wk, this result was not reproduced at other time points or in further experiments.) The XS-SX level in the acid-soluble fraction was also not different between diabetic and normal animals (normal, 0.05 ± 0.01 μmol/g liver; diabetic, 0.06 ± 0.01 μmol/g liver). However, after 1 wk of diabetes, there was a significant decrease in the efflux of both total GSH and GSSG to the bile. Their levels declined further with the increased duration
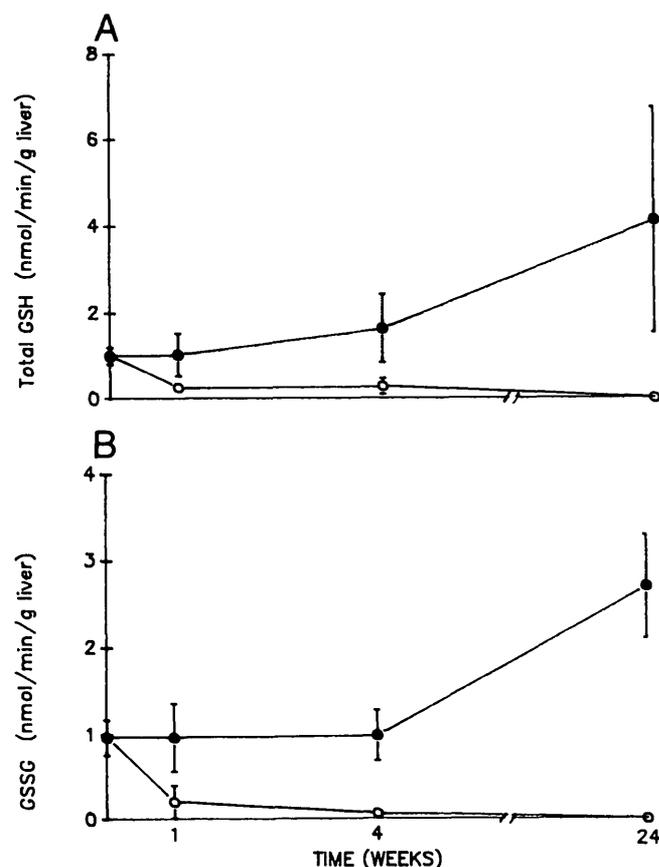
of diabetes, often reaching undetectable levels by 24 wk of diabetes (Fig. 1). The activity of hepatic GGT in diabetic animals was also significantly higher than normal after 24 wk of diabetes (normal, 0.46 ± 0.1 nmol · min^{-1} · mg^{-1} protein; diabetic, 1.09 ± 0.3 nmol · min^{-1} · mg^{-1} protein; $P < 0.01$).

Insulin treatment of diabetic rats for 3 wk reduced blood glucose to near-normal levels (Fig. 2). This degree of diabetic control was able to completely prevent the decreased biliary efflux of GSSG and to normalize substantially the efflux of total GSH. In addition, the increased activity of GGT in the liver of diabetic animals was abolished by insulin treatment (Fig. 3).

Restricting food intake of diabetic animals to the level consumed by normal rats caused a fall in hepatic total GSH (normal, 5.63 ± 0.55 μmol/g liver; diabetic, 5.20 ± 0.45 μmol/g liver; food-restricted diabetic, 3.67 ± 1.31 μmol/g liver; $P < 0.01$).

Fasting of normal animals for 72 h decreased body weight of the rats by a mean of 12%. This reduced the level of hepatic total GSH and GSSG but had no effect on their efflux to the bile or GGT activity (Table 2).

The superoxide dismutase activities in the liver of diabetic animals ($n = 7$) were 25.6 ± 3.9 U/mg protein (total), 11.9 ± 4.3 U/mg protein (Mn dependent), and 13.6 ± 6.3 U/mg protein (Cu-Zn dependent). These levels were higher than the corresponding values in normal animals (19.1 ± 5.9, 7.9 ± 2.4, and 11.2 ± 5.1 U/mg protein, respectively), but the differences did not reach statistical significance.

The hepatic level of malondialdehyde in the diabetic animals was 0.21 ± 0.02 nmol/g liver, significantly lower than that in normal animals (0.30 ± 0.03 nmol/g liver).

FIG. 1. Biliary efflux of total glutathione (GSH; A) and oxidized glutathione (GSSG; B) in normal (●) and diabetic (○) rats.

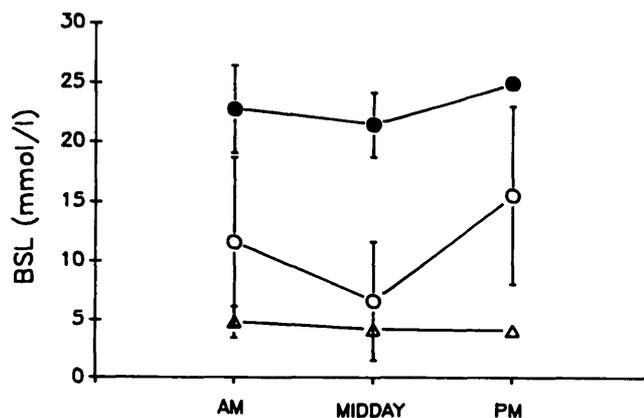
FIG. 2. Blood glucose levels (BSL) in normal (△), diabetic (●), and insulin-treated diabetic (○) rats.
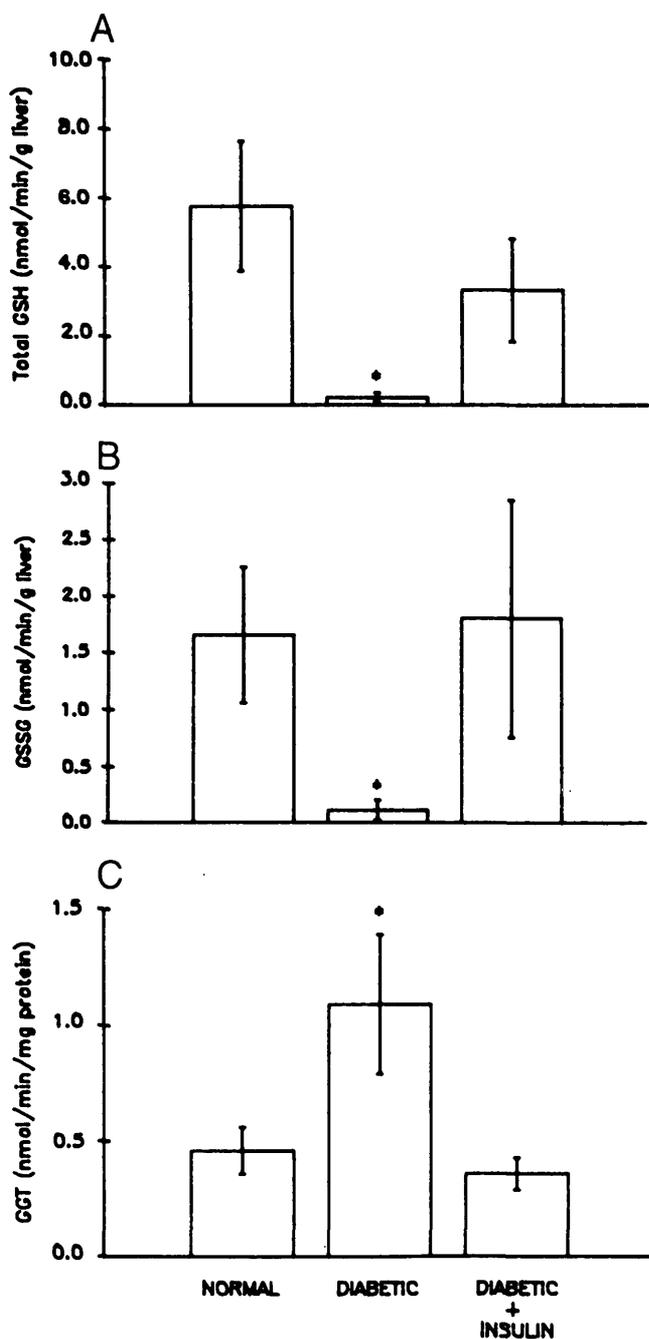
FIG. 3. Effects of 3 wk of insulin treatment from onset of diabetes on biliary efflux of total glutathione (GSH; A), oxidized glutathione (GSSG; B), and hepatic γ-glutamyltransferase (GGT; C) activity. *P < 0.001.

DISCUSSION

Glutathione is a ubiquitous compound with many important functions. It participates in the protection of sulfhydryl groups and the detoxification of electrophilic substances. It serves as a reservoir of cysteine (15,16). It also plays a vital role in the regulation of the redox state and prevention of cell damage by oxidative stress. This last action is of potential physiological significance, because reduction of intracellular glutathione due to removal of cystine in the culture medium leads to the premature death of fibroblasts unless the medium is supplemented with antioxidants (3). Hepatocytes exposed to chemicals that deplete cellular glutathione showed increased lipid peroxidation followed by cell death (4).

Because of its importance, it is not surprising that glutathione metabolism is carefully regulated at many levels. The relative proportion of the reduced and oxidized forms of glutathione is governed by the oxidative state and the activities of glutathione peroxidase and glutathione reductase. The buildup of oxidized glutathione can be minimized by the formation of XS-SX. Glutathione can also be translocated across cell membrane, where it is cleaved by the enzyme GGT. After this step, its constituent amino acids can be taken up to be resynthesized into glutathione to replenish tissue stores. Alternatively, the translocated glutathione can enter the circulation and be taken up by other organs possessing a γ-glutamyl cycle. Quantitatively, the most important of these organs is the kidney, which reabsorbs and conserves most of the filtered glutathione. By a complex interplay of these regulatory steps, tissue glutathione in various organs is maintained at a level sufficient to fulfill its functions as an antioxidant and as a source of cysteine.

Changes in hepatic glutathione metabolism in response to increased oxidative stress have been studied by several methods, including the perfusion of liver with t-butyl hydroperoxide and the chronic feeding of animals with ethanol (17,18). In this situation, increased consumption of reduced glutathione leads to a decline in its level in favor of the oxidized moiety, which can be reduced enzymatically. The levels of oxidized glutathione can also be channeled by conjugation to form mixed disulfides and by enhancement of biliary excretion. Collectively, these steps have the net effect of minimizing a buildup of oxidized glutathione. This occurs at the expense of a decrease in total glutathione resulting from its increased consumption and disposal. This pattern of changes in glutathione metabolism in response to oxidative stress is not mimicked by our findings in the liver of diabetic rats. In diabetes, there was no change in the hepatic mixed disulfide level, and the biliary excretion of glutathione was strikingly diminished, reaching unmeasurable levels after 24 wk of diabetes. There was also no decline in the hepatic glutathione level or change in the proportion of oxidized GSSG to total GSH, provided the animals were allowed to feed ad libitum. Moreover, there was a major increase in the activity of the hepatic GGT in diabetes. Previous studies have demonstrated that a loss in the activity of this enzyme, as a result of either an inborn error of metabolism or administration of an inhibitor such as D-γ-glutamyl-(o-carboxy) phenyl diazide, leads to an inability of tissue to recover the constituent amino acids of glutathione, resulting in its increased loss in the urine (19,20). The in-

TABLE 2
Effects of 72-h fasting of normal rats on glutathione metabolism

	Normal	Fasted
Hepatic total glutathione	8.24 ± 0.28	4.44 ± 0.10
Hepatic oxidized glutathione	0.18 ± 0.07	0.09 ± 0.05
Hepatic protein-bound mixed disulfides	0.36 ± 0.04	0.36 ± 0.06
Hepatic γ-glutamyltransferase	27.3 ± 7.3	27.2 ± 10.8
Bile total glutathione	5.2 ± 3.4	6.04 ± 1.32
Bile oxidized glutathione	3.48 ± 0.84	3.09 ± 0.52

Values are means ± SD.

creased hepatic GGT activity that we observed in diabetes probably represents a compensatory mechanism to conserve glutathione. Because GGT has been localized in the biliary canaliculi (21), it is possible that the decreased excretion of glutathione by the biliary route in diabetes is a result of increased activity of the γ-glutamyl cycle at this level. Whether the combined effects of increased GGT activity and decreased biliary glutathione excretion could have masked a fall in hepatic glutathione level in diabetes remains to be tested.

Because we cannot readily invoke increased oxidative stress to explain the pattern of changes we observed, the mechanisms underlying the alterations in hepatic glutathione metabolism in diabetes remain to be determined. Food deprivation alone is not a sufficient explanation, because the changes in biliary glutathione efflux and hepatic GGT activity are not observed when normal animals are starved. However, there is a fall in hepatic glutathione level when normal or diabetic rats are restricted in their food intake. Thus, the decline in hepatic glutathione in diabetes reported by Loven et al. (7) is likely to be a result of the food restriction of diabetic animals used in their protocol. Because the changes in hepatic glutathione metabolism persist long after the injection of streptozocin and insulin is able to substantially reverse them, it seems unlikely that streptozocin toxicity plays a dominant role.

The hypothesis that there is increased oxidative stress in diabetes is an important one, because oxidative damage could be a contributing factor to the development of diabetic complications. Although increased products of lipid peroxidation have been found in the circulation of diabetic patients and animals (2), there is no unanimous agreement that increased oxidative stress occurs as a general phenomenon in diabetes. In this study, the levels of hepatic superoxide dismutase activity and malondialdehyde also did not show a clear increase in oxidative stress. The changes of hepatic glutathione metabolism in diabetes are not typical of those seen in states of increased oxidative stress. Many factors, including nutritional status, can affect tissue glutathione level. Moreover, compensatory mechanisms may mask any change in tissue turnover of glutathione. Thus, glutathione should only be accepted as an index of the tissue oxidative state when these factors have been taken into consideration.

ACKNOWLEDGMENTS

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