

Glutathione Redox State Is Not the Link Between Polyol Pathway Activity and *Myo*-Inositol-Related Na⁺-K⁺-ATPase Defect in Experimental Diabetic Neuropathy

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SUMMARY

Decreased glutathione levels in the ocular lens have been invoked as a possible cause for the decreased lenticular Na⁺-K⁺-ATPase in diabetes because both are corrected by aldose reductase inhibitors, and the Na⁺-K⁺-ATPase is known to be susceptible to oxidation inactivation. Because an analogous Na⁺-K⁺-ATPase defect that is prevented by aldose reductase inhibitors has been described in diabetic peripheral nerve, we examined the effect of streptozocin (STZ) diabetes and aldose reductase inhibition on reduced (GSH) and oxidized (GSSG) glutathione levels in crude homogenates of rat sciatic nerve. Neither GSSG nor GSH levels were altered by 2 or 8 wk of untreated diabetes or by aldose reductase inhibition. Because the defect in Na⁺-K⁺-ATPase is fully expressed by 4 wk of STZ diabetes, we conclude that altered glutathione redox state plays no detectable role in the pathogenesis of this defect in diabetic peripheral nerve. DIABETES 1986; 35:1282–85.

Recent in vivo and in vitro studies have identified a set of interrelated metabolic abnormalities in target tissues for diabetic complications that are attributable to elevated ambient blood glucose concentrations.¹ In combination, these metabolic changes induce a variety of biochemical and biophysical alterations in nerve that may be highly relevant to the pathogenesis of diabetic peripheral neuropathy. Acutely reversible slowing of nerve conduction in diabetes in the rat has been localized to an alteration of nerve Na⁺-K⁺-ATPase activity.² This defect is prevented by oral *myo*-inositol (MI) supplementation or aldose reductase inhibition that normalizes nerve MI content in diabetes.^{3,4} The mechanism(s) linking altered nerve polyol and metabolism to decreased MI and Na⁺-K⁺-ATPase has

yet to be elucidated. Alterations in the cytoplasmic redox potential could serve as a hypothetical link between polyol pathway activity, MI metabolism, and Na⁺-K⁺-ATPase activity (Figs. 1 and 2). Increased polyol pathway activity alters the NADPH-to-NADP⁺ ratio in several tissues because reduction of glucose to sorbitol requires NADPH as a cofactor.^{5,6} The NADPH:NADP⁺ redox potential is linked to glutathione via the reversible NADP⁺-requiring glutathione reductase reaction. Glutathione in its reduced form (GSH) is a well-known antioxidant.⁷

A 60% decrease in the GSH content of the lens in diabetes is prevented by treatment with an aldose reductase inhibitor.⁸ Oxidation of GSH in lens has been associated with a secondary inhibition of lens Na⁺-K⁺-ATPase activity that has been speculatively attributed to increased formation of disulfide bridges in the enzyme.⁹ Increased polyol pathway activity in the diabetic nerve might diminish protective GSH levels, thus allowing oxidation inactivation of critical membrane proteins such as Na⁺-K⁺-ATPase or a Na⁺-dependent MI cotransporter.¹⁰ *Myo*-inositol depletion is thought to lead to alterations of phosphoinositide metabolism that could perpetuate the depletion of MI through further impairment of Na⁺-K⁺-ATPase¹¹ (Fig. 2). We therefore hypothesized that an alteration in the ratio of GSSG to total glutathione (GSSG + GSH) might impair function of the Na⁺-K⁺-ATPase and/or the Na⁺-dependent MI transporters in diabetic peripheral nerve. Thus, we measured GSH and GSSG in crude homogenates of sciatic nerve from untreated and sorbinil-treated diabetic rats and nondiabetic controls. Neither diabetes nor sorbinil treatment altered the content of these metabolites in peripheral nerve, suggesting that the mechanism of decreased Na⁺-K⁺-ATPase activity and decreased MI content is not oxidation inactivation due to altered glutathione content in diabetic peripheral nerve.

MATERIALS AND METHODS

Animal model. Cesarean-delivered, barrier-sustained, age-matched male Wistar rats (initial weight 180–200 g) were maintained throughout the study on standard rat chow and were given free access to food and water. Diabetes, defined

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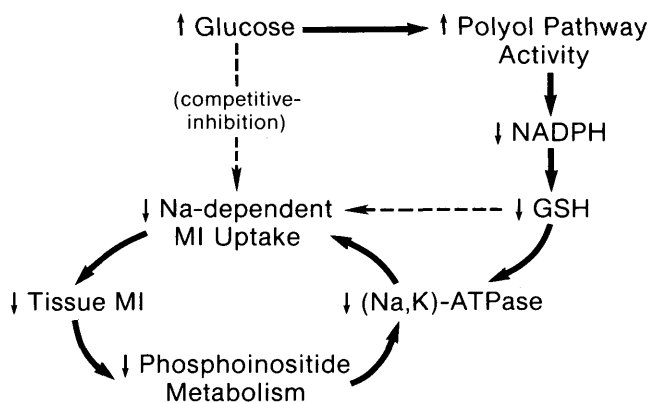


FIG. 1. Postulated relationship between hyperglycemia polyol pathway, reduced glutathione (GSH), myo-inositol (MI), and Na⁺-K⁺-ATPase in diabetes. Glucose at hyperglycemic concentrations increases polyol pathway activity. When glucose is enzymatically converted to sorbitol by this path, NADPH is depleted, leading to diminished GSH; this contributes to reduction of Na⁺-K⁺-ATPase activity possibly mediated by sulfhydryl bond interactions, which may also act to diminish Na⁺-dependent MI uptake. Hyperglycemia itself, through competitive inhibition, decreases Na⁺-dependent MI uptake. Self-reinforcing metabolic cycle of continued depletion of MI, Na⁺-K⁺-ATPase activity, and phosphoinositide metabolism is thus postulated.

by nonfasting plasma glucose concentrations >300 mg/dl, was induced with streptozocin (STZ; Upjohn, Kalamazoo, MI), 60 mg/kg i.v. injected in 0.10 ml of 0.01 M citrate buffer, pH 5.5, into the tail vein of rats fasted overnight. Sorbinil (Pfizer, Groton, CT) was suspended in distilled water (final concentration 4–5 mg/ml) as previously described¹² and was administered daily by gavage at a dose of 20 mg/kg.

Tissue and plasma collection. At the conclusion of the 2- and 8-wk studies, nonfasted animals were verified to be diabetic and were anesthetized with pentobarbitol sodium. Midhigh segments of right and left sciatic nerves were isolated and dissected free of adherent muscle and epineurium with strict hemostasis. The nerves were firmly blotted to minimize any residual retention of blood and were then weighed and immediately processed for enzymatic GSH determination or fluorometric GSH and oxidized glutathione (GSSG) determinations.

Analytic techniques. Plasma glucose was determined in a Beckman glucose analyzer II (Beckman, Fullerton, CA). Reduced glutathione was measured in freshly prepared crude

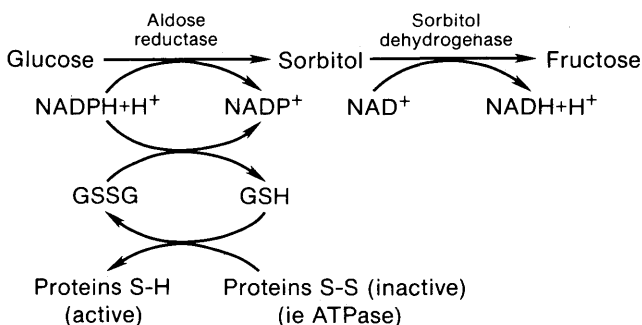


FIG. 2. Hypothesized role for glutathione in diabetic neuropathy. Polyol pathway, activity of which is increased in diabetes through aldose reductase, converts glucose into sorbitol utilizing NADPH. Significant depletion of NADPH might lead to deplete reduced form of glutathione (GSH) that protects against oxidation inactivation of critical membrane proteins, i.e., Na⁺-K⁺-ATPase.

homogenates of whole sciatic nerve with the spectrophotometric enzymatic method of Tietze.¹³ Briefly, nerve segments were homogenized at 4°C in 0.1 ml 1% (vol/vol) picric acid with a motor-driven glass-on-glass homogenizer. Five to 10 µl of homogenate were assayed for cyclic formation of 2-nitro-5-thiobenzoic acid (TNB) in 1.02 ml of a reaction mixture containing 800 µl 0.75 mM NaH₂PO₄-H₂O, 6.3 mM EDTA, pH 7.5, and 10 µl glutathione reductase (dialyzed 24 h in the same buffer). After 3 min incubation at 30°C, TNB was measured in a Varian (Palo Alto, CA) Cary Model 210 spectrophotometer at 410 nm. Standard curves were determined daily with freshly prepared solutions of GSH in distilled water. Reduced and oxidized glutathione were determined fluorometrically with a modification of the methods of Hissin and Hilf and Mokrash and Teschke.¹⁵ Nerve segments were freshly homogenized as described previously in 200 µl of 0.5 M formic acid. Reduced glutathione was assayed utilizing the pH-dependent formation of the fluorescent o-phthalaldehyde (OPT) GSH complex at pH 8. Five to 10 µl of homogenate were assayed in 1.3 ml of reaction mixture in acid-washed test tubes containing 0.1 ml 37% (vol/vol) buffered formalin, 0.1 M Na₂H₂PO₄, 5 mM EDTA, pH 8, and 0.1 mg/L OPT in methanol. After the reaction stabilized, GSH-OPT was determined with a Farrand A₄ fluorometer with Corning primary filter 5860 and secondary Corning filters 4303 and 3387. Oxidized glutathione was similarly determined in the same homogenates, utilizing the pH-dependent formation of OPT-GSSG fluorophore at pH 13 in the presence of N-ethylmaleimide (NEM); 1.3 ml of reaction mixture containing 0.005 ml 0.04 M NEM, 0.100 ml 37% (vol/vol) buffered formalin, 0.1 mg/L OPT in methanol, and 0.5 M NaOH was assayed. Fresh GSSG standards were prepared daily. All reagents were obtained from Sigma (St. Louis, MO) and were of the highest available purity unless otherwise indicated. Acids were obtained from Fisher (Fairlawn, NJ). Fluorometric scanning demonstrated no enhancement or quenching of OPT-GSH or OPT-GSSG fluorescence by 0.5 M NaOH or 0.5 M formic acid. Recovery studies showed the optimal pH range for the determination of GSH to be 8 and GSSG to be 12–13. The amount of NEM selected (0.005 ml of 0.04 M) completely eliminated GSH interference with the GSSG assay. Recovery of added GSH and GSSG was >95% for the spectrophotometric and >80% for the fluorometric assays. Spectrophotometric and fluorometric measurements of GSH in individual samples correlated with an *r* value >.75.

The ability of these assays to detect small variations in GSH content of rat sciatic nerve was verified with buthionine sulfoxamine (BSO) (Chemical Dynamics, South Plainfield, NJ), a compound known to cause small but significant changes in tissue GSH.¹⁶ Three doses of BSO, 90 mg/100 g

TABLE 1
Glutathione content of controls and buthionine sulfoxamine-treated normal Wistar rats

N	Body wt (g)	Glucose (mg/dl)	Reduced glutathione (µg/g nerve)
Control 6	203 ± 2	107 ± 4	122 ± 12
BSO 8	199 ± 2	96 ± 4	86 ± 10

Spectrophotometric measurements are means ± SE.

TABLE 2
Body weight, plasma glucose, and reduced glutathione content of rat sciatic nerves after 2 wk of STZ diabetes

N	Body wt (g)	Plasma glucose (mg/dl)	Reduced glutathione (μg/g nerve)
Control 12	364 ± 5	148 ± 3	276 ± 18
Untreated diabetic 11	242 ± 7	525 ± 28	264 ± 13
Sorbinil-treated diabetic 13	236 ± 12	464 ± 17	242 ± 12

Spectrophotometric measurements are means ± SE.

body wt s.c., were given to normal male Wistar rats, mean weight 200 g (controls received matched quantity of distilled water vehicle). The first injection was given at 16 h, the second at 15 h, and the final dose at 3 h before death, at which time sciatic nerves were immediately processed for GSH and GSSG measurements as described previously.

Statistics are presented as means ± SE, and significance of difference was calculated by Student's *t* test.

RESULTS

Validation of assay: effect of BSO treatment on glutathione content of rat sciatic nerves. Treatment with 3 doses of 90 mg/100 g BSO resulted in statistically significant 29 and 36% decreases, respectively, in spectrophotometrically (Table 1) and fluorometrically determined GSH (not shown). This compares to a 7% decrease measured in brain and a 33% decrease in skeletal muscle measured after single-dose BSO,¹⁶ and gives values similar to those obtained in greyhound sciatic nerve.¹⁷ Thus, small changes in GSH were detectable by the assays employed in these studies.

Effect of 2 wk of STZ diabetes and sorbinil treatment on spectrophotometrically determined GSH content of rat sciatic nerve. At the conclusion of the 2-wk study period, STZ diabetic rats demonstrated a 33% decrease in body weight compared with age-matched controls (Table 2). Sorbinil treatment had no effect on the reduction in body weight. Untreated diabetics had significantly higher plasma glucose than controls, and, although there was a trend for lower plasma glucose in sorbinil-treated diabetics, this was not statistically significant.

Levels of GSH were somewhat higher in the rats that were several weeks older than those used in the validation study. Two weeks of untreated STZ diabetes produced a statistically insignificant 4% decrease in the amount of GSH in sciatic nerve homogenates compared with nondiabetic controls.

Sorbinil treatment produced a further but again insignificant 12% decrease in sciatic nerve GSH in diabetic rats. Thus, neither untreated diabetes nor administration of an aldose reductase inhibitor produced changes in nerve GSH content after 2 wk.

Effect of 8 wk of STZ diabetes and sorbinil treatment on spectrophotometric and fluorometric GSH and fluorometric redox ratios in rat sciatic nerves. To determine if more prolonged diabetes affected nerve GSH, or if the effect of diabetes was expressed primarily as a change in the GSSG/GSSG + GSH ratio, an 8-wk study was performed during which both GSH and GSSG were measured fluorometrically.

At the end of the 8-wk study period, STZ diabetic rats demonstrated a 38% decrease in body weight compared with age-matched normal controls. Sorbinil treatment had no effect on the reduction in body weight (Table 3). Plasma glucose levels were significantly elevated in the diabetic animals compared with controls; although there was a trend for lower plasma glucose in the sorbinil treated group, it was not statistically significant. Fluorometric GSH levels in these older rats were somewhat higher. Approximately 2.5% of total glutathione was in the oxidized form. Neither the concentration of GSH or GSSG nor the ratio of GSSG to total glutathione was changed by 8 wk of untreated STZ diabetes or sorbinil administration. Thus, neither diabetes nor aldose reductase inhibition altered GSH, GSSG, or the GSSG/GSSG + GSH ratio in peripheral nerve.

DISCUSSION

Nerve conduction impairment in acutely diabetic rats is attributable to an MI-related defect in nerve Na⁺-K⁺-ATPase.² This defect in MI and Na⁺-K⁺-ATPase is corrected by sorbinil. The mechanisms relating this defect to polyol pathway activity and mediating its correction by aldose reductase inhib-

TABLE 3
Body weight, plasma glucose, GSH, GSSG, and GSSG/GSSG ± GSH ratio in 8-wk STZ rat sciatic nerves

N	Body wt (g)	Plasma glucose (mg/dl)	GSH (μg/g)	GSSG (μg/g)	GSSG/GSSG + GSH (%)
C 10	492 ± 14	159 ± 10	299 ± 16	6.89 ± 0.95	2.25 ± 0.28
D 9	303 ± 15	523 ± 41	267 ± 15	6.50 ± 0.74	2.38 ± 0.37
D + S 9	300 ± 27	467 ± 27	277 ± 35	6.06 ± 0.67	2.14 ± 0.24

Fluorometric measurements are means ± SE. GSH, reduced glutathione; GSSG, oxidized glutathione.

itors are unknown. One mechanism by which the polyol pathway might be implicated in the pathogenesis of this defect would be through alteration of cytoplasmic redox state due to NADPH and GSH depletion, thus leaving the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ subject to oxidative inactivation (Fig. 1). Oxidative inactivation of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ occurs through modification of its sulfhydryl groups,¹⁸ possibly by a glutathione-mediated mechanism. Diminished GSH in the diabetic lens is correctable with aldose reductase inhibitors.⁸ Oxidation of GSH in lens has been associated with secondary inhibition of lens $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity, speculatively attributed to direct action of GSH on formation of disulfide bridges in the enzyme.¹⁹ Decreased GSH levels have been hypothesized to play a role in the pathogenesis of experimental cataract formation.²⁰

If GSH availability is responsible for maintaining functionally important sulfhydryl groups on vital membrane proteins (i.e., $\text{Na}^+ - \text{K}^+ - \text{ATPase}$), then alterations in GSH and/or GSSG should precede measurable decreases in the activity of these proteins. Levels of GSH are decreased dramatically in the lens after 2 wk of STZ diabetes⁸; the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ defect is fully expressed in sciatic nerve after 4 wk of STZ diabetes³ and is known to persist to 12 wk.² Neither diabetes nor sorbinil treatment altered GSH at 2 or 8 wk of diabetes nor did they alter GSH, GSSG, or the GSSG/GSH + GSH ratio at 8 wk of diabetes. The discordance between the effect of diabetes on glutathione content in lens versus our data in nerve may be due to differences in the content in enzymes involved with glutathione synthesis, maintenance of the redox state, differences in transport mechanisms, or differential susceptibility to oxidant stresses between these target tissues. An alternative explanation might be depletion of metabolites due to leakage mediated by osmotic effects believed to be important in the lens but insignificant in nerve.²¹ Hence, in contrast to the lens where diabetes decreases and aldose reductase inhibition corrects diminished GSH content, neither diabetes nor sorbinil treatment significantly alters GSH or GSSG content in STZ diabetic peripheral nerve. Therefore we conclude that alteration of glutathione content or the redox state cannot play a major role in the pathogenesis of the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ defect in diabetic peripheral nerve.

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REFERENCES

- Greene, D. A., Lattimer, S. A., Ulbrecht, J., and Carroll, P.: Glucose-induced alterations in nerve metabolism: current perspective on the pathogenesis of diabetic neuropathy and future directions for research and therapy. *Diabetes Care* 1985; 8:290-99.
- Greene, D. A., Yagihashi, S., Lattimer, S. A., and Sima, A. A. F.: Nerve $\text{Na}^+ - \text{K}^+ - \text{ATPase}$, conduction and *myo*-inositol in the insulin deficient BB rat. *Am. J. Physiol.* 1984; 247:E534-39.
- Greene, D. A., and Lattimer, S. A.: Impaired rat sciatic nerve sodium-potassium ATPase in acute streptozocin diabetes and its correction by dietary *myo*-inositol supplementation. *J. Clin. Invest.* 1983; 72:1058-63.
- Greene, D. A., and Lattimer, S. A.: Action of sorbinil in diabetic peripheral nerve: relationship of polyol (sorbitol) pathway inhibition to a *myo*-inositol mediated defect in sodium-potassium ATPase activity. *Diabetes* 1984; 33:712-16.
- Morrison, A. D., Clements, R. S., Travis, S. B., Oski, F., and Winegrad, A. I.: Glucose utilization by the polyol pathway in human erythrocytes. *Biochem. Biophys. Res. Commun.* 1970; 40:199-205.
- Travis, S. F., Morrison, A. D., Clements, R. S., Winegrad, A. I., and Oshi, F. A.: Metabolic alterations in the human erythrocyte produced by increases in glucose concentrations. *J. Clin. Invest.* 1971; 50:2104-12.
- Meister, A.: New aspects of glutathione biochemistry and transport: selective alteration of glutathione metabolism. *Fed. Proc.* 1984; 43:3031-42.
- Gonzalez, A. M., Sochor, M., and McLean, P.: The effect of an aldose reductase inhibitor (sorbinil) on the level of metabolites in lenses of diabetic rats. *Diabetes* 1983; 32:482-85.
- Whikehart, D. R., and Soppet, D. R.: The effects of glutathione and adenosine on plasma ATPases of the corneal endothelium. A hypothesis on the stimulatory mechanism of perfused glutathione upon deturgescence. *Curr. Eye Res.* 1981; 1:451-55.
- Greene, D. A., and Lattimer, S. A.: Sodium- and energy-dependent uptake of *myo*-inositol by rabbit peripheral nerve. *J. Clin. Invest.* 1982; 70:1009-18.
- Greene, D. A., and Lattimer, S. A.: Protein kinase c agonists acutely normalize decreased ouabain-inhibitable respiration in diabetic rabbit nerve: implications for diabetic complications. *Diabetes* 1986; 35:242-45.
- Finogold, D., Lattimer, S. A., Nolle, S., Bernstein, M., and Greene, D. A.: Polyol pathway activity and *myo*-inositol metabolism: a suggested relationship in the pathogenesis of diabetic neuropathy. *Diabetes* 1983; 32:988-92.
- Tietze, F.: Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal. Biochem.* 1969; 27:502-22.
- Hissin, P. J., and Hilf, R.: A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* 1976; 74:214-26.
- Mokrasch, L. C., and Teschke, E. J.: Glutathione content of cultured cells and rodent brain regions: a specific fluorometric assay. *Biochem.* 1984; 140:506-509.
- Larsson, A., Holmgren, A., Orrenius, S., and Mannervik, B.: *Functions of Glutathione Biochemical, Physiological, Toxicological and Clinical Aspects.* New York, Raven, 1982.
- Chvapil, M., Kern, J. M., Misiorowski, R., and Weinstein, P. R.: Endogenous-antioxidants and rate of malondialdehyde formation in central and peripheral nervous systems. *Exp. Neurol.* 1982; 78:765-74.
- Esmann, M., and Norby, J. G.: A kinetic model for *N*-ethylmaleimide inhibitor of the ($\text{Na}^+ + \text{K}^+$)ATPase from rectal glands of squalus acanthias. *Biochim. Biophys. Acta* 1985; 812:9-20.
- Epstein, D. L., and Kinoshita, J. H.: The effect of diamide on lens glutathione and lens membrane function. *Invest. Ophthalmol. Vis. Sci.* 1970; 18:468-75.
- Ansara, K. H., and Srivastava, S. K.: Role of glutathione in the prevention of cataractogenesis in rat lenses. *Curr. Eye Res.* 1982-3; 4:271-75.
- Greene, D. A.: Metabolic abnormalities in diabetic peripheral nerve: relation to impaired function. *Metabolism* 1983; 32:118-23.