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Modifying a standard method allows simultaneous extraction of RNA and protein, enabling detection of enzymes in the rat retina with low expressions and protein levels

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Abstract

The aim of the study was to evaluate messenger RNA and protein expression in limited amounts of tissue with low protein content. The Chomczynski method was used for simultaneous extraction of RNA, and protein was modified in the protein isolation step. Template mass and cycling time for the complementary DNA synthesis step of real-time reverse transcription–polymerase chain reaction (RT-PCR) for analysis of catalase, copper/zinc superoxide dismutase, manganese superoxide dismutase, the catalytic subunit of glutamylcysteine ligase, glutathione peroxidase 1, and the endogenous control cyclophilin B (CypB) were optimized before PCR. Polymerase chain reaction accuracy and efficacy were demonstrated by calculating the regression (R^2) values of the separate amplification curves. Appropriate antibodies, blocking buffers, and running conditions were established for Western blot, and protein detection and multiplex assays with CypB were performed for each target. During the extraction procedure, the protein phase was dissolved in a modified washing buffer containing 0.1% sodium dodecyl sulfate, followed by ultrafiltration. Enzyme expression on real-time RT-PCR was accomplished with high reliability and reproducibility (R^2, 0.990-0.999), and all enzymes except for glutathione peroxidase 1 were detectable in individual retinas on Western blot. Western blot multiplexing with CypB was possible for all targets. In conclusion, connecting gene expression directly to protein levels in the individual rat retina was possible by simultaneous extraction of RNA and protein. Real-time RT-PCR and Western blot allowed accurate detection of retinal protein expressions and levels.

1. Introduction

Studies on rat retina for gene expression and protein analysis are challenged by the limited amounts of tissue, and it is therefore necessary to use analytical methods that can perform with precision and scientific accuracy in small tissue samples. Recently, we demonstrated that quantitative competitive polymerase chain reaction (QC-PCR) with a modified DNA mimic method for measurements of messenger RNA (mRNA) is sensitive enough for this purpose and thus appropriate when the amount of sample RNA is limited and/or the gene expression is low [1]. An alternative PCR method, real-time reverse transcription–PCR, has the additional advantage of being able to perform the PCR cycling and corresponding measurement of the PCR-product accumulation simultaneously during the exponential phase of the reaction. The use of a fluorogenic probe during the process gives rise to an exponentially increasing fluorescence proportionate to the initial amount in the sample as the process proceeds. The fluorescence is subsequently computer analyzed [2,3]. The technique is faster and more specific than QC-PCR. It requires no post-PCR manipulations and allows inter-experimental comparison [4-6].

The purpose of this study was to detect the mRNA expressions and protein levels of endogenous antioxidant enzymes, that is, catalase (CAT), copper/zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), the catalytic subunit of glutamylcysteine ligase

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(GCLc), glutathione peroxidase 1 (GPx1), and the endogenous control cyclophilin B (CypB), in the limited amount of tissue available from one normal rat retina. The first aim of this study was to develop a method for simultaneous extraction of RNA and protein by modifying the protein isolation step of an existing model according to Chomczynski [7], thereby reducing the total amount of tissue needed for analysis. The second aim was to apply optimal real-time RT-PCR and establish a Western blot method for the detection of gene expression and protein levels of these enzymes.

2. Materials and methods

2.1. Animals

Female Wistar rats (body weight, 200-250 g) from Taconic (Lille Skensved, Denmark) were killed by carbon dioxide, the eyes were immediately enucleated, lenses removed, and the retinas were gently peeled off from the pigment epithelium, snap-frozen on dry ice, and stored at −80°C. 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. The Animal Ethics Committee of Malmö/Lund approved the study.

2.2. Simultaneous extraction of RNA and protein

Each retina was homogenized in 1 mL of TRI reagent (Sigma, Schnelldorff, Germany) supplemented with 5μL of Polyacryl Carrier (Molecular Research Center, Cincinnati, OH) on a rotor-stator Polytron (PT1200, Kinematica, Cincinnati, OH). After addition of 100 μL of 1-bromo-3-chloropropane (BCP, Sigma), the sample was vortexed and left for 15 minutes before phases were separated by centrifugation at 12 000 g for 15 minutes at 4°C. The aqueous phase (RNA) and the red organic phase (protein) were then transferred to new tubes, whereas the interphase (DNA) was discarded. The RNA was precipitated with 500 μL of isopropanol at 12 000 g for 10 minutes at 4°C. The pellet was washed in 75% ethanol, centrifuged at 7500 g for 5 minutes at 4°C, and left to dry in the air before it was dissolved in 50μL of DEPC-H_2O supplemented with 60 U of RNasin Plus RNase Inhibitor (Promega/SDS Biosciences, Falkenberg, Sweden). Any residual DNA was precipitated from the organic phase with 100% ethanol followed by a 2000 g centrifugation for 5 minutes at 4°C. The organic phase consisting of proteins, BCP, and phenol was resolved in 14 mL of wash buffer (31.25 mmol/L Tris-HCl, pH 6.8, and detergent, 0.1% 2.4 sodium dodecyl sulfate [SDS]) by vortexing. After centrifugation at 3000 g for 5 minutes at 23°C, the resulting upper phase consisting of phenol, protein, and wash buffer was transferred to a 5-kd MWCO Amicon-15 (Millipore, Molsheim, France) ultrafiltration tube, whereas the lower BCP phase was discarded. The Amicon-15 tube was then centrifuged at 3000 g for 30 minutes at 23°C. The filtrate was discarded, and the same wash step was performed 3 times with 14 mL of new wash buffer added each time. The protein solution (~250 μL retentate) was transferred to a new tube, and 5 μL of 50× protease inhibitor was added (Complete Protease Inhibitor Cocktail, Roche, Bromma, Sweden). Total RNA quantification was performed on a spectrophotometer (Eppendorf Biophotometer, VWR International, Stockholm, Sweden). Total protein quantification was performed using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). RNA and protein samples were stored at −80°C until analysis.

2.3. Real-time reverse transcription–polymerase chain reaction

Complementary DNA (c-DNA) was synthesized using 200 U SuperScript II RNase H^- RT (Invitrogen Life Technologies, Paisley, UK) and 250-ng random hexamer primers for 2 hours at 42°C. Two micrograms of RNA was the amount of template yielding the most efficient cDNA outcome in a dilution series trial. Likewise, the 2-hour running time was chosen as a result of optimization trials. Expression of CAT, CuZnSOD, MnSOD, GCLc, GPx1, and CypB mRNA levels was analyzed using real-time RT-PCR on a 7900 HT system (Applied Biosystems, Stockholm, Sweden). Primers and TaqMan probes were designed using the PrimerExpress 2.0 software (Applied Biosystems), spanning over an intron-exon boundary, blasted for specificity (nBLAST, National Center for Biotechnology Information, Rockville, MD) [8], and supplied by MWG Biotech (Ebersberg, Germany). For each reaction, 5 ng cDNA, 900 nmol/L primer, 250 nmol/L TaqMan probe, and 1× TaqMan Universal PCR Master Mix were loaded in duplicate. Probes were dually labeled with 6-FAM (reporter) on the 5′ end and tetramethylrhodamine (TAMRA, quencher) on the 3′ end. All sequences are 5′→3′:

1. CAT
   Forward: CCC GAG TCC AGG CTC TTC T
   Reverse: CGG CCT GTA CGT AGG TGT GA
   Probe: ACC AGT ACA ACT CCC AGA AGC CTA AGA ATG CA
2. CuZnSOD
   Forward: GCG GTC CAG CGG ATG A
   Reverse: GTC CTT TCC AGC AGC CAC AT
   Probe: AGG CAT GTT GGA GAC CTG GGC
3. MnSOD
   Forward: TCA GGA CCC ACT GCA AGG A
   Reverse: GCG TGC TCC CAC ACA TCA
   Probe: CCA CAG GCC TTA TTC CAC TGA TGG G
4. GCLc
   Forward: AGG AGA ACA TCA GGC TCT TTG C
   Reverse: GTG CTC TGG CAG TGT GAA TCC

Probe: CGA TAA CTT CAT TTC CCA GGC TAG GCT GC

5. GPx1
   Forward: CTC GGT TTC CCG TGC AAT
   Reverse: CAT ACT TGA GGG AAT TCA GAA TCT CTT
   Probe: ATT CTT GCC ATT CTC CTG ATG TCC GAA CT
6. CypB
   Forward: GGA GAT GGC ACA GGA GGA AA
   Reverse: CCA TAG TGC TTC AGC TTG AAG TTC T
   Probe: AGC ATC TAT GGT GAG CGC TTC CCA GA

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The total protein levels obtained from one retina measured with the standard BCA protein method [9] were between 0.5 and 1.0 μg/μL. Protein samples were mixed with the modified Laemmli buffer [10] to yield the final concentrations: 31.25 mmol/L Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol. The prepared samples were kept on ice during the pipetting procedure, vortexed, and spinned down before heat denaturation for 5 minutes at 95°C. Thereafter, the samples were mixed while kept on ice, spinned down, and applied in duplicate to 10.5% and 4% precast Criterion Tris-HCl gradient gel (Bio-Rad Laboratories, Sundbyberg, Sweden), each in a volume of 20 μL corresponding to 5 μg total protein. Precision Plus Protein Standard All Blue (Bio-Rad Laboratories) and the chemiluminescent Magic Mark XP Western Protein Standard (Invitrogen Life Technologies) were applied to the gel. The gel was run in 4°C running buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS) using Criterion Cell Tank (Bio-Rad Laboratories) for 1 hour at 200 V (constant voltage) or until the front dye reached the bottom of the gel and subsequently stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories) as well as with a more sensitive silver staining method (GelCode Color Silver Stain Kit, Pierce) according to the instructions of the manufacturer. The qualities of the protein samples were confirmed by the staining procedures, showing a protein pattern ranging from small to large protein sizes.

2.5. Western blot transfer

The gel was immersed in 4°C transfer buffer according to Towbin (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol) [11] with the addition of 0.1% SDS. A hydrophobic polyvinylidene difluoride (PVDF) membrane (0.2 μm, Immune-Blot PVDF membrane, Bio-Rad Laboratories) capable of retaining small amounts of proteins very strongly [12] was immersed in methanol and equilibrated in 4°C transfer buffer. A sandwich containing the gel, PVDF membrane, filter papers, and fiber pads was prepared, and any air bubble trapped was carefully removed. The sandwich was placed in a Criterion Blotter Tank (Bio-Rad Laboratories) containing 4°C transfer buffer and an ice block and was run for 2 hours at 50 V (constant voltage). For the confirmation of successful transfer of proteins to the membrane, the gel was stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories) to verify the absence of proteins and the membrane with the reversible Ponceau S Staining Solution (Sigma) to verify their presence.

2.6. Immunoprobing

Nonspecific binding sites were blocked by incubation of the PVDF membrane in an excess of blocking buffer. The different blocking buffers tested are shown in Table 1. The blocking step was performed for 1 hour at room temperature at gentle shaking. In a first procedure, 10 mL of the primary antibody diluted properly for the antibody used was added to a 60-mL tube (Sarstedt, Landskrona, Sweden) fit for the membrane. Air bubbles were carefully removed, and the membrane was incubated overnight at 4°C on a roller mixer. The primary antibody was poured off, and after one quick rinse in 40-mL washing buffer, Tris-buffered saline (TBS; pH 7.6), and 0.1% Tween 20 (TBS-T), the membrane was washed for 10 minutes at room temperature on a roller mixer 3 times. The second procedure with horseradish peroxidase (HRP)–conjugated secondary antibody, chosen and diluted to suit the concentration of the primary antibody and the washing procedure, was performed as described above, except that the membrane was incubated for 1 hour at room temperature. In the third procedure, the membrane was incubated for 5 minutes at room temperature on a roller mixer with 10 mL of light-sensitive substrate working solution (SuperSignal West Dura Extended Duration Substrate, Pierce) for chemiluminescent detection of HRP and thereafter wrapped in a polypropylene bag and kept in dark

Table 1
Blocking buffers used during the optimization trials

Blocking buffer	GPx1	CAT	CuZnSOD	MnSOD	GCLc	CypB
TBS-T with 0.5% gelatin	++	+	+	+	+	+
TBS-T with 5% nonfat dry milk	+	+	(+)	+	Not tested	+
TBS-T with 3% Top-Block (Fluka)	+	+	+	+	Not tested	+
Western blocker (Sigma)	+	+	+	Not tested	Not tested	+
SEA BLOCK (Pierce)	Not tested	−	−	−	Not tested	−

Test results are noted as positive (+), negative (−), or not tested.

until detection. The chemiluminescent detection of the protein standard and targets was performed using Fuji LAS3000 charge coupled device camera (Fujifilm, Stockholm, Sweden). Exposure times were chosen depending on targets to be detected.

2.7. Optimization of antibodies for detection of antioxidants and endogenous control

Five micrograms of total protein was applied to gels. Primary antibody dilutions were tested within a range of 1:200 to 1:5000 and secondary antibody appropriate for the primary antibody in dilutions within a range of 1:10 000 to 1:250 000. All antibodies, both primary and HRP-conjugated secondary antibodies, were commercially available. Each target was subsequently tested for the multiplexing ability with CypB.

3. Results

3.1. Real-time reverse transcription–polymerase chain reaction

The amplification curves and the regression values of the standard curves for real-time RT-PCR are shown in Fig. 1. Detection of all the antioxidant enzymes as well as the endogenous control CypB was accomplished with good reliability and reproducibility (R^2 = 0.990-0.999) and an efficacy between 99.89% and 101.20% calculated on the slope for each amplification plot (Fig. 1).

3.2. Optimization of antibodies for detection of proteins and endogenous control

3.2.1. Catalase

Three commercial CAT antibodies were tested. Catalase was not detectable using the antibody from USBiological (Swampscott, MA) or the monoclonal antibody from LabFrontier (Seoul, South Korea), whereas a dilution of 1:2000 of the polyclonal antibody (LF-PA0060) from LabFrontier gave the CAT band. The secondary antirabbit HRP antibody (I1904-41P) from USBiological was used at a dilution of 1:100 000. The samples were positioned at 60 kd. The exposure time needed for the detection was between 5 and 20 minutes (data not shown). Compatible blocking buffers turned out to be TBS-T with 0.5% gelatin, TBS-T with 5% nonfat dry milk, TBS-T with 3% Top-Block (Fluka, Stockholm, Sweden), and Western blocker (Sigma) (Table 1). The membrane was completely blocked by SEA BLOCK (Pierce) at a dilution of 1:10 in TBS and was consequently of no use.

3.2.2. Copper/zinc superoxide dismutase

The primary antibody (S8060-15; 1:1500) and the secondary antisheep HRP antibody (I1904-59C; 1:50 000 or 1:100 000), both from USBiological, showed the CuZn-

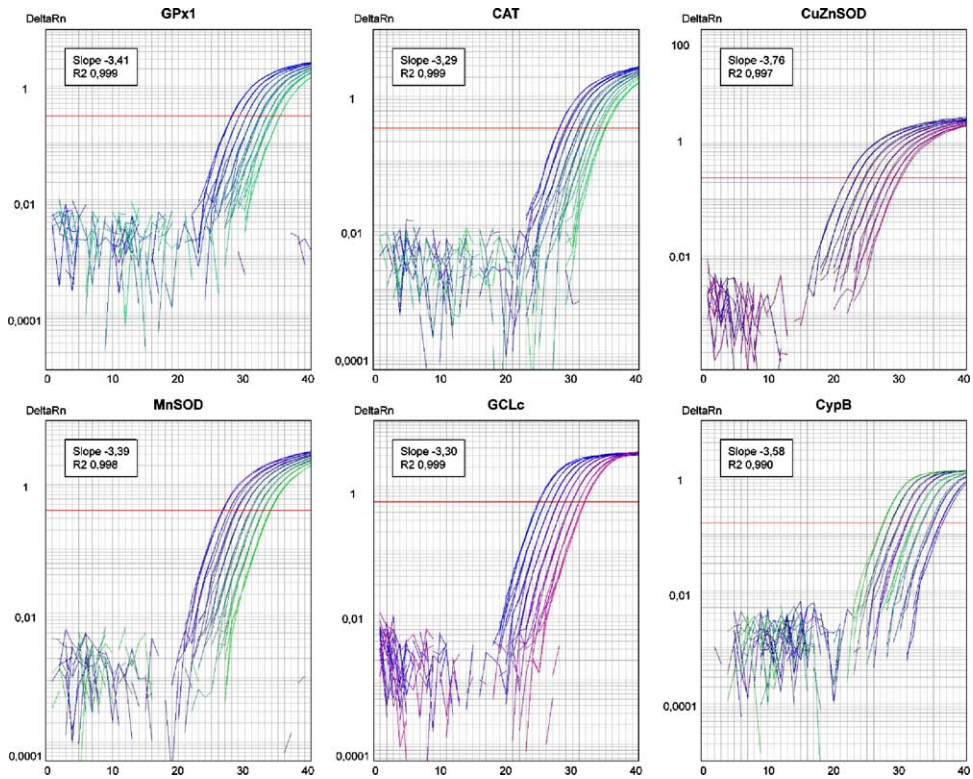

Fig. 1. Amplification plots for each target. Each plot shows the result of 40 PCR cycles (x-axis). ΔRn (y-axis) represents the reporter fluorescence normalized toward the internal background fluorescence of the assay (ROX fluorescent dye). The R^2 value, representing the reliability and reproducibility of the assay (1.00 = 100%), and the slope, representing its efficacy (−3.33 = 100%), are given.

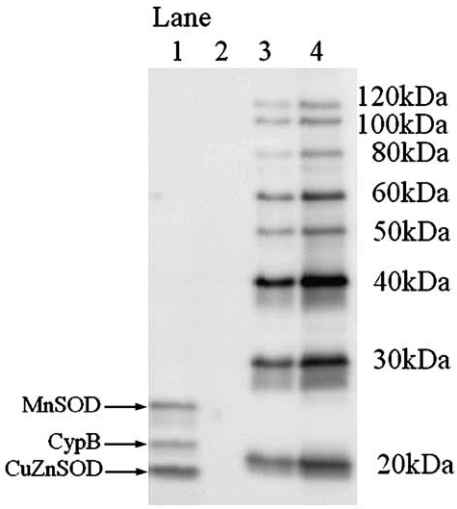

Fig. 2. Detection of MnSOD, CypB, and CuZnSOD on Western blot. Lane 1 contained 5 μg total protein of single rat retinal extract, lane 2 contained a negative control (ie, double distilled water), and lanes 3 and 4 contained protein standard.

SOD band at 16 kd at an exposure time between 1 and 10 minutes (Fig. 2). CuZnSOD was not detectable when the secondary antibody from DakoCytomation (Glostrup, Denmark) was used. TBS-T with 0.5% gelatin, TBS-T with 3% Top-Block (Fluka), or Western blocker (Sigma) was appropriate as blocking buffers (Table 1). A weak signal was detected using TBS-T with 5% nonfat dry milk, and no signal was detected using SEA BLOCK (1:10, Pierce).

3.2.3. Manganese superoxide dismutase

The primary antibody (S8060-10A; 1:1000) and the secondary antirabbit HRP antibody (I1904-41P; 1:50 000 or 1:100 000), both from USBiological, gave the MnSOD band at 25 kd at an exposure time of 1 to 10 minutes (Fig. 2). TBS-T with 0.5% gelatin, TBS-T with 5% nonfat dry milk, or TBS-T with 3% Top-Block (Fluka) was used as a blocking buffer (Table 1). No signal was detected using SEA BLOCK (1:10, Pierce) or Western blocker (Sigma).

3.2.4. Glutamylcysteine ligase

The GCLc (glutamylcysteine synthetase [GCS]) band at 73 kd was detectable using the primary antibody (RB-1697-P1; 1:1000) from NeoMarkers (LabVision, Newmarket, Suffolk, UK) and the secondary antirabbit HRP antibody (I1904-41P; 1:50 000) from USBiological, at an exposure time between 5 and 10 minutes (data not shown). The blocking buffer used was TBS-T with 0.5% gelatin (Table 1). No other blocking buffers were tested.

3.2.5. Glutathione peroxidase 1

Four commercial GPx1 antibodies were tested. GPx1 was not detectable using one of the two antibodies from USBiological or the antibody from Biogenesis (Poole, UK), whereas the antibody (LF-PA0019; 1:2000) from LabFrontier gave the GPx1 band. The secondary antibody used was antirabbit HRP (I1904-41P; 1:50 000 or 1:100 000) from USBiological. The exposure time needed for the detection was between 1 and 10 minutes. TBS-T with 0.5% gelatin gave a stronger signal than TBS-T with 5% nonfat dry milk. Other blocking buffers giving a signal were Western blocker (Sigma) and TBS-T with 3% Top-Block (Fluka) (Table 1). Ten normal rat retinal tissues were homogenized to obtain a sample with a high total protein concentration. A serial dilution ranging from 200 to 3.125 μg total protein demonstrated that GPx1 was detectable at a level between 12.5 and 25 μg.

3.2.6. Cyclophilin B

The primary CypB antibody (PA1-027; 1:50 000) from Affinity BioReagents (Sydney, Australia) was a strong antibody that gave a very clear band at 23 kd, which was at a higher position than Affinity BioReagents was specifying in their product sheet (19 kd). The secondary antibody used was antirabbit HRP (I1904-41P; 1:100 000 or 1:250 000) from USBiological. The exposure time needed was between 30 seconds and 5 minutes (Fig. 2). TBS-T with 5% nonfat dry milk, TBS-T with 0.5% gelatin, Western blocker (Sigma), or TBS-T with 3% Top-Block (Fluka) gave adequate blocking results, whereas SEA BLOCK (1:10, Pierce) gave no signal (Table 1).

3.3. Multiplexing with cyclophilin B

The molecular weights of the targets were separable from that of CypB, enabling multiplexing on Western blot for controllable template loading and possible normalization toward the endogenous control.

4. Discussion

In the present study, real-time RT-PCR and Western blot were applied to rat retinal tissue after simultaneous extraction of RNA and protein, giving a means for evaluation of the mRNA expression and concentration of antioxidant enzymes in this limited amount of tissue. Simultaneous extraction of RNA and protein is tissue-saving and allows direct coupling of gene expression to its corresponding protein product at the same time in the individual retina. In the present study, the protein precipitation step described in the Chomczynski [7] model had to be excluded because the dissolution of the protein pellet by the use of 1% SDS or the suggested alternatives turned out to be impossible. Modification of the SDS buffer, the use of other detergents, or heating of the sample did not solve this problem. In the present modified protocol, the precipitation step was therefore bypassed by keeping the organic phenol–BCP phase containing the proteins in solution, subsequently resolved in a modified wash buffer, centrifuged to separate the protein and BCP phases, and ultimately ultrafiltrated to refine the proteins. The centrifugation step had to be performed above 20°C; if not, the SDS from the wash buffer crystallized. Dilution of the concentrated phenol in wash buffer and removal of BCP

after the initial centrifugation step were necessary because the concentrated phenol and BCP each on its own would other wise damage the Amicon-15 tube. The red dye from the organic phase was trapped on the surface of the filter, but did not seem to block the filtration. As the weight of a rat retina is only 6 to 10 mg and the TRI Reagen protocol assumes 50 to 100 mg of starting material, we supplemented the TRI Reagent with Polyacryl Carrier before homogenization to facilitate isolation of RNA from small samples (<10 mg tissue), as recommended by the manufacturer. The Polyacryl Carrier supports the precipitation, thereby increasing the RNA yield. According to the Molecular Research Center [9], it is also recommended to use BCP instead of chloroform for the phase separation, making the DNA interphase more compact and thereby reducing DNA contamination of the aqueous RNA phase. Moreover, BCP is heavier, less toxic, and less volatile than chloroform.

In a recent study, [1] we demonstrated that QC-PCR with a modified DNA mimic method for measurements of mRNA is appropriate for the analysis of small tissue samples or low gene expression. In the present study we established the use of real-time RT-PCR, which combines reverse transcription with a fluorescence measuring technique as described previously [2,3]. It is fast, allows a high throughput, and demands no post-PCR manipulations. It is a highly reliable method with small inter-experimental standard deviations and more specific than QC-PCR. The weak link of the method, however, is still the transcription step from RNA to cDNA. Reverse transcription is performed by a viral polymerase, that is, reverse transcription, only able to decode double-stranded nucleotide strands, making it necessary to add a DNA primer for the production of cDNA, which is identical to the gene strand with the exception of introns. The synthesis of cDNA, however, involves excess reaction components such as DNA contamination products, nucleotides (deoxyribonucleotide triphosphate), and RT primers, which, along with debris from chemical compounds, may give rise to false measurement results, necessitating the use of an internal control [2-6]. In the present study, cDNA synthesis was optimized by choosing 2 μg of template mass (instead of 1 μg) and comparatively long running time for the reverse transcription step (2 hours instead of 1 hour), yielding optimal cDNA output on dilution series and normalization toward an endogenous control, CypB, which was continuously monitored during all of the following processes. CypB was chosen instead of other more widely used endogenous controls, such as β-actin, GAPDH, and CypA, which are more often up- or down-regulated in various cellular processes, for example, cellular stress and apoptosis, which can influence the interpretation of the results. CypB is a ubiquitous enzyme involved in the catalysis of protein folding, protein trafficking, and nucleolytic degradation, but it is less prone to genetic regulation, allowing better reproduction of experiments [13].

The mRNA expression was coupled to its biological effects in each retina by detection of the antioxidant enzymes on Western blot. Except for GPx1, all antioxidant enzymes as well as CypB were detectable on Western blot, which is, however, a less sensitive method with larger standard deviations and hence a need for larger quantities than real-time RT-PCR. Detection of GPx1 in one retina only was not possible. The inability to detect GPx1 on Western Blot in one retina only suggests a low protein content of GPx1 in the retina compared with the other antioxidant enzymes, which is in accordance with the results by Yan et al [14].

For the multiplexed Western blot assays, the blocking buffer used was TBS-T with 0.5% gelatin, because it gave a satisfactory band signal regardless of the target to be detected. The expected protein size according to the manufacturer of the CypB antibody is 19 kd for rat. The calculated theoretical protein size of the primary structure for rat CypB is 23.0 kd [15], which is in accordance with the obtained result.

In the present study, we have shown that it is possible to extract RNA and proteins simultaneously from the rat retina by modulating an existing protocol according to Chomczynski [7], thereby reducing the amount of tissue needed for analysis and yielding adequate RNA and protein substrate from the same individual retina. Detection of mRNA expression of all the relevant antioxidant enzymes and the endogenous control was thereafter safely accomplished by the use of real-time RT-PCR, and the corresponding protein levels were measured on Western blot; but to detect GPx1, putting together several retinas was required.

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References

[1] Agardh CD, Israelsson B, Thuesen-Olesen B, et al. Application of quantitative competitive polymerase chain reaction for measurements of mRNA from antioxidative enzymes in the diabetic rat retina and kidney. Metabolism 2002;51:1279-84.

[2] Pagliarulo V, George B, Beil SJ, et al. Sensitivity and reproducibility of standardized-competitive RT-PCR for transcript quantification and its comparison with real time RT-PCR. Mol Cancer 2004;3:5.

[3] Peinnequin A, Mouret C, Birot O, et al. Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. BMC Immunol 2004;5:3.

[4] Overbergh L, Valckx D, Waer M, et al. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. Cytokine 1999;11:305-12.

[5] Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002;29:23-39.

[6] Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 2002; 30:503-12.

[7] Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 1993;15:532-4, 536-7.
[8] National Center for Biotechnology (NCBI), nBLAST. Homepage: http://www.ncbi.nlm.nih.gov/.
[9] Molecular Research Center, Inc. TRI reagent—RNA/DNA/protein isolation reagent. Cat. no. TR 118, 2003. Homepage: http://www.mrcgene.com.
[10] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
[11] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350-4.
[12] Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem 1987; 262:10035-8.
[13] Stürzenbaum SR, Kille P. Control genes in quantitative molecular biological techniques: the variability of invariance. Comp Biochem Physiol B Biochem Mol Biol 2001;130:281-9.
[14] Yan T, Jiang X, Zhang HJ, et al. Use of commercial antibodies for detection of the primary antioxidant enzymes. Free Radic Biol Med 1998;25:688-93.
[15] Swiss Institute of Bioinformatics, Swiss-Prot Group, Geneva, Switzerland. Homepage: http://www.expasy.org/sprot/.