# Glutathione Levels Are Reduced in Diabetic Rat Retina But Are Not Influenced by Ischemia Followed by Recirculation

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**Free radicals have recently been proposed to play a role in the development of diabetic retinopathy. The aim of the present study was to examine whether the abnormal metabolism caused by diabetes and by ischemia followed by recirculation interferes with a free radical enzyme defense system in the retina, ie, glutathione. Diabetes mellitus was induced by injecting streptozotocin ([STZ] 60 mg/kg body weight [BW] intraperitoneally). After 2 and 6 months, respectively, glutathione levels were measured in the retina and compared against those of age-matched normal control rats. Retinal ischemia was induced by careful ligation of the vessels and the accompanying optic nerve behind the left eye bulb. The right eye served as a control. After 90 minutes of ischemia, retinal circulation was reestablished by removing the ligature. Two-month-old diabetic rats were kept for an additional 3 days and normal rats for 5 minutes, 15 minutes, or 3 days before they were killed for measurement of glutathione. Retinal levels of glutathione were significantly lower in 6-month diabetic compared with 2-month diabetic rats (16.6 ± 2.9 *v* 19.0 ± 2.2 nmol/mg protein, $P < .05$) and 6-month normal control rats (16.6 ± 2.9 *v* 21.0 ± 2.1 nmol/mg protein, $P < .001$). Ischemia followed by recirculation did not influence the total tissue level of glutathione either in 2-month-old diabetic rats or in normal rats. The present study indicates that the abnormal metabolism caused by diabetes, rather than by changes in retinal circulation, results in an impaired defense mechanism against free radicals, a factor that may be of importance for the development of diabetic retinopathy. However, since glutathione levels in the present study were measured in the whole retina, it cannot be excluded that particular cell types, such as vascular cells, show an alteration in glutathione that is masked by the glutathione levels in the other nonvascular cells of the retina. Studies using other techniques are needed to further explore this subject.**



THE PATHOGENESIS of diabetic retinopathy is still not clear. Recently, some clinical and experimental approaches have suggested that reactive oxygen species might be involved.[1-3] Diabetic patients with microangiopathy have increased plasma levels of diene conjugates compared with patients without such complications.[4] In the diabetic rat retina, the content of lipid peroxides is increased[5,6] and the activity of superoxide dismutase (SOD) is decreased,[7] as are the glutathione levels,[8] implying the presence of reactive oxygen species and/or reduced defense systems. It has also been indicated that ischemia followed by recirculation might contribute to free radical production in other tissues.[9] In diabetes mellitus, the retina is exposed to hyperglycemia in combination with abnormal blood circulation and oxygenation,[10,11] thus potentially increasing the risk for free radical production.

Glutathione is present in most mammalian cells and plays an important role in many biological processes. Glutathione also participates in the cellular defense system against oxidative stress by reducing disulfide linkage of proteins and other cellular molecules or by scavenging free radicals and reactive oxygen intermediates.[12]

Glutathione is needed to maintain both the integrity of the cell membrane and the thiol-disulfide status of the cell. A reduction of the glutathione concentration in red blood cells makes them vulnerable to hemolysis, especially in conditions leading to oxidative stress. Other cells are probably also affected under these conditions.[13]

The aim of the present study was to examine whether the abnormal metabolism present in diabetes interferes with free radical enzyme defense systems in the rat retina, and whether ischemia followed by recirculation further influences tissue levels of glutathione.

## MATERIALS AND METHODS

### *Animals*

All rats were purchased from Möllegaard's Breeding Center (Copenhagen, Denmark). Female Sprague-Dawley rats (body weight [BW], 200 to 250 g), referred to as normal, were used about 1 week after delivery. Slightly older male rats (BW, 290 to 325 g) were injected intraperitoneally (60 mg/kg BW) with streptozotocin ([STZ] Zanosar; Upjohn, Kalamazoo, MI) and kept for a 2- or 6-month period. Blood glucose levels were measured using a reflectometer (One Touch II; Orion Diagnostic, Trosa, Sweden). When the blood glucose level was 15 mmol/L or less 1 week after STZ, the injection was repeated once (30 mg/kg BW). If the blood glucose after 2 weeks was still 15 mmol/L or less, the animals were excluded. During the 2- or 6-month period, blood glucose levels were measured once every week to ensure that high blood glucose was maintained until the rats were used for the experiments. After 2 (n = 10) or 6 (n = 6) months, the rats were killed by decapitation during $CO_2$ anesthesia. Control rats (n = 10 and n = 6, respectively) matched for sex and age were kept for the same time and under the same laboratory conditions as STZ-injected rats.

### *Ischemia Induction*

The effect of ischemia was studied in normal rats, in rats with STZ-induced diabetes for 2 months, and in matched control rats. The animals were anesthetized with Mebumal (Apoteksbolaget, Umeå, Sweden) 9.72 mg/mL plus chloral hydrate 42.5 mg/mL, magnesium sulfate 86.25 mmol/L, 10% (vol/vol) ethanol, and 40% (vol/vol) propylene glycol, 0.3 mL/100 g BW. The pupils were dilated with 1% Cyclogyl (cyclopentolat; Alcon, Puurs, Belgium). Retinal blood flow was observed directly using a corneal contact lens and a microscope. Retinal ischemia was induced by careful ligation of the vessels and the accompanying optic nerve behind the left eye bulb.[14] The right eye served as a control. The ligature was tightened until complete

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cessation of the retinal blood flow could be observed. After 90 minutes, retinal blood flow was restored as the ligature was removed. Normal animals were killed during $CO_2$ anesthesia after 5 minutes (n = 12), 15 minutes (n = 12), or 3 days (n = 10). Diabetic rats (n = 7) and matched control rats (n = 9) were killed after 3 days of recirculation.

### *Glutathione Analyses*

After decapitation, each eye was immediately enucleated, the lens was removed, and the retina was gently peeled away from the pigment epithelium and placed in 500 μL ice-chilled 10-mmol/L sodium phosphate buffer, pH 8.0. Within 1 hour, 30 μL 0.1-mol/L dithiothreitol was added and the retina was homogenized in a Duall tissue grinder (Kebo-Lab, Stockholm, Sweden). The homogenate was incubated at 37°C for 15 minutes, and 100 μL 15% sulfosalicylic acid was added. After 30 minutes at 4°C, centrifugation (12,000 × *g* for 5 minutes at 4°C) was performed and the supernatant was frozen at −70°C until analysis, no more than 2 days later. This procedure measures the total amount of glutathione, whether disulfide-bound or not. The determination of the total amount of glutathione was performed as previously described.[15] In short, the analyses were made using a high-performance liquid chromatographic method with isocratic reversed-phase ion-pair liquid chromatography at pH 2.4, postcolumn derivatization with 4.4-dithiopyridine, and colorimetric detection at 324 nm. The total imprecision (coefficient of variation [CV]) was less than 5%. To test the influence of matrix differences in the homogenate and the calibration sample, glutathione was added to the homogenates. The recovery of added glutathione was between 94% and 102%. The cellular content of glutathione is expressed as nanomoles per milligram of cell protein. Protein was analyzed according to the method of Lowry et al.[16]

### *Statistics*

Student's two-tailed *t* test was used for statistical evaluation of the results. The data are presented as the mean ± SD.

## RESULTS

### *Animal Characteristics*

For diabetic and control rats, BW and blood glucose values at death are shown in Table 1.

### *Glutathione Levels in STZ-Induced Diabetic Rat Retina*

Retinal glutathione levels were lower in 6-month diabetic rats compared with age-matched controls (16.6 ± 2.9 *v* 20.9 ± 2.1 nmol/mg protein, $P = .0004$) and also compared with 2-month diabetic rats (16.6 ± 2.9 *v* 19.0 ± 2.1 nmol/mg protein, $P = .0279$) (Fig 1).

### *Retinal Glutathione Levels After Retinal Ischemia Followed by 5 Minutes, 15 Minutes, or 3 Days of Recirculation in Normal Rats*

The effects of total ischemia for 90 minutes followed by a variable recirculation time were initially studied in normal rat retina (Fig 2). After 5 minutes, 15 minutes, or 3 days of recirculation, there was no change in the retinal glutathione level for ischemic rats compared with paired control rats, (24.1 ± 7.5 *v* 25.6 ± 6.5 nmol/mg protein, $P = .5947$, 16.6 ± 4.0 *v* 19.2 ± 3.4, $P = .1036$, and 30.6 ± 6.9 *v* 30.8 ± 3.8, $P = .3150$, respectively).

**Table 1. Animal Characteristics at Death**

| Rat Group | BW (g) | Blood Glucose (mmol/L) |
|---|---|---|
| 2-mo STZ (n = 10) | 303 ± 56 | 19.6 ± 4.5 |
| 2-mo control (n = 10) | 493 ± 26 | 3.5 ± 0.2 |
| 6-mo STZ (n = 6) | 324 ± 41 | 22.4 ± 1.9 |
| 6-mo control (n = 6) | 578 ± 35 | 3.4 ± 0.4 |

NOTE. Results are the mean ± SD.

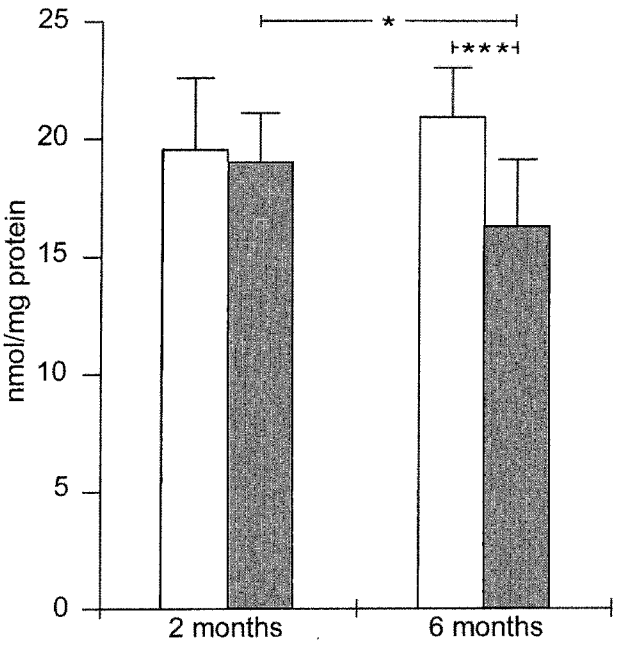


**Fig 1. Glutathione levels in STZ-induced diabetic rat retina (mean ± SD). (▨) Diabetic animals; (□) matched control animals. *$P < .05$, ***$P < .001$.**

### *Retinal Glutathione Levels After Retinal Ischemia in 2-month Diabetic Rats*

The effect of ischemia followed by 3 days of recirculation in 2-month diabetic rats was studied and compared with that of the

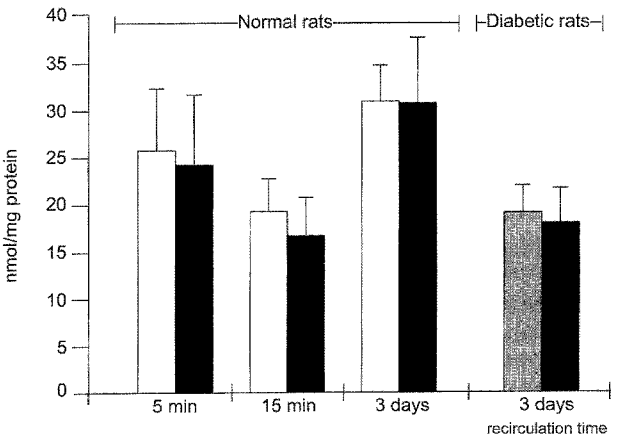


**Fig 2. Retinal glutathione levels in retinal ischemia followed by 5 minutes, 15 minutes, or 3 days of recirculation in normal rats and 3 days of recirculation in 2-month diabetic rats (mean ± SD). (■) Ischemic; (□) paired normal controls; (▨) diabetic controls.**

matched control rats (Fig 2). Retinal glutathione levels did not differ between ischemic and paired control rats, either in the diabetic animals or in the matched controls ($18.0 \pm 3.6$ *v* $19.1 \pm 2.9$ and $17.3 \pm 3.3$ *v* $18.3 \pm 1.8$ nmol/mg protein, $P = .5571$ and $P = .3990$, respectively).

## DISCUSSION

Diabetes mellitus tends to increase oxidative stress in both humans and animals, and increased oxidative stress may play a role in the development of late diabetic complications.[1] Mechanisms that contribute to increased oxidative stress in diabetes may include increased nonenzymatic glycosylation, autoxidative glycosylation, and an alteration in sorbitol pathway activity.[1] The vertebrate retina has several features that render it vulnerable to damage from free radicals, such as abundant mitochondria, which may leak activated oxygen species, and a high percentage of polyunsaturated fatty acids in the photoreceptor membranes, which are susceptible to lipid peroxidation.[17] Vitamin E (α-tocopherol) is known as an antioxidant against hydroxyl radicals, and vitamin E deficiency may cause a degeneration of retinal rod outer segments and loss of photoreceptor cells in rats.[18]

Glutathione acts as an antioxidant and helps to maintain the normal redox potential within cells. It protects the cell from the toxic effects of reactive peroxides and free radicals, serves in the storage and transport of cysteine moieties, and also participates in catalytic processes and transhydrogenation reactions.[19] An active system for maintaining glutathione in its reduced form is normally present in the retina, and the retina has the ability to regulate the glutathione concentration by a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent process.[20] In the present study, retinal glutathione levels were significantly lower in 6-month diabetic rats compared with 2-month diabetic and 6-month normal control rats. In different experiments, there was a slight variation of the total glutathione concentration. This was not due to the method used for glutathione determination, which has a low imprecision (CV <5%). Likewise, there was no deviation in accuracy during the experimental period as assessed by internal controls. The experimental conditions, sample preparation, and biological variation might have contributed to the slight variation in the glutathione concentration observed in different experiments. Therefore, in each experiment with diabetic rats, matched controls were always included. Thus, the results of the present study indicate an impaired defense against oxidative stress in the diabetic rat retina. Experimentally induced diabetes by toxic agents per se may result in an increased formation of oxygen radicals, as previously shown for alloxan.[21] However, in the present study, diabetes was induced by STZ, the actions of which do not seem involved in the direct formation of reactive oxygen radicals.[22,23]

The mechanisms underlying the glutathione loss from the retina in diabetes are not yet clear. It might be that the abnormal metabolism in diabetes increases the consumption and/or decreases the synthesis of glutathione. Others have reported that enzymes of glutathione synthesis are not impaired in the retina of diabetic rats, whereas enzymes of the glutathione redox cycle become subnormal. Also, these defects can be corrected by antioxidant (vitamin C plus vitamin E) therapy.[24,25] The redox imbalance induced by elevated glucose levels may be the result of an increased oxidation of sorbitol to fructose coupled with a reduction of oxidized nicotinamide adenine dinucleotide ($NAD^+$) to reduced nicotinamide adenine dinucleotide (NADH) in the sorbitol pathway and increased nonenzymatic glycosylation and autoxidative glycosylation.[26] Competition for NADPH due to the accelerated oxidation of sorbitol to fructose by NAD-dependent sorbitol dehydrogenase during hyperglycemia may impair this ability. Reduced levels of tissue glutathione, as shown in this study, may also be caused by a lower activity of glutathione reductase,[27] an enzyme that regenerates oxidized glutathione in a NADPH-dependent reaction. Our finding of subnormal glutathione levels in the retina in diabetes, consistent with results from other groups,[8,28] indicates a relatively impaired reduction capability and potentially increased oxidative stress within the retina, which in turn might contribute to the development of diabetic retinopathy.

The hypothesis that the generation of reactive oxygen species contributes to ischemia-reperfusion damage in the brain was suggested years ago.[9,29] It has also been shown that ischemia causes a decrease in the cerebral cortical concentration of glutathione, with a further decrease during subsequent recirculation following a 30-minute period of either complete or pronounced incomplete ischemia, indicating an impaired defense against oxidative stress.[30] Accumulating evidence from experimental studies indicates that reactive oxygen species may also mediate ischemic retinal injury, eg, SOD diminished swelling in the inner plexiform layer induced by ischemia followed by recirculation,[31] and catalase and SOD reduced the changes due to ischemia and recirculation[32] registered on an electroretinogram. In hypoxic tissues, vascular changes are closely linked to an increase in NADH/$NAD^+$ and associated metabolic imbalances. An increase in cytosolic free NADH/$NAD^+$ is also observed in tissues exposed to elevated glucose levels (in vivo or in vitro) at normal tissue $po_2$. Furthermore, the retinal circulation has been shown to be modulated by changes in actual blood glucose. Thus, retinal blood flow was shown to be decreased in patients with insulin-dependent diabetes mellitus with no retinopathy, and acute elevations in blood glucose resulted in an increased retinal blood flow.[33,34] The diabetic state, which is characterized by fluctuating levels of blood glucose, may thus mimic a relative ischemic-recirculation condition. One aim of the present study was therefore to examine whether ischemia followed by recirculation influences glutathione levels in the retina and whether a possible influence is more marked in retinas with impaired glucose metabolism. However, when studying different recirculation times, we did not find any change in the glutathione level of ischemic retinas in normal or diabetic rats, indicating that ischemia followed by recirculation does not contribute to or further aggravate oxidative stress in diabetes, as measured by glutathione levels in this study.

In summary, the results of the present study indicate that the abnormal metabolism caused by diabetes, rather than changes in retinal circulation, results in impaired defense mechanisms against oxidative stress.

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