Application of Quantitative Competitive Polymerase Chain Reaction for Measurements of mRNA From Antioxidative Enzymes in the Diabetic Rat Retina and Kidney

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This present study applied quantitative competitive polymerase chain reaction (QC-PCR) in the analyses of mRNA expression of the endogenous antioxidative enzymes CuZn superoxide dismutase (SOD), MnSOD, catalase, and glutathione peroxidase in tissue samples from the retina and kidney cortex of diabetic rats. RNA was extracted from snap-frozen retinas and pieces of the kidney cortex of male Wistar rats with streptozotocin (STZ)-induced diabetes and control rats. The mRNA levels were analyzed using QC-PCR. The animals were kept in the laboratory for 1 and 6 months, respectively, and fed a normal or probucol- (1% wt/wt) enriched diet. By using QC-PCR, relative mRNA levels of all antioxidative enzymes could be estimated in the retina and kidney cortex. In the retina, the relative catalase mRNA concentration was about 1/10 that of the other enzymes. After 6 months of diabetes, there was a 100% increase of the catalase (median, 0.012 [range, 0.008 to 0.017] *v* 0.006 [0.005 to 0.010]; *P* = .011) and a 50% increase of the glutathione peroxidase mRNA levels (0.88 [0.44 to 1.12] *v* 0.52 [0.31 to 0.79]; *P* = .044). In the kidney cortex, the relative glutathione peroxidase mRNA level was 10 to 15 times higher, and catalase mRNA level about half of those of CuZnSOD and MnSOD. After 1 month of diabetes, there was an increase only of the glutathione peroxidase mRNA levels, by 170% (17.59 [6.19 to 29.49] *v* 6.96 [2.34 to 9.04]; *P* = .047). We conclude that quantification of mRNA can provide difficulties when the amount of sample RNA is limited and/or the gene expression is low. The present study shows QC-PCR to be useful as a tool for measuring expression of mRNA not only in the kidney cortex but also in small tissue samples like the retina. Our results indicate modestly increased mRNA expression of catalase and glutathione peroxidase in the retina and likewise modestly increased mRNA expression of glutathione peroxidase in the kidney cortex of rats with STZ-induced diabetes. Extended studies, also including enzyme activities, are needed before any effect of hyperglycemia on the overall enzyme activity can be established.

STUDIES ON gene expression in the retina of small animals like the rat may present problems due to the limited amount of tissue available, particularly if the gene expression is low. In those cases, a method like quantitative competitive polymerase chain reaction (QC-PCR) could be useful due to its sensitivity.[1] To generate quantitative data, external standards are frequently applied that mimic or closely imitate RNA species with respect to primer binding and other variables affecting PCR amplification. Both DNA and RNA mimics have been used. The RNA standard approach is often considered more precise, because it controls for variation in both the reverse transcriptase (RT) and PCR reaction.[2] There are, however, many disadvantages with the synthetic RNA standard approach.[3] First, more steps are needed to perform this assay and, consequently, the procedure is labor-intensive and expensive. Second, a preliminary RT-PCR titration study is necessary to quantify the standard and each RNA species simultaneously. Third, to meet all those steps more RNA is required. This may not be possible with limited sample sources. Fourth, it is not possible to ensure that all samples have the same RNA starting levels, which can lead to huge errors when quantifying the target mRNA levels. A different approach is to use a DNA standard that acts as a competitor to the target cDNA, although it is added after the reverse transcription of the target mRNA.[4] Therefore, to circumvene the variation in reverse transcription, a housekeeping gene is also measured. A first aim of the present study was to apply QC-PCR, using a modified DNA mimic method, to limited amounts of tissue by examining the mRNA expression of endogenous antioxidative enzymes in retina of individual rats.

A second aim of the study was to investigate whether diabetes could induce any changed expression of the endogenous antioxidative enzymes CuZn superoxide dismutase (SOD), MnSOD, catalase, and glutathione peroxidase in 2 target organs, the retina and kidney. In addition, if so, whether probucol, a free radical scavenger,[5-7] influenced the transcription level of those enzymes. Diabetic retinopathy and nephropathy are the most severe microvascular consequences of long-term hyperglycemia but the exact pathogenic mechanisms are not yet fully understood. Hyperglycemia induces a variety of metabolic changes, among others an increased intracellular production of reactive oxygen species (ROS).[8,9] Under normal circumstances, ROS are usually rendered harmless by endogenous scavengers. The superoxide radical is converted to hydrogen peroxide by SODs, which in turn is converted to hydrogen and water by catalase and glutathione peroxidase (GSH-Px). Any imbalance between ROS and antioxidant defenses can create a state of oxidative stress, which results in disturbances in the cell metabolism including DNA strand breakage, damage to membrane ion transporters and/or specific proteins, depletion of nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP), and peroxidation of lipids.[10] Glucose can generate

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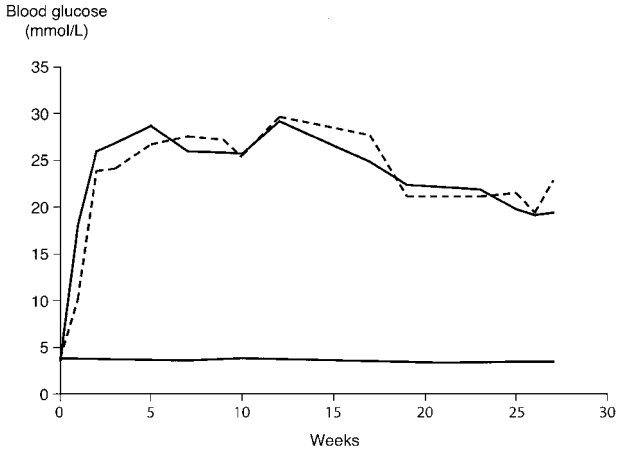

Fig 1. Blood glucose levels in diabetic and control rats throughout the study. (Upper full line) Diabetic rats on normal diet; (dashed line) diabetic rats on probucol; (lower full line) control rats.

ROS by enhancing metal-catalyzed autooxidation yielding hydrogen peroxide, hydroxyl radical, and ketoaldehyde, increasing tissue lipid peroxidation, and inactivating nitric oxide.[11] In addition, glucose can inactivate the antioxidant enzyme defenses by nonenzymatic glycation.

MATERIALS AND METHODS

Animals

Male Wistar rats (M&B A/S, Lille Skensved, Denmark), body weight 250 to 300 g, were injected intraperitoneally with streptozocin (STZ) (60 mg/kg body weight in 0.9% sodium chloride, pH 3.5 to 4.5). In 19% of the animals, the blood glucose level was less than 15 mmol/L after 4 days, and the injection was repeated once. Only rats in which blood glucose levels were≥ 15 mmol/L were included. Survival rate throughout the entire study was 78%. Diabetic (n = 26) as well as strain-matched control animals (n = 12) received either a normal diet (n = 13 and 12, respectively) or addition of probucol (1% wt/wt) in the pellets in diabetic animals (n = 13). Probucol was given from the day of STZ injection and onwards. The amount of probucol ingested was in diabetic rats approximately 0.35 g/d and in control rats 0.20 g/d. All animals had free access to food and tap water throughout the study. When signs of dehydration appeared and the rats lost body weight, 2 to 4 IU of insulin (Ultratard 1001 E/mL, Novo Nordisk A/S, Bagsvaerd, Denmark) was injected subcutaneously, initially twice, later 6 days a week. Blood glucose levels during the course of the study are shown in Fig 1. After 1 month, diabetic animals had a body weight of 261 ± 24 g compared to 376 ± 35 g for nondiabetic ($P < .001$). After 6 months, the corresponding figures were 320 ± 31 and 495 ± 31, respectively ($P < .001$). Probucol did not influence the body weight. After 1 and 6 months, respectively, 1 kidney and 1 eye were removed during deep CO_2 anesthesia and the animals were killed by decapitation.

All animals were treated according to the principles for the care and use of animals in Ophthalmic and Vision Research approved by the Association for Research in Vision and Ophthalmology (ARVO). The Animal Ethics Committee of Malmö/Lund approved the study.

Tissue Preparation

The kidney was immediately put in liquid nitrogen. The lens was rapidly removed from the enucleated eye, and the retina gently peeled off from the pigment epithelium and put in liquid nitrogen. Both the retina and kidney were stored at -80°C until further analyses.

RNA Extraction

Total cellular RNA was isolated from the retina and 1 piece of the kidney cortex (30 to 40 mg) of each rat by the guanidine thiocyanate/phenol/chloroform extraction method described by Chomczynski and Sacchi,[12] using a Polytron (PT 12000, Kinematica AG, Littau-Lucerne, Switzerland) instead of a glass Teflon homogenizer. RNA was quantitated by ultraviolet light absorbency at 260 nm and the integrity was verified by 2 bands of 18S and 28S, respectively, using electrophoresis on a 2% agarose gel stained with ethidium bromide (EtBr). RNA was kept frozen in RNase-free water at -80°C until used.

RT-PCR

Total RNA was transcribed into cDNA using the MMLV Reversal Transcriptase (Superscript II RT, Life Technologies, Grand Island, NY). A total of 1,000 ng RNA was mixed with 500 ng oligo $(dT)_{18}$-primer. Denaturation took place at 70°C for 10 minutes, and 200 U Superscript II RT was added to a total volume of 30 μL. The samples were incubated at 42°C for 50 minutes and thereafter, the transcriptase was inactivated at 70°C for 15 minutes.

Primers

Primers specific for the different enzymes studied are listed in Table 1. The enzyme sequences were obtained from Puga and Oates[13] (CuZnSOD), Ho et al[14] (MnSOD), Nakashima et al[15] (catalase), and Ho et al[16] (GSH-Px). The sequence of cyclophilin, the housekeeping gene used as internal control, was obtained from the database National Center for Biotechnology Information (NCBI; accession no. M19533).

Mimic Preparation

A mimic competing for the same primers as target cDNA was constructed using a PCR Mimic Construction Kit (PT1521-1, Clontech Laboratories, Palo Alto, CA). The competitive template was constructed from 2 ng of v-erb*B* fragment and composite primers performing 16 cycles of PCR for 45 seconds at 94°C, 45 seconds at 52°C, and 45 seconds at 72°C (PTC-100, MJ Research, Watertown, MA). The first composite primers consisted of sense and antisense primer sequences for CuZnSOD, coupled to 20-bp long sequences complementary to 20 bp at each end of a 200-bp long neutral sequence. The second

Table 1. Oligonucleotides of 5′ Primers and 3′ Primers of Five Target Genes

	5′ Primers	3′ Primers	mRNA (bp)	Mimic (bp)
Cyclophilin (housekeeping gene)	5′-CAGACAAAGTTCCAAAGACAGCAG-3′	5′-CTTGCCATTCCTGGACCCAAAACG-3′	377	296
CuZnSOD (exon 1)	5′-CAGGATTAACTGAAGGCGAGCATG-3′	5′-CAATCACACCACAAGCCAAGCGGC-3′	342	248
MnSOD (exon 2-4)	5′-CGTCACCGAGGAGAAGTACCACGA-3′	5′-CAGCCTGAACCTTGGACTCCCACA-3′	252	344
Catalase (exon 9-13)	5′-CACTCACCGCCACCGCCTGGGACC-3′	5′-TTTTCCCTTGGCAGCTATGTGAGA-3′	484	392
GSH-Px (exon 1-2)	5′-GTTCGGACATCAGGAGAATGGCAA-3′	5′-GGTTGCTAGGCTGCTTGGACAGCA-3′	368	440

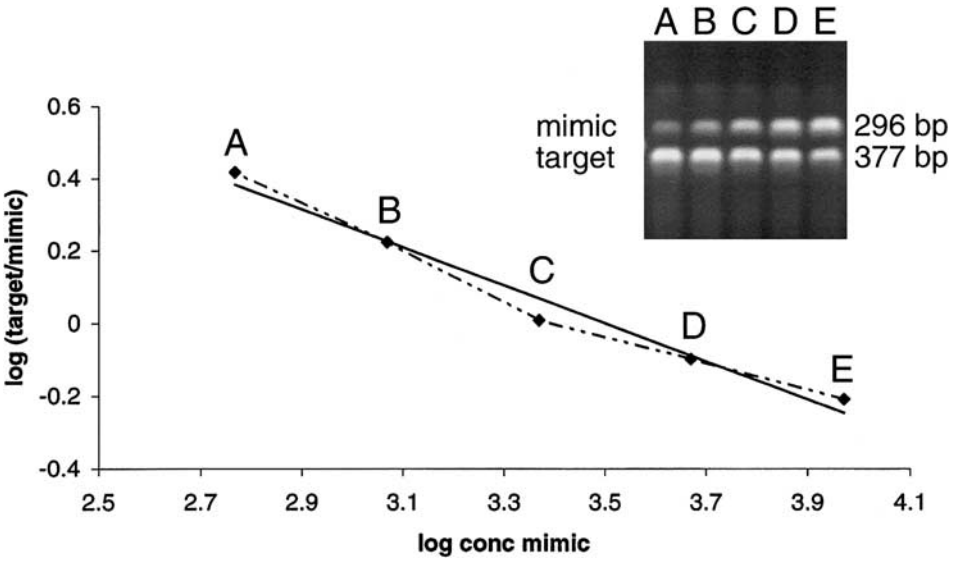

Fig 2. QC-PCR products of 1 sample with serial dilutions of competitor mimic cDNA and a constant volume of target cDNA for cyclophilin. The log target mimic intensity, quantified by Kodak 1D 2.0.0 software, was plotted against log mimic concentration representing a series of dilutions of the mimic. The log number of molecules at log target/mimic = 0 was 3.50.

composite primers were constructed using a 24-bp long sequence complementary to the CuZnSOD primers in the construct and to the 24 bp of the cyclophilin sense and antisense primers. The procedure was repeated for the third (MnSOD), fourth (catalase), and fifth (GSH-Px) primers, thus creating 1 mimic construct for all cDNA species. The DNA mimic was cloned in pGEM-T (Promega, Madison, WI) and linearized by the restriction enzyme *Hinc*II (Appligene, Illkirch, France). After purification of the plasmid, the final concentration was determined at 260 nm and diluted aliquots were stored at -20°C until used.

Amplification Efficiency Test

Before the mimic construct was accepted, an amplification test was performed. PCR tests demonstrated equal amplification efficiency of and equal primer competition between the mimic and all five cDNA targets (data not shown).

QC-PCR

In a first step, 2 μL of a 10-fold serial dilution of the mimic and 0.121 μL cDNA corresponding to 4 ng of total RNA was mixed with 4 pmol of each primer (sense and antisense), 0.5 U Taq DNA polymerase, 0.2 mmol/L dNTP, and 1.5 mmol/L $MgCl_2$ in buffer in a total volume of 20 μL. Denaturation, annealing, and polymerase extension were performed for 35 cycles and for 45 seconds at 94°C, 30 seconds at 62°C, and 60 seconds at 72°C, respectively. In a second step, the same procedure was performed with 2-fold serial dilutions of the mimic. The PCR products, 20 μL, were separated on a 3% agarose gel, then stained with EtBr and the specific bands were visualized with ultraviolet light. Photography (Fig 2), detection, and quantitation of the mimic and target cDNA were carried out using Kodak 1D 2.0.0 software (Eastman Kodak Co, France).

cDNA Concentration

After correcting for intensity variations due to different molecular sizes between cDNA and mimic PCR products (72 to 94 bp), the ratio between cDNA and the mimic PCR product was calculated. The log ratio target/mimic was plotted against the log mimic concentration in a diagram. The cDNA concentration for each enzyme was defined as the log mimic concentration at log ratio = 0 (Fig 2). This inverted log value, representing the actual number of molecules, was divided by corresponding figure for cyclophilin, thus representing the mRNA enzyme concentrations.

Coefficients of Variation

In the kidney, we performed 6 separate assays on 1 kidney sample and 1 assay on 8 identical samples. The intra-assay and interassay coefficients of variation were 8.8% and 14.4%, respectively.

Statistics

SPSS 9.0 for Windows (SPSS Inc, Chicago, IL) was used for statistical calculations. Student's 2-tailed *t* test was applied when testing differences between groups regarding body weight and blood glucose levels, Mann-Whitney's *t* test for differences regarding mRNA expression. A *P* value less than .05 was considered significant.

RESULTS

Blood Glucose and Body Weight

After 1 month of diabetes, blood glucose levels were 19.9 ± 2.0 mmol/L and 3.5 ± 0.3 mmol/L (mean ± SD) in diabetic and control rats, respectively. Diabetic rats had lost 16.7 ± 19.5 g, whereas control rats had gained 84.5 ± 9.9 g. After 6 months of diabetes, blood glucose levels were 19.1 ± 3.9 mmol/L and 3.5 ± 0.3 mmol/L, respectively. Diabetic rats had gained 42.2 ± 24.6 g compared with 216.0 ± 28.3 g in control rats. These parameters were not different in the corresponding subgroups of rats treated with probucol.

mRNA in the Retina

The relative retinal mRNA concentrations in diabetic as well as in control rats are given in Table 2. The catalase mRNA level in the retina was low, about 1/10 of those of the other enzymes, but there was a 100% increase after 6 months of diabetes (P = .011). Probucol had no influence on the increased catalase expression. There was also an increase of the relative GSH-Px mRNA concentration, 50%, after 6 months of diabetes (P = .044). Normalization was achieved with probucol.

Table 2. Relative mRNA Concentrations of Free Radical–Protecting Enzymes in the Retina of Rats With Diabetes for 1 or 6 Months on Normal or Probucol-Enriched Diet and in Age-Matched Control Rats

Enzyme	Diabetes, 1 Month (n = 7)	Diabetes, 1 Month, PB (n = 6)	Control, 1 Month (n = 6)	Diabetes, 6 Months (n = 6)	Diabetes, 6 Months, PB (n = 7)	Control 6 Months (n = 6)
CuZnSOD	0.20 (0.16-0.29)	0.25 (0.13-0.29)	0.25 (0.15-0.36)	0.17 (0.12-0.36)	0.18 (0.11-0.48)	0.17 (0.16-0.24)
MnSOD	0.67 (0.41-1.18)	0.49 (0.28-0.83)	0.68 (0.53-0.84)	0.69 (0.38-0.94)	0.54 (0.38-1.47)	0.56 (0.27-1.71)
Catalase	0.009 (0.006-0.019)	0.009 (0.004-0.011)	0.009 (0.008-0.018)	0.012 (0.008-0.017)*	0.011 (0.006-0.013)	0.006 (0.005-0.010)
GSH-Px	0.58 (0.38-0.80)	0.42 (0.24-0.67)	0.56 (0.46-0.83)	0.88 (0.44-1.12)†	0.56 (0.43-0.62)‡	0.52 (0.31-0.79)

NOTE. Values are medians (range).
Abbreviation: PB, probucol.
*P = .011, catalase diabetes 6 months *v* control.
†P = .044, GSH-Px diabetes 6 months *v* control.
‡P = .042, GSH-Px diabetes 6 months PB *v* diabetes 6 months.

mRNA in the Kidney

The relative mRNA kidney cortex concentrations are given in Table 3. The GSH-Px mRNA concentration was higher, 10 to 15 times, and catalase mRNA concentration lower, about half of those of CuZnSOD and MnSOD. After 1 month of diabetes, mRNA expression of GSH-Px was 170% increased in diabetic compared with control animals (P = .047), but probucol did not reduce its expression. After 6 months of diabetes, the expression of GSH-Px was similar in all experimental groups.

DISCUSSION

In the present study we have used the QC-PCR technique in the analyses of mRNA concentrations in vivo in 2 organs of the rat, the retina and kidney. Since the amount of retinal tissue in each individual animal is small, it was of interest to apply this method to retina due to its sensitivity, and hence its particular usefulness when the amount of sample RNA is limited and/or the gene expression is low.[1] Application of the QC-PCR method revealed that all the antioxidant enzymes could be demonstrated in the retina as well as in the kidney. Intra- and interassay coefficients of variation were similar to those reported by others.[17]

The disadvantage of currently used DNA standard methods is that variations of reverse transcriptase may result in erroneous estimates of mRNA abundance. To circumvene this problem we used a modified DNA mimic method that corrects for reverse transcriptase differences. To reduce variation of the efficiency in the reverse transcription, cyclophilin cDNA from each target RNA sample was measured and used as an internal reference for all the measured target cDNAs. Cyclophilin was chosen as the housekeeping gene relevant for this assay, because it has been shown that insulin can affect transcription of the commonly used β-actin.[18]

The competitor DNA can be constructed in several ways, for example, by introducing a deletion in target cDNA[19] or using a nonhomologous sequence as a template.[20] In this study, we aimed at constructing a mimic DNA that would fulfil the criteria of equal amplification efficiency between target cDNA and the mimic DNA. The mimic DNA was constructed with several primer sets adding one after another with a difference between the target cDNA and the mimic DNA of only 72 to 94 bp, thus producing a mimic DNA that would fulfill the amplification efficiency criteria.

There is evidence for an increased diabetes-induced oxidative stress in the retina as well as in the kidney, which occurs prior to retinal histologic vessel changes or early in the course of nephropathy. Hence, it was of interest to test whether the expression of antioxidant enzymes in those tissues changed after exposure to hyperglycemia. It has previously been found that after 6 to 8 weeks of STZ-induced diabetes in rats, retinal thiobarbituric acid reactive substances (TBARS) are elevated in the retina[21] and kidney.[22] Lal et al[23] demonstrated elevated TBARS already after 10 days in the kidney. In addition, Obrosova et al[24] recently found evidence for increased lipid peroxidation in the retina after 6 weeks of diabetes by demonstrating an accumulation of 4-hydroxyalkenal, an early marker for oxidative stress.

In diabetes, defense mechanisms against oxidative stress seem to be impaired, and in the diabetic retina, reduced enzyme activities of glutathione reductase, GSH-Px, and SOD were reported.[24,25] After 6 to 8 weeks of diabetes, glutathione levels (GSH) were not yet depleted,[24,26] whereas this seemed to be the case after 6 months of diabetes.[26,27] Pericyte loss is one con-

Table 3. Relative mRNA Concentrations of Free Radical–Protecting Enzymes in the Kidney Cortex of Rats With Diabetes for 1 or 6 Months on Normal or Probucol-Enriched Diet and in Age-Matched Control Rats

Enzyme	Diabetes, 1 Month (n = 6)	Diabetes, 1 Month, PB (n = 6)	Control, 1 Month (n = 6)	Diabetes, 6 Months (n = 6)	Diabetes, 6 Months, PB (n = 7)	Control 6 Months (n = 6)
CuZnSOD	0.80 (0.15-2.00)	0.35 (0.17-1.36)	0.55 (0.41-0.58)	1.08 (0.51-2.94)	0.96 (0.35-1.09)	0.87 (0.50-0.95)
MnSOD	0.98 (0.16-1.77)	0.90 (0.61-1.24)	1.05 (0.83-1.37)	1.32 (0.56-2.43)	0.98 (0.26-2.61)	1.12 (0.32-2.08)
Catalase	0.48 (0.04-1.02)	0.53 (0.11-1.22)	0.58 (0.17-0.90)	0.46 (0.38-1.09)	0.66 (0.21-1.29)	0.46 (0.35-0.73)
GSH-Px	17.59 (6.19-29.49)*	13.34 (5.04-30.64)	6.96 (2.34-9.04)	11.31 (5.87-15.01)	19.28 (5.50-24.56)	10.75 (5.74-17.31)

NOTE. Values are medians (range).
*P = .047, GSH-Px diabetes 1 month *v* control.

sequence of diabetic retinopathy and it has been suggested that increased oxidative stress with diminished antioxidant defenses might be of importance for the programmed cell death in pericytes that occurs in diabetes. In humans, Li et al[28] demonstrated an upregulation of GSH-Px, a downregulation of CuZnSOD, and unchanged expression of MnSOD and catalase in pericytes in postmortem eyes of diabetic patients with more than 18 years of diabetes.

In the present study on the expression of 4 different endogenous scavenger enzymes in the retina after 1 and 6 months of diabetes, respectively, the results suggest that steadily increasing blood glucose levels during the first month of diabetes do not change the mRNA levels. Hence, the results do not support an augmented expression of those enzymes in the rat retina after 1 month of hyperglycemia. After an additional 5 months, a modestly increased expression of catalase and GSH-Px could be demonstrated. Although a less than 2-fold increase is too low to establish overexpression of those enzymes, it is of interest to note that probucol normalized the mRNA GSH-Px levels. We have previously demonstrated that probucol can normalize a diabetes-induced reduction of GSH/cysteine ratio in the retina of rats with diabetes for 6 months.[27] Increased mRNA expression and activities of antioxidative enzymes may represent signs of increased oxidative stress and normalized expression and activity of some antioxidative enzymes by antioxidants[21,24] may thus provide further support for diabetes-induced oxidative stress. However, it remains to be shown that oxidative stress is the primary cause of retinal microvascular abnormalities like pericyte loss and endothelial cell proliferation. We have previously not been able to demonstrate any reduced diabetes-induced formation of acellular capillaries by probucol, whereas we could show that probucol postponed, although it did not inhibit, cataract formation in this animal model.[26]

In the present study, the expression of GSH-Px in the kidney cortex was modestly increased only after 1 month of diabetes and the expressions of all other enzymes were similar to controls both at 1 and 6 months of diabetes. This is partly in accordance with the results of Reddi and Bollineni,[29] who found an increased, although not quantified, expression of GSH-Px, but in addition, they could demonstrate an enhanced expression of CuZnSOD. Lal et al[23] reported a 50% increased expression of CuZnSOD, after only 2 days of diabetes. Taken together, it thus seems as if enhanced expressions of antioxidative enzymes may occur early in the diabetic kidney, earlier than in the retina.

In summary, the present study shows that QC-PCR can be a useful tool for measuring the expression of mRNA not only in the kidney cortex but also in small tissue samples like the retina. The results from the present study indicate modestly increased mRNA expression of catalase and GSH-Px in the retina and likewise modestly increased mRNA expression of catalase in the kidney cortex of rats with STZ-induced diabetes. Extended studies, also including enzyme activities, are needed before any effect of hyperglycemia on the overall enzyme activity can be established.

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