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Gene expression profiling of diabetic and galactosaemic cataractous rat lens by microarray analysis

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Abstract *Aims/hypothesis:* Osmotic and oxidative stress is associated with the progression and advancement of diabetic cataract. In the present study, we used a cDNA microarray method to analyse gene expression patterns in streptozotocin-induced diabetic rats and galactose-fed cataractous lenses. In addition, we investigated the regulation and interaction(s) of anti-oxidant protein 2 and lens epithelium-derived growth factor in these models. *Methods:* To identify differential gene expression patterns, one group of Sprague–Dawley rats was made diabetic with streptozotocin and a second group was made galactosaemic. Total RNA was extracted from the lenses of both groups and their controls. Labelled cDNA was hybridised to Atlas Rat Arrays. Changes in gene expression level were analysed. Real-time PCR and western analysis were used to validate the microarray results. *Results:* The expression of 31 genes was significantly modulated in hyperglycaemic lenses compared with galactosaemic lenses. Notably, transcript and protein levels of B-cell leukaemia/lymphoma protein 2 and nuclear factor- κ B were significantly elevated in rat lenses at 4 weeks after injection of streptozotocin. At a later stage, mRNA and protein levels of *TGF- β* were elevated. However, levels of mRNA for *IGF-1*, lens epithelium-derived growth factor and anti-oxidant protein 2 were diminished in streptozotocin-induced diabetic cataract. *Conclusions/interpretations:* These results provide evidence that progression of sugar cataract involves oxidative- and *TGF- β* -mediated signalling. These pathways may

promote abnormal gene expression in the hyperglycaemic and galactosaemic states and thus may contribute to the symptoms associated with these conditions. Since oxidative stress seems to be a major event in cataract formation, supply of anti-oxidant may postpone the progression of such disorders.

Keywords cDNA microarray · Diabetic cataract · Galactose cataract · Oxidative stress · Polyol pathway

Abbreviations AOP2: Anti-oxidant protein 2 · AR: Aldose reductase · bcl2: B-cell leukaemia/lymphoma protein 2 · Ct: Cycle threshold · Hsp: Heat shock protein · LEC: Lens epithelial cell · LEDGF: Lens epithelium-derived growth factor · NF- κ B: Nuclear factor- κ B · ROS: Reactive oxygen species · STZ: Streptozotocin

Introduction

Diabetic eye disease develops as a complication of long-term diabetes and is the most common cause of blindness. Diabetic cataract is a leading cause of vision loss in younger patients. Surgery is the only treatment for diabetic cataract, but after surgery the progression of diabetic retinopathy is often accelerated by the breakdown of the blood–aqueous barrier [1]. Furthermore, anterior capsular contraction [2, 3] and posterior capsular opacification [4] are induced earlier in diabetic patients than in non-diabetic patients after cataract surgery and intra-ocular lens implantation. The development of more effective therapies requires a precise understanding of the molecular mechanisms that cause disease, providing a basis for a rational approach to therapy.

Hyperglycaemia triggers the activation of the aldose reductase (AR)/sorbitol pathway, non-enzymatic glycation of lens fibres and oxidative stress [5–8]. In the lens, osmotic stress imposed by sorbitol accumulation has long been believed to be the major factor in the progression of diabetic cataract [6–11]. Activation of the AR/sorbitol pathway also increases oxidative stress by increasing levels of hydrogen peroxide [12] and free radicals [13]. The involvement of the

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polyol pathway in generation of hyperglycaemia-induced oxidative stress has been demonstrated in diabetic lenses [7, 8, 14]. We have reported that human lens epithelial cells overexpressing AR are susceptible to apoptosis under hyperglycaemic conditions, and that supply of an AR inhibitor and/or anti-oxidant protein 2 (AOP2) was able to protect these cells from hyperglycaemia-induced apoptosis [15]. We have used streptozotocin (STZ)-induced diabetes in rats as an animal model of type 1 diabetes, and galactose-fed rats have been studied as animal models for sugar cataracts [7, 8, 11]. Lenticular polyol accumulation was the primary event in these models for the induction of cataractogenesis, and the progression of sugar-induced cataract depended on the level of serum glucose or galactose [7]. However, disease severity increased as the diabetes progressed, and nearly all diabetic patients are susceptible to the development of cataract.

Among the several studies of the mechanism of progression of diabetic cataract and its complications, none has explored the profiling of global gene expression. Importantly, oxidative stress and osmotic stress are both believed to be involved in the pathogenesis of diabetic as well as sugar cataract, with a major role proposed for osmotic stress in the latter disorder [14–16]. Analysis of individual genes has begun to define some critical stages in the progression of hyperglycaemia- or galactocaemia-induced cataractogenesis, yet the complex interaction(s) of extracellular signals and responding genetic networks that control lens physiology are largely unstudied. Since global changes in gene expression underlie developmental processes in pathogenesis, we expect, by using cDNA microarray analysis, to reveal signalling pathways and biomolecules important for the development of diabetic cataract.

In the present study, we used cDNA microarrays from Clontech (Palo Alto, CA, USA) to analyse systematically the genes that are modulated and also to define the genes that are common to both aetiologies or specific for one of them. The design of our study is as follows. First, we used lenses from the early stages of galactocaemic and hyperglycaemic cataracts and their respective age-matched controls. Secondly, we compared both spatial and temporal expression profiles in each group to define more fully the common or specific genes characteristic of each condition. Thirdly, we validated our profiles by demonstrating the galactocaemic- and/or glycaemic-specific expression of certain genes by real-time PCR and western blot analysis. The present study provides novel and detailed insights into gene networks activated in lenses or lens epithelial cells (LECs) facing galactocaemic and hyperglycaemic assault. Furthermore, knowledge of dysregulated gene expression, together with detailed studies on the relevant signalling pathways and their uniqueness in both cataract groups, may provide opportunities for the rational development of drugs to delay and prevent the onset of these conditions.

Materials and methods

Animals All animal experiments followed the recommendations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We used 4-week-old Sprague–Dawley albino rats, which were obtained from a local animal dealer (Clea Japan, Osaka, Japan). Diabetic cataract was induced in 20 rats by a single intraperitoneal injection of 80 mg/kg body weight STZ (Sigma, St. Louis, MO, USA) in 0.05 mmol/l citrate buffer (pH 4.5) after they had been fasted overnight [17]. These STZ-injected rats were given free access to regular chow consisting of 25% (w/w) protein, 53% carbohydrate, 6% fat and 8% water (Oriental Yeast, Osaka, Japan) for 4 or 12 weeks (STZ4W and STZ12W groups). At the fourth week after injection of STZ, all rats had blood glucose levels over 33.4 mmol/l. To induce sugar cataract, ten rats were given free access to 50% D-galactose mixed with regular chow for 4 or 14 days without injection of STZ (Gal4d and Gal14d groups). The eyes of these animals were monitored daily to assess the progression of cataract, using a previously published method [13]. At predefined times, the animals were killed and RNA was taken from their lenses. RNA from age-matched animals was used as control.

RNA extraction from lens At 4 weeks after STZ injection, and on day 4 of galactose feeding, five rats from each group were killed with CO₂. Lenses were extracted from these rats and immediately frozen in liquid nitrogen. We also used 8-week-old rats for the study, and the gene profiles were compared. Total RNA from each sample was extracted with the Atlas Glass Total RNA Isolation Kit (Clontech) according to the manufacturer's protocol. Lenses from control rats were used to isolate RNA that served as control. Samples of RNA were set aside for real-time PCR to verify the results obtained from the microarray analysis. Agarose gel electrophoresis was performed to assess RNA integrity. Each isolated total RNA had a 28S/18S ratio of 1.5.

RNA labelling and hybridisation The Atlas Glass Fluorescent Labeling Kit (Clontech) was used for RNA labelling. Briefly, for each labelling, 20 µg of total RNA was reverse-transcribed in the presence of CDS primer mix and MMLV reverse transcriptase. Cy3 fluorescent dye (Amersham Pharmacia, Piscataway, NJ, USA) was coupled to the first-strand cDNA. Labelled cDNA was hybridised to Atlas Glass Rat 1.0 Arrays (Clontech) according to the manufacturer's protocol. Briefly, labelled cDNA was mixed with pre-incubated GlassHyb Hybridisation Solution at 50°C. Hybridisation solution containing labelled cDNA was hybridised to the glass array in the hybridisation chamber at 50°C. After overnight hybridisation with the labelled probe, each glass slide was washed once in GlassHyb Wash Solution, twice in 1×SSC (15 mmol trisodium citrate and 150 mmol NaCl) and once in 0.1×SSC at room temperature. Air-dried slides were scanned with a fluorescent slide scanner. Scanned images were analysed with ArrayGauge software (Fuji Photo Film, Tokyo, Japan).

Microarray procedure and statistical analysis Atlas Glass Rat 1.0 Microarrays were purchased from Clontech and used for the experiment. Briefly, the array contains 1,081 genes, represented by well-characterised single-strand cDNA fragments and excluding expressed sequence tags, which can often be redundant and misrepresentative, and is printed on a DNA Ready Type II slide. Each rat gene is represented by an 80-base oligonucleotide.

Atlas Iris software (Clontech) was used to normalise the 1,081 genes for overall background and to compare the experimental slides. The fold change(s) in gene expression in the galactose or STZ groups vs the control group was calculated, and genes that were differentially expressed to a statistically significant extent were identified with the Atlas Iris software.

Real-time PCR To validate the gene expression patterns obtained from microarray analysis, relative quantification of mRNA was performed with LightCycler-RNA Master SYBRGreen I (Roche, Mannheim, Germany). At 4 and/or 12 weeks after STZ injection, five rats from each group were killed with CO₂. To avoid amplification of genomic DNA sequences, all RNA samples were treated with DNase I and diluted to 100 ng/μl. All reactions were carried out in a single tube reaction setup on the LightCycler system (Roche). The following temperature profile was used: 20 min at 61°C for reverse transcription and 30 s at 95°C for denaturation of cDNA/RNA hybrid, followed by 45 cycles of 5 s at 95°C, 5 s at 55°C and 13 s at 72°C. The cycle threshold (Ct) was used to calculate relative amounts of target RNA. Table 1 summarises the sequences and sources of primers used in this study. Samples that did not reach the threshold line were considered not to be above background. The Cts of target genes were normalised to the levels of beta-actin as an endogenous control in each group. The average Ct for each gene is calculated by subtracting the Ct of the sample RNA from that of the control RNA for the same time measurement. This value is known as the ΔCt and reflects the relative expression of the treated sample

compared with the control, and becomes the exponent in the calculation for amplification $2^{\Delta C_{t\text{control}} - \Delta C_{t\text{sample}}}$, the equivalent to fold change in expression. Unpaired Student's *t*-tests were used for statistical analysis.

Western blot analysis At 4 and 12 weeks after STZ injection (STZ4W and STZ12W groups) and on days 4 and 14 of feeding of 50% galactose (Gal4d and Gal14d groups), five rats from each group were killed with CO₂. Tissue lysates of lenses were prepared in ice-cold RIPA buffer as described previously [18]. The protein concentration of each supernatant was determined by the Lowry assay [19]. Protein (20 μg) was loaded and run on a 10 to 20% SDS-PAGE gradient gel and transferred to a PVDF membrane (BioRad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% milk, and were incubated overnight at 4°C with anti-mouse B-cell leukaemia/lymphoma protein 2 (bcl-2) monoclonal Ab, anti-rabbit bax polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-rabbit lens epithelium-derived growth factor (LEDGF) polyclonal Ab (described elsewhere [18]), and anti-rabbit AOP2 polyclonal Ab (dilution 1:1,000–4,000). After washing, the membranes were incubated with anti-mouse or anti-rabbit IgG labelled with horseradish peroxidase (diluted 1:2,000; Santa Cruz Biotechnology) and visualised by enhanced chemiluminescence according to the manufacturer's protocol (Santa Cruz Biotechnology). The same dilutions of absorbed Abs to the various molecules were used as negative controls. Anti-rabbit actin Ab (Santa Cruz Biotechnology) was used to show that an equal amount of protein was loaded in each lane.

Results

Cataracts in STZ-injected and galactose-fed rats Some peripheral vacuoles in the equatorial cortical regions were observed in the STZ4W group and the Gal4d group. Cortical cataract progressed to the entire lens surfaces in

Table 1 Primer sets used for real-time PCR

Gene name	Primer sequence	Product size (bp)
NF-κB	Forward; 5' tcttcgactacgcggttacgggag 3' Reverse; 5' catcatccaccttcacgctcagct 3'	127
Bcl-2	Forward; 5' gggatgcctttgtggaacta 3' Reverse; 5' ctactgtgtgccaggat 3'	138
TGF-β3	Forward; 5' aatctgccacagagaggcaccgc 3' Reverse; 5' catgtacatactttcagccaat 3'	138
IGF-binding protein	Forward; 5' acaccgagtctgcttcaact 3' Reverse; 5' gaacctgtgtcaggcagga 3'	138
IGF-1	Forward; 5' ccgctgaagcctacaagtc 3' Reverse; 5' gggaggctcctcctcaattc 3'	144
AOP2	Forward; 5' ccagagtttgccaaggagac 3' Reverse; 5' tccaatcaactgctgctgtc 3'	166
LEDGF	Forward; 5' ggggttacgtcaacctctga 3' Reverse; 5' ttggccttttagcatgtcc 3'	114
Beta actin	Forward; 5' tgtaaccaactgggacgatatg 3' Reverse; 5' attagagaaggcgtgctga 3'	203

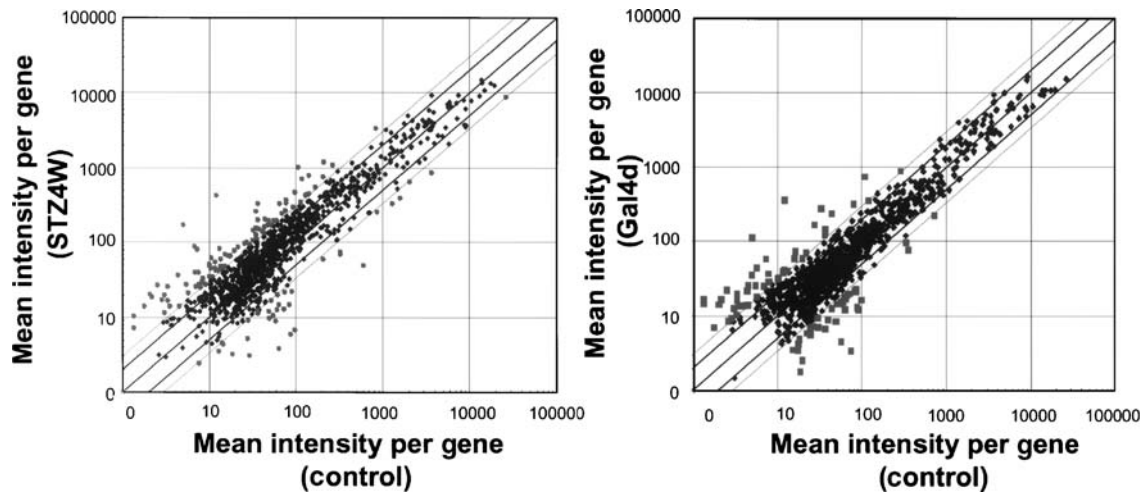


Fig. 1 Analysis of gene expression profile. The scatter plots show a representative experiment, using rat lens cDNAs from the normal group and from the streptozotocin group at 4 weeks (*STZ4W*) or the galactose group at 4 days (*Gal4d*). Horizontal and vertical axes

represent normal and experimentally generated signals, shown in *blue* and *red*, respectively, on a logarithmic scale. Genes used for subsequent analysis are described in “Materials and methods”

the STZ12W and Gal14d groups. These lenses were used for gene expression profiling and were compared within and between the groups.

Analysis of gene expression profile Scattergrams of mRNA expression demonstrating the differences between the control lens and those from the STZ4W and Gal4d groups are shown in Fig. 1. The changes in the expression of the majority of genes were within the 3-fold range (Fig. 1). Changes greater than 2.5-fold were seen in 83 genes in the STZ4W group and 29 genes in the Gal4d group (see Electronic Supplementary Material [ESM], link on page 1 of this article). In the STZ4W group, 64 genes were significantly upregulated and 19 genes were significantly downregulated (see ESM). In the Gal4d group, 22 genes

were significantly upregulated and 7 genes were significantly downregulated (see ESM). Differentially regulated genes in these rat lenses could be classified into 17 groups: (1) cell surface antigens; (2) transcription factors, activators and repressors; (3) cell cycle; (4) cell adhesion receptors/proteins; (5) extracellular transport/carrier proteins; (6) stress-response proteins; (7) membrane channels and transporters; (8) trafficking/targeting proteins; (9) metabolism; (10) apoptosis-associated proteins; (11) DNA-binding and chromatin proteins; (12) cell receptors (by ligands); (13) cell signalling and extracellular communication proteins; (14) intracellular transducers/effectors/modulators; (15) protein turnover; (16) cell receptors (by activities); and (17) unclassified function.

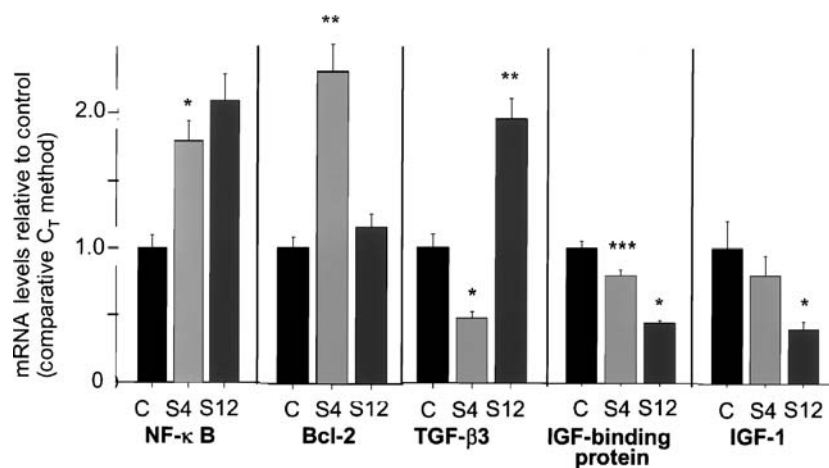


Fig. 2 Real-time PCR confirming the gene expression profiles obtained from microarray results in STZ rats with various durations of treatment (*S4*, 4 weeks streptozotocin; *S12*, 12 weeks streptozotocin). Total RNA was isolated as described elsewhere and PCR was conducted with gene-specific primers (see Table 1) according to the manufacturer’s protocol. A higher level of expression of NF- κ B (*S4*) ($*p < 0.003$) and TGF- β 3 (*S12*) ($**p < 0.001$) was observed, whereas

the expression level of TGF- β 3 (*S4*) was decreased ($*p < 0.003$). Notably, the expression level of *bcl2* was sharply increased in the *S4* group ($**p < 0.001$) and normalised in the *S12* group by real-time PCR. IGF-binding protein and IGF-1 expression levels showed declining patterns in the *S4* and *S12* groups ($*p < 0.003$; $***p < 0.01$). *C* control (black bars)

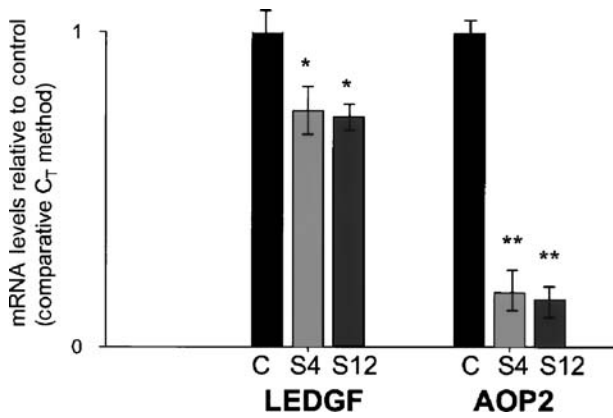


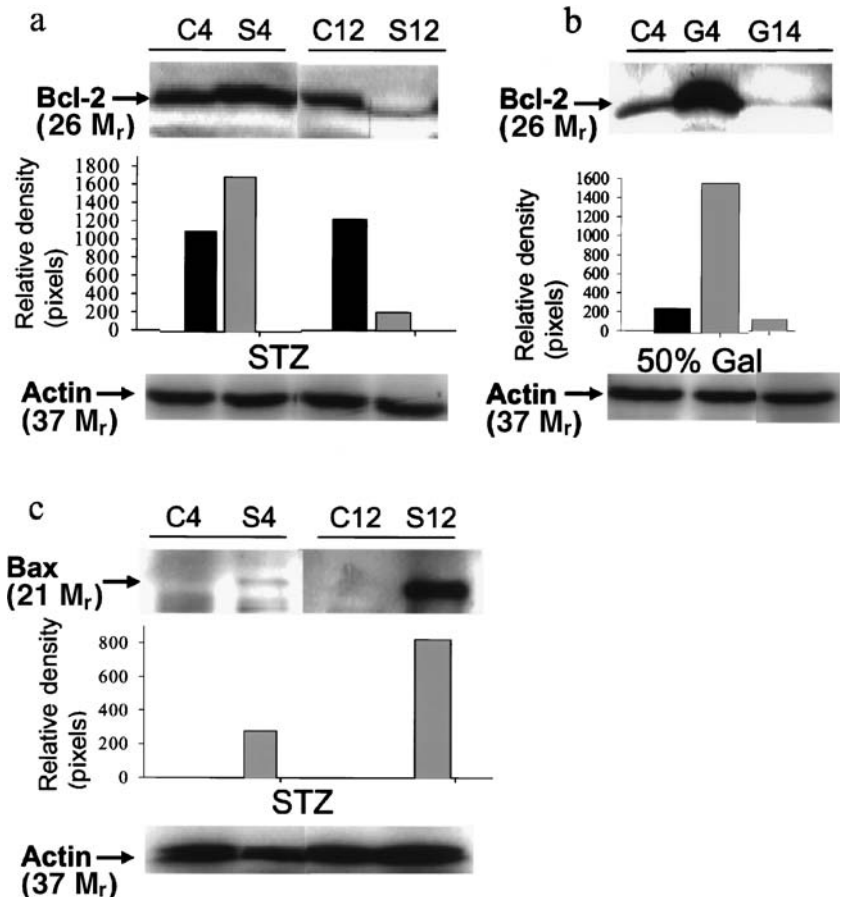
Fig. 3 Real-time PCR showing downregulation of LEDGF and AOP2 mRNAs in STZ-induced diabetic rats. Real-time PCR was conducted with specific LEDGF and AOP2 primers (see “Materials and methods” and Table 1) to assess their expression. Expression of LEDGF mRNA was reduced in the S4 (4 weeks streptozotocin) and the S12 (12 weeks streptozotocin) groups ($*p < 0.01$). Notably, a dramatic reduction in AOP2 mRNA in the S4 and S12 groups compared with control (C, black bar) demonstrates the higher oxidative environment in the diabetic condition ($**p < 0.001$)

Twenty-three genes that were up- or downregulated by more than 2.5-fold relative to the control were common to both the STZ4W and Gal4d groups. However, the most highly upregulated gene common to both groups was *bcl-2*, an anti-apoptotic-associated protein, of which the

expression was more than 30-fold higher than the control. The transcriptional factor nuclear factor- κ B (NF- κ B), an activator of the *AR* gene [20], was upregulated in both groups (see ESM). Expression levels of *TGF- β 1* and *TGF- β 3*, *IGF-1* and *IGF-binding protein* were diminished in both the STZ4W and/or Gal4d groups. Analysis of the gene expression patterns of the STZ and galactose groups demonstrated a common pattern of expression of certain genes, specifically those associated with oxidative stress. However, our study also demonstrated that the role of oxidative stress is predominant in the STZ group, whereas osmotic and oxidative stress are implicated in cataractogenesis in the galactose group.

Real-time PCR analysis of mRNA expression Next, we verified the up- or downregulation of expression of selected genes in both groups by real-time PCR. *NF- κ B*, *bcl-2*, *TGF- β 3*, *IGF-binding protein* and *IGF-1* were selected on the basis of the results from the microarray study in the STZ4W group (Fig. 2). *NF- κ B* mRNA was upregulated at the 4th and 12th weeks after STZ injection ($p < 0.003$). On the other hand, *TGF- β 3* mRNA expression was downregulated at the 4th week ($p < 0.001$) and upregulated at the 12th week ($p < 0.002$) (Fig. 2). *IGF-binding protein* and *IGF-1* were downregulated at the 4th and 12th weeks ($p < 0.01$) after STZ injection (Fig. 2). Real-time PCR showed that *bcl-2* mRNA expression was upregulated at the 4th week ($p < 0.001$) and normalised at the 12th week (Fig. 2). Next,

Fig. 4 Western blot analysis showing expression levels of *bcl2* and *bax* protein in rat lenses with STZ- and galactose-induced cataracts. **a** Representative protein blot showing elevated expression of *bcl2* protein at 4 weeks (lane S4) compared with control (lane C4) and reduced expression at 12 weeks (lane S12). **b** Experiment showing an increased level of *bcl2* protein in lenses from galactose-fed rats on day 4 of feeding (lane G4) and a diminished level on day 14 (lane G14); the level of *bcl2* was restored in lenses of rats receiving an aldose reductase inhibitor (*ARI 14*). **c** Western blot showing increased expression of *bax* in lenses of rats with STZ-induced diabetic cataracts at week 4 (lane S4) and further increased expression at week 12 (lane S12) compared with control (lanes C4 and C12), which had no expression. This result suggests the predominance of apoptotic events. Expression of actin in all groups was almost the same, showing that an equal amount of protein was loaded in each lane



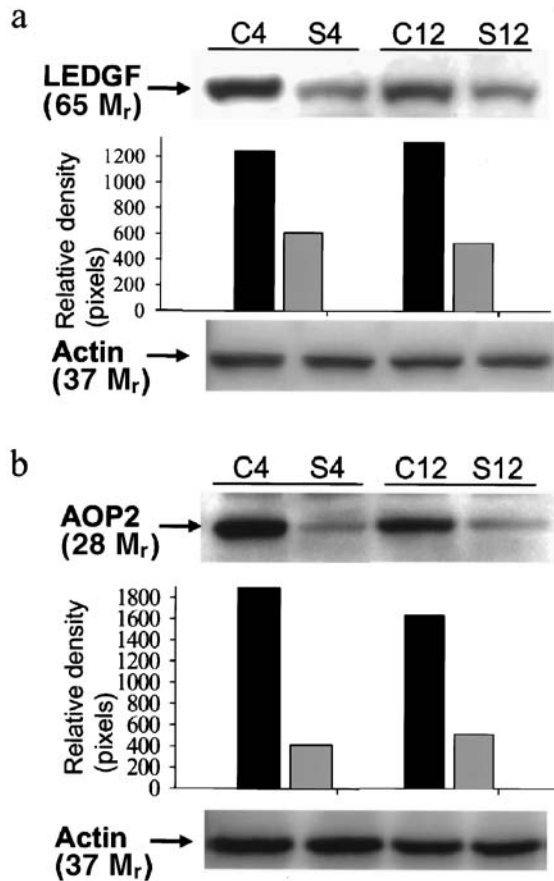


Fig. 5 Western blot showing expression patterns of LEDGF and AOP2 proteins in lenses of rats with STZ-induced diabetic cataracts. **a** Equal amounts of protein were loaded onto each lane, blotted on nitrocellulose membranes, probed with anti-LEDGF-specific antibody and visualised as described in “Materials and methods”. Reduced expression of LEDGF protein was observed in both groups (lanes S4 and S12) compared with the control (lanes C4 and C12). **b** Representative western blot showing diminution of AOP2 protein in both groups (lanes S4 and S12) compared with the control (lanes C4 and C12), suggesting a higher prevalence of an oxidative environment during progression of the hyperglycaemic state. Expression of actin in all groups was almost equal showing that an equal amount of protein was loaded in each lane

we examined transcription levels of the oxidative-stress-associated as well as apoptosis-associated genes *LEDGF* and *AOP2* [21–27]. We found that *LEDGF* and *AOP2* mRNAs were downregulated in rat lenses at the 4th and 12th weeks after STZ injection (Fig. 3) (*LEDGF*: $p < 0.01$; *AOP2*: $p < 0.001$), suggesting that supply of anti-oxidant protein may prevent hyperglycaemia-induced complication(s).

Protein blot analysis of oxidative-stress-associated and apoptosis-related genes in STZ-injected and/or galactose-fed rats Since elevation of gene transcription is not always associated with gene translation, we examined the protein levels of selected relevant biomolecules, including the apoptosis-related *bcl-2* and *bax*, the apoptosis- and oxidative-stress-related transcriptional factor *LEDGF*, and *AOP2*, also known as peroxiredoxin 6. Our analysis revealed elevated *bcl-2* protein expression in lenses at the 4th week after

STZ injection and the 4th day of 50% galactose feeding, but reduced expression at the 12th week after STZ injection and the 14th day of 50% galactose feeding (Fig. 4a, b). In contrast, the pro-apoptotic protein *bax* was upregulated in STZ rats, especially at the 12th week after STZ injection (Fig. 4c). *LEDGF* and *AOP2* were downregulated in STZ rat lenses (Fig. 5a, b). There was no significant difference in *LEDGF* and *AOP2* protein expression levels between the STZ4W and STZ12W groups (Fig. 5a, b).

Discussion

Oxidative stress may result from an increase in free radical production and/or a decrease in anti-oxidant defenses because of environmental stressors [28]. High glucose levels attenuate the function of LECs through the generation of free radicals [13, 16, 29], suggesting a possible pathophysiological linkage between the high level of glucose and balance of anti-oxidants. Our study revealed decreased expression of *AOP2* mRNA and protein in the lenses of rats with diabetes- or galactose-induced cataracts. This is consistent with our previous observations that hyperglycaemia causes a decrease of this protein in LECs and that these cells subsequently undergo apoptosis.

However, the aim of this study was to define more precisely the novel family of genes that provide a sustained response to deleterious signals from reactive oxygen species (ROS) generated during hyperglycaemia or from osmotic stress due to hyperglycaemia and galactocaemia. To do so, we obtained lenses from rats with glucose-induced (STZ) or galactose-induced cataracts at different stages, isolated total RNA and performed microarray analysis. Using this approach, we identified several subsets of genes that continue to respond to ROS signalling. For example, the transcription factors *NF- κ B* and *API1* are redox active and are highly expressed during hyperglycaemia. Of the 1,081 genes analysed, 83 genes in the STZ group and 29 genes in the galactose group were significantly up- or downregulated at the 4th week after STZ injection and on the 4th day of galactose feeding (see ESM). In the STZ group, 64 genes showed more than 2.5-fold upregulation and 19 genes showed more than 2.5-fold downregulation compared with the control. In the galactose group, 22 genes showed more than 2.5-fold upregulation and 7 genes showed more than 2.5-fold downregulation compared with the control. In the STZ group, upregulated genes were classified into 16 groups and downregulated genes were classified into 7 groups (see ESM). In the galactose group, upregulated genes were classified into ten groups and downregulated genes were classified into three groups (see ESM).

Although all the genes (listed in ESM) may be of interest, it was not possible to study all of them. Lenticular polyol accumulation initiated by AR has been considered a principal factor in the aetiology of sugar cataract, such as STZ cataract and galactosaemic cataract [6–9, 30]. Among the 22 upregulated genes in the galactose group, 16 genes were upregulated similarly in the STZ group. Among the seven downregulated genes in the galactose group, three genes

were downregulated similarly in the STZ group. Diabetes in STZ-treated rats results from the STZ-mediated destruction of the insulin-producing beta cells of the pancreas [17, 31]. Gene expression changes in STZ rats could be induced not only by AR-mediated osmotic or oxidative stress but also by insulin depletion or by factors mediated by beta cell destruction. Thus, special attention was paid to the set of genes that showed more than 4-fold up- or downregulation in both groups, and mRNA expression levels of these genes were confirmed by real-time PCR. Moreover, sorbitol is a polyol osmolyte synthesised from glucose by the enzyme AR. High plasma glucose concentrations increase intracellular glucose concentrations in cells permeable to glucose and force an increase in sorbitol synthesis by mass action [32]. The accumulation of sorbitol may damage cells in the eye, in part by inducing a reciprocal loss of other volume regulatory solutes such as myo-inositol and taurine [33]. In LECs, the accumulation of sorbitol accompanies the ultrastructural changes that commonly occur in diabetes and is believed to cause cell swelling as an initial event in cataract formation. Thus, there are several factors that may be involved in gene transcription. Under hypertonic conditions, however, sorbitol accumulation occurs and is accompanied by upregulation of the transcription and translation of the gene encoding for the sodium-dependent myo-inositol cotransporter [34]. Evidence to date implicating the polyol pathway in the pathogenesis of diabetic complications has been considered. The present study demonstrates that various redox-active genes are upregulated, and future studies should investigate how these genes are associated with specific stages of the disease. Moreover, the gene for the transcriptional factor NF- κ B was upregulated in both groups in microarray analysis, and real-time PCR showed time-dependent increases in its mRNA expression level after STZ injection (ESM; Fig. 2). It has been reported that hypertonic induction of *AR* mRNA is induced by activation of NF- κ B and its binding to the osmotic response element of the *AR* promoter [20, 35, 36]. AR-dependent metabolism is essential for cell death mediated by cytokines and high glucose, and the inhibition of AR prevents redox-sensitive events preceding the activation of protein kinase C and NF- κ B [36]. However, increased levels of *NF- κ B* transcript in sugar cataract may be related to the induction of AR expression and of apoptosis in the lens.

TGF- β 1 and *TGF- β 3* were downregulated in the STZ4W and/or Gal4d groups and *TGF- β 3* was upregulated in the STZ12W group. TGF- β has been reported to induce morphological changes similar to those in human anterior subcapsular cataract. Anterior and posterior subcapsular cataracts are typically observed in diabetic patients. TGF- β , a potent inducer of matrix protein synthesis, may be a candidate mediator of the morphological changes observed in diabetic cataract. In addition, our results indicate activation of TGF- β signalling in diabetic and galactocaemic conditions (Fig. 3). We believe that NF- κ B signalling is suppressed by TGF- β . Furthermore, hyperglycaemia-induced oxidative stress (ROS/H₂O₂) and ROS themselves are known inducers of bioactive TGF- β [37, 38]. A decline in

anti-oxidants has also been reported in diabetes, providing a further reason for TGF- β activation [14, 16].

In humans, increases in serum and vitreous IGF-1 levels have been found in patients with poorly controlled diabetes [39]. However, *IGF-1* and *IGF-1-binding protein* were downregulated in the STZ4W and STZ12W groups, but not in the Gal4d group. The level of mRNA for *IGF-1* in diabetic rats differs among tissues, and expression of *IGF-1* mRNA in liver and stomach was lower in STZ-diabetic rats than in normal rats [39, 40]. Our data show that lenticular expression of *IGF-1* and *IGF-1-binding protein* mRNAs was lower in diabetic rats than in normal animals. Insulin depletion in STZ-diabetic rats may be the cause of the lower *IGF-1* level in the lens. In the present study, mRNA for the anti-apoptotic protein *bcl-2* was upregulated more than 30-fold in both groups by microarray analysis (ESM), and we confirmed this overexpression of *bcl-2* in sugar cataract by real-time PCR in the STZ4W and STZ12W groups. Interestingly, *bcl-2* transcripts were 2.2-fold upregulated at 4 weeks in the STZ-treated rats and decreased to about the control level by 12 weeks (Fig. 3). However, as *bcl-2* expression was found to be upregulated, we wondered about the data obtained from real-time PCR (2.5-fold) and microarray (30-fold) on *bcl-2* expression. This discrepancy may be due to the presence of splicing variants or some other technical problems. In some cell lines, *bcl-2*-overexpressing cells show greater resistance to various pro-oxidants than do mock-transfected cells [41–43]. However, in other cell lines, expression of *bcl-2* does not protect the transfected cells against oxidative stress [44, 45]. In rabbit LECs, *bcl-2*-transfected cells are more susceptible to H₂O₂-induced apoptosis than are control cells [46]. In the present study, upregulation of *bcl-2* was observed in early (4 weeks) cortical cataracts in STZ-treated rats. It is not clear whether an anti-apoptotic pathway is active in these early cataracts, but upregulation of the apoptotic protein *bax* and normalised levels of *bcl-2* were observed in cataractous lenses 12 weeks after STZ treatment. It has been reported that *bcl-2* increases the susceptibility of rabbit LECs to H₂O₂-induced apoptosis, an effect mediated by downregulation of the α B-crystalline gene as a result of changed activity of LEDGF [46]. LEDGF is a newly identified transcriptional factor [25, 47–49] and is homologous to p75, a known coactivator of transcription and pre-mRNA splicing [50–52]. LEDGF mRNA and protein are activated and upregulated by heat and oxidative stress and enhance cellular survival [22]. In our study, LEDGF and AOP2 mRNA and protein levels were downregulated in lenses from STZ-treated rats after 4 and 12 weeks. We have reported that AOP2 can protect against hyperglycaemia-induced apoptosis in human LECs [15]. Downregulation of LEDGF and AOP2 would increase oxidative stress in lenses from STZ-treated rats. In turn, increased oxidative and osmotic stress would induce apoptosis and lens fibre swelling of LECs, leading to sugar cataract in STZ-treated rats. It is our intention to determine whether or not upregulation of *bcl-2* can induce downregulation of AOP2.

We conclude from this study that a large number of factors are involved in the initiation and progression of sugar

cataract. However, the expression patterns of several genes, such as *NF- κ B*, *bcl-2* and *bax*, were similar in the STZ-treated and galactose-fed groups. From these results, we speculate that activation of the polyol pathway may lead to osmotic and oxidative stress that regulates gene expression in lenses from STZ-treated and galactose-fed rats. Importantly, these findings indicate that the induction and progression of diabetic and galactocaemic cataract may be associated with abnormal redox signalling caused by the presence of TGF- β . However, the differential expression of several genes in hyperglycaemic and galactocaemic cataract suggests the involvement of separate and novel signalling pathways in their pathogenesis. These findings may suggest mechanisms that lead to the induction of survival-inhibitory genes in diabetic cataractous lenses, thereby providing an approach for reproducing the effects of survival genes such as *LEDGF* and anti-oxidant genes such as *AOP2*. In turn, application of this knowledge may lead to therapies capable of postponing the onset or progression of diabetic cataract.

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