Eye (2007) 21, 238–245

www.nature.com/eye

LABORATORY STUDY

Expression of antiapoptotic and proapoptotic molecules in diabetic retinas

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Abstract

Purpose **To investigate the expression of the antiapoptotic and proapoptotic markers in diabetic retinas.**

Methods **In total, 12 donor eyes from six subjects with diabetes mellitus, and 10 eyes from five nondiabetic subjects without known ocular disease serving as control subjects were examined. Immunohistochemical techniques were used with antibodies directed against cyclooxygenase-2 (Cox-2), Akt (protein kinase B), Mcl-1, Bad, cytochrome *c*, apoptosis-inducing factor (AIF), tumour necrosis factor receptor-1-associated death domain protein (TRADD), and Fas-associated death domain protein (FADD).**

Results **In retinas from all subjects without diabetes, cytoplasmic immunoreactivity for the antiapoptotic molecules Cox-2, Akt, and Mcl-1 was noted in ganglion cells. Cytoplasmic immunostaining for Cox-2 was also noted in the retinal pigment epithelial cells. Weak immunoreactivity for the mitochondrial apoptogenic proteins cytochrome *c*, and AIF was noted in the inner segments of photoreceptors, in the inner one-third of the outer plexiform layer, in cells in the inner nuclear layer, in the inner plexiform layer, and in ganglion cells. There was no immunoreactivity for the other antibodies tested. All diabetic retinas showed *de novo* cytoplasmic immunoreactivity for Bad in ganglion cells, and in occasional cells in the inner nuclear layer. Upregulation of cytochrome *c* and AIF immunoreactivity was noted. Cox-2, Akt, and Mcl-1 immunoreactivity was not altered in the diabetic retinas. There was no immunoreactivity for TRADD, and FADD.**

Conclusions **Ganglion cells in diabetic and nondiabetic retinas express the antiapoptotic molecules Cox-2, Akt, and Mcl-1. Retinal ganglion cells express the proapoptotic molecule Bad in response to diabetes-induced neuronal injury. Diabetic retinas show upregulation of the mitochondrial proteins cytochrome *c*, and AIF.**

Eye (2007) **21**, 238–245. doi:10.1038/sj.eye.6702225; published online 20 January 2006

Keywords: retina; diabetes; apoptosis; mitochondria

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Received: 4 March 2005
Accepted in revised form: 22 September 2005
Published online: 20 January 2006

Cell death by apoptosis, a tightly orchestrated event under the control of genetic programs, appears to play a prominent role in the development and pathogenesis of diabetic complications. Several *in vitro* and *in vivo* studies demonstrated that oxidative stress induced by hyperglycaemia leads to oxidative injury of neurons, which in turn activates the death pathways implicated in neuronal apoptosis.[1–3] Retinas from diabetic rats showed increased oxidative stress, and administration of antioxidants inhibited the development of retinopathy.[4–6] Increased apoptosis of neural retinal cells in experimental diabetes in rats and diabetes mellitus in humans was recently documented. This cell death by apoptosis gives rise to a chronic neurodegeneration in which neurons are lost in the diabetic retinas before other histopathology is detectable.[7,8]

The molecular mechanisms that regulate neuronal survival and apoptosis in the retinas from human subjects with diabetes mellitus have not been completely identified. In a previous study, we demonstrated that ganglion cells in diabetic retinas expressed the apoptosis-promoting factors caspase-3, Fas, and Bax, suggesting that these cells are the most vulnerable population.[9] A number of additional

mediators that are either antiapoptotic or proapoptotic have been identified.[10] They include among others the serine/threonine protein kinase Akt (also known as protein kinase B), cyclooxygenase-2 (Cox-2), Mcl-1, Bcl-2-associated death promoter (Bad), cytochrome *c*, apoptosis-inducing factor (AIF), tumour necrosis factor receptor-1-associated death domain protein (TRADD), and Fas-associated death domain protein (FADD).

Akt has been shown to protect neuronal cells against apoptosis by influencing the activity of several transcription factors implicated in regulating cell survival.[11–16] Several studies showed that Cox-2 functions as a survival factor by protecting cells from apoptosis.[17–21] Overexpression of Mcl-1, a member of the Bcl-2 family,[22] delays apoptosis by a broad array of agents.[18,21,23–26] Bad is a proapoptotic member of the Bcl-2 gene family that promotes apoptosis by binding to and inhibiting functions of antiapoptotic proteins Bcl-2 and Bcl-xL.[10,27] Mitochondria play a key role in the control of apoptotic cell death. Early during the apoptotic process, mitochondria can release several apoptogenic proteins, such as cytochrome *c* and AIF, into the cytosol.[27,28] The death domain containing adaptors TRADD and FADD act as pivotal proteins in the mechanism of ligand-induced programmed cell death (apoptosis) originating at the Fas (CD 95/APO-1) and tumour necrosis factor type 1 receptors. Recruitment of caspase-8 through TRADD and FADD results in caspase activation and subsequent apoptosis.[29]

Identifying the molecular mechanisms that regulate apoptosis of neural cells in diabetic retinas is beneficial for the development of therapeutic strategies that may provide a way to delay or prevent the onset of retinal neural cell death in patients with diabetes. Therefore, we used immunohistochemical techniques to study the expression of the apoptosis-related markers Akt, Cox-2, Mcl-1, Bad, cytochrome *c*, AIF, TRADD, and FADD in the retinas from diabetic patients and in the retinas from subjects without diabetes.

Methods

Study population

We obtained 12 human donor eyes postmortem from six subjects with diabetes mellitus. No subject had a history of retinal photocoagulation. We also obtained 10 eyes from five persons with no history of diabetes or of ocular disease, as determined by gross pathologic examination. Donor eyes were obtained and used in the study in accordance with the provisions of the Declaration of Helsinki for research involving the human tissue. Immediately after the specimens arrived in the laboratory, an incision was made 3 mm posterior to the limbus, and the cornea, iris, lens, and vitreous were gently removed. The retina and uveal tissue were dissected from the surrounding tissue, fixed in 4% paraformaldehyde, and embedded in paraffin.

Immunohistochemical staining

After deparaffinization, endogenous peroxidase was abolished with 2% hydrogen peroxide in methanol for 20 min, and nonspecific background staining was blocked by incubating the sections for 5 min in normal swine serum. For Cox-2 detection, the sections underwent heat-induced antigen retrieval with a microwave oven (three 5-min cycles in 10 mM Tris-EDTA buffer (pH 9) at 650 W). For Akt, Mcl-1, cytochrome *c*, AIF, TRADD, and FADD detection, antigen retrieval was performed by boiling the sections in 10 mM Tris-EDTA buffer (pH 9) for 30 min. Subsequently, the sections were incubated with the monoclonal and polyclonal antibodies listed in Table 1. The specificity of the antibodies used is indicated in Table 2. Optimum working concentration and incubation time for the antibodies were determined earlier in pilot experiments. For Cox-2, Akt, Mcl-1, cytochrome *c*, and Bad immunohistochemistry, the sections were incubated for 30 min with goat anti-rabbit or anti-mouse immunoglobulins conjugated to peroxidase-labelled

Table 1 Monoclonal and polyclonal antibodies used in the study

Primary antibody	*Dilution*	*Incubation time (min)*	*Source*[a]
• Anti-cyclooxygenase-2 (H-62) (pc)	1 : 100	Overnight	Santa Cruz Biotechnology Inc.
• Anti-Akt (E-3) (mc)	1 : 20	30	Santa Cruz Biotechnology Inc.
• Anti-Mcl-1 (S-19) (pc)	1 : 50	30	Santa Cruz Biotechnology Inc.
• Anti-Bad (C-7) (mc)	1 : 20	30	Santa Cruz Biotechnology Inc.
• Anti-cytochrome *c* (A-8) (mc)	1 : 50	30	Santa Cruz Biotechnology Inc.
• Anti-apoptosis-inducing factor (D-20) (pc)	1 : 50	30	Santa Cruz Biotechnology Inc.
• Anti-TRADD (C-20) (pc)	1 : 20	30	Santa Cruz Biotechnology Inc.
• Anti-FADD (S-18) (pc)	1 : 20	30	Santa Cruz Biotechnology Inc.

pc = polyclonal antibodies; mc = monoclonal antibodies.
[a]Location: Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

dextran polymer (EnVision$^+$; Dako, Carpinteria, CA, USA). For AIF, TRADD, and FADD immunohistochemistry, the sections were incubated for 30 min with the biotinylated secondary antibody and reacted with the avidin-biotinylated peroxidase complex (Dako). The reaction product was visualized by incubation for 10 min in 0.05 M acetate buffer at pH 4.9, containing 0.05% 3-amino-9-ethylcarbazole (Sigma-Aldrich, Bornem, Belgium) and 0.01% hydrogen peroxide, resulting in bright-red immunoreactive sites or 0.06% 3.3′-diaminobenzidine (Sigma) and 0.01% hydrogen peroxide resulting in brown immunoreactive sites. The slides were faintly counterstained with Harris haematoxylin. Finally, the sections were rinsed with distilled water and coverslipped with glycerol.

Omission or substitution of the primary antibody with an irrelevant antibody of the same species and staining with chromogen alone were used as negative controls. Sections from patients with colorectal carcinoma and breast cancer were used as positive controls. The sections from the control patients were obtained from patients treated at the University Hospital (University of Leuven, Belgium), in full compliance with the tenets of the Declaration of Helsinki.

All sections were examined by two independent observers (AMA, KG). One of them (KG) was unaware of the origin of the specimens. In case of disagreement, the results obtained by the blinded observer were used. Disagreement between the two observers was less than 5%. The staining was graded on the basis of the presence or absence of immunoreactivity, intensity of immunoreactivity, thickness of the staining, and the homogeneous or heterogenous character of staining.

Table 2 Specificity of the antibodies used in the study

Primary antibody	*Specificity*
• Anti-cyclooxygenase-2	Raised against a recombinant protein corresponding to amino acids 50–111 mapping near the amino terminus of cyclooxygenase-2 of human origin
• Anti-Akt	Raised against amino acids 229–556 of protein Kinase B kinase of human origin
• Anti-Mcl-1	Raised against a recombinant protein corresponding to an amino-acid sequence mapping within an internal region of Mcl-1 of human origin
• Anti-Bad	Raised against a recombinant protein corresponding to amino acids 1–168 representing full-length Bad of human origin
• Anti-cytochrome *c*	Raised against amino acids 1–104 of cytochrome *c* of horse origin
• Anti-apoptosis-inducing factor	Raised against a peptide mapping at the carboxy terminus of apoptosis-inducing factor of mouse origin
• Anti-TRADD	Raised against a peptide mapping at the carboxy terminus of TRADD of human origin
• Anti-FADD	Raised against a peptide mapping at the amino terminus of FADD of mouse origin

Results

Donor's age and sex, type of diabetes, duration of diabetes, presence or absence of arterial hypertension, retinopathy status, cause of death, and death-to-enucleation times are summarized in Table 3. Nonproliferative diabetic retinopathy was documented to be present in two cases (Cases 1 and 2). The condition of the retina was unknown in three cases (Cases 3, 4 and 5). In these eyes, there were no histopathological

Table 3 Subjects characteristics

Case number	*Age/sex*	*Type of diabetes*	*Duration of diabetes (years)*	*Arterial hypertension*	*Retinopathy status*	*Cause of death*	*Death to enucleation time (h)*
Subjects with diabetes							
1	75 F	NIDDM	14	+	NPDR	Coronary artery disease	17
2	70 M	NIDDM	20	−	NPDR	Renal failure	9
3	58 M	NIDDM	16	−	Unknown	Intracranial bleeding	10
4	67 F	NIDDM	18	−	Unknown	Coronary artery disease	21
5	68 F	IDDM	33	+	Unknown	Myocardial infarction	9
6	73 F	NIDDM	15	+	Absent	Brain stroke	10
Subjects without diabetes							
7	52 M	−	−		−	Colon cancer metastasis	17
8	42 M	−	−		−	Unknown	15
9	79 M	−	−		−	Coronary artery disease	17
10	64 F	−	−		−	Coronary artery disease	18
11	70 M	−	−			Rupture of aortic aneurysm	18

NIDDM = noninsulin-dependent diabetes mellitus; IDDM = insulin-dependent diabetes mellitus; NPDR = nonproliferative diabetic retinopathy.

changes suggestive of diabetic retinopathy. One case (Case 6) had no retinopathy.

There was no staining in the negative control slides (Figure 1) and when the chromogen alone was applied. A summary of the results is given in Table 4. Similar findings were noted in retinas from all subjects without diabetes. Retinas from subjects without diabetes showed granular cytoplasmic immunoreactivity for Cox-2 in ganglion cells (Figure 2a) and in a few cells in the inner nuclear layer. Cytoplasmic immunostaining for Cox-2 was also noted in the retinal pigment epithelial cells and in the pigmented and nonpigmented layers of the ciliary body epithelium (data not shown). Granular cytoplasmic immunoreactivity for Akt, and Mcl-1 was noted in ganglion cells. Figure 2a shows a representative result of the expression of these mediators in ganglion cells. Occasional cells in the inner nuclear layer showed cytoplasmic immunoreactivity for Mcl-1. Weak granular immunoreactivity for cytochrome *c*, and AIF was noted in the inner segments of photoreceptors, in the inner one-third of the outer plexiform layer, in the cytoplasm of cells in the inner nuclear layer and ganglion cells, and in the inner plexiform layer. Figure 2b shows a representative result of the expression of these proteins. There was no immunoreactivity for Bad, TRADD, and FADD.

Similar findings were noted in all diabetic retinas. *De novo* cytoplasmic immuno-reactivity for Bad was observed in ganglion cells and in few cells in the inner nuclear layer (Figure 3a). Upregulation of granular cytochrome *c*, and AIF immunoreactivity was noted in the inner segments of photoreceptors, in the inner one-third of the outer plexiform layer, in cells in the inner nuclear layer, in the inner plexiform layer, and in

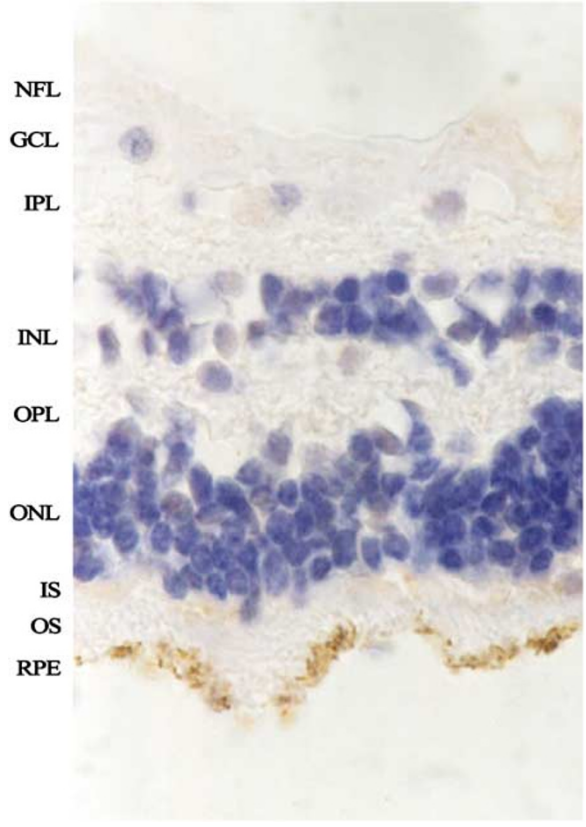

Figure 1 Photomicrograph of a retina from a diabetic subject. Negative control slide that was treated identically with an irrelevant antibody, showing no labelling. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments of photoreceptor cells; OS, outer segments of photoreceptor cells; RPE, retinal pigment epithelium. (Original magnification ×100).

Table 4 Summary of staining results

Antibody	*Retinas from five subjects without diabetes*[a]	*Retinas from six subjects with diabetes*[a]
● Anti-cyclooxygenase-2	+(10/10)	+(12/12)
● Anti-Akt	+(10/10)	+(12/12)
● Anti-Mcl-1	+(10/10)	+(12/12)
● Anti-Bad	−(10/10)	+(12/12)
● Anti-cytochrome *c*	+(10/10)	+++(12/12)
● Anti-apoptosis-inducing factor	+(10/10)	+++(12/12)
● Anti-TRADD	−(10/10)	−(12/12)
● Anti-FADD	−(10/10)	−(12/12)

−, no staining; +, weak staining; ++, intense staining; +++, very intense staining.
[a]Both eyes were examined in each patient.

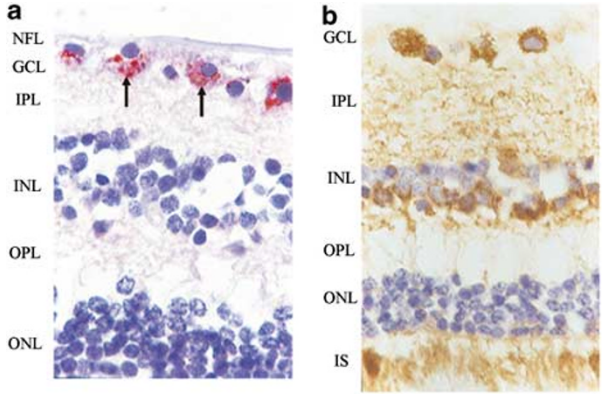

Figure 2 Photomicrographs of a retina from a nondiabetic subject that was immunostained for cyclooxygenase-2, showing immunoreactivity in ganglion cells (arrows) (a), and apoptosis-inducing factor, showing immunoreactivity in inner segments of photoreceptors, in the inner one-third of outer plexiform layer, in cells in the inner nuclear layer, in the inner plexiform layer, and in ganglion cells (b). Abbreviations are defined in Figure 1 legend. (Original magnification ×100).

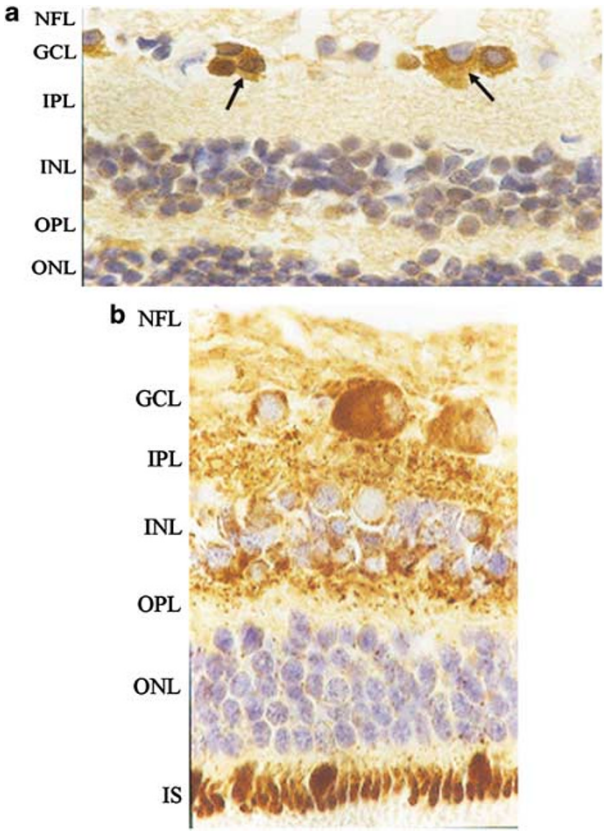

Figure 3 Photomicrographs of a retina from a diabetic subject that was immunostained for Bad, showing immunoreactivity in ganglion cells (arrows) (a), and apoptosis-inducing factor, showing upregulation of apoptosis-inducing factor immunoreactivity (b). Abbreviations are defined in Figure 1 legend. (Original magnification $\times 100$).

ganglion cells. Figure 3b shows a representative result of the expression of these proteins. The distribution and intensity of immunoreactivity for Cox-2, Akt, and Mcl-1 was not altered in the diabetic retinas compared with retinas from subjects without diabetes. There was no immunoreactivity for TRADD and FADD.

Discussion

In the present study, we demonstrated the following points: (1) retinal ganglion cells constitutively expressed the antiapoptotic molecules Akt, Cox-2, and Mcl-1; (2) diabetes induced a *de novo* expression of the proapoptotic molecule Bad in retinal ganglion cells; and (3) diabetic retinas showed upregulation of the mitochondrial proteins cytochrome *c*, and AIF.

The serine/threonine protein kinase Akt, a downstream effector of phosphatidylinositol (PI) 3-kinase (PI 3-kinase), appears to play a key role in mediating neuronal cell survival and neuroprotection.[30] Orike *et al*[11] demonstrated that the survival of adult sympathetic neurons in the absence of neurotrophic factors depends on PI 3-kinase/Akt signalling. Several studies showed that PI 3-kinase/Akt pathway plays a major role in mediating the survival response of retinal ganglion cells after axotomy to a variety of growth factors.[12–14] In addition, apoptosis of retinal neurons induced by serum deprivation was reduced by insulin via activating the PI 3-kinase/Akt pathway.[15] Furthermore, retinal PI 3-kinase/Akt signalling pathway was activated by optic nerve clamping and had a neuroprotective effect on injured retinal ganglion cells.[16] In agreement with our results, immunoreactivity for phosphorylated Akt was detected in retinal ganglion cells in the injured retina.[16]

Cox enzymes mediate the production of prostaglandins from arachidonic acid. Two Cox isoforms Cox-1 and Cox-2 have been identified. Cox-1 is constitutively expressed in most tissues and is believed to be responsible for maintenance levels of prostaglandins for various housekeeping functions. In contrast, Cox-2 is the product of an immediate early gene that is rapidly inducible and tightly regulated. Cox-2 expression can be induced in various tissues by pathologic stimuli, such as bacterial lipopolysaccharide, proinflammatory cytokines, growth factors, hormones, and tumour promoters. Even though it is called the inducible isoform, Cox-2 is constitutively expressed in brain, spinal cord, testis, tracheal epithelia, and macula densa of kidney. In these tissues, Cox-2 is present without obvious stimulatory processes and is considered to be the dominating Cox isoform. Cox-2 has been shown to contribute constitutively to the physiologic regulation and development in these highly differentiated organ systems.[17]

Recent data showed that Cox-2 and the prostaglandins resulting from its enzymatic activation are involved in the control of cellular growth, angiogenesis, apoptosis, and development of neoplasia. Selective Cox-2 inhibitor attenuated the retinal angiogenesis that accompanies retinopathy of prematurity, and normal retinal development.[31] These findings indicate that Cox-2 plays an important role in both developmental and pathologic retinal angiogenesis. In addition, several studies suggested a role of Cox-2 in neuronal function in the brain.[32,33] Furthermore, several studies have established a direct role for Cox-2 in rendering cells resistant to apoptosis by upregulating the expression of Mcl-1 through activation of the PI 3-kinase/Akt-dependent pathway.[18,21] In the present study, cytoplasmic immunoreactivity for Cox-2 was observed in retinal ganglion cells, in the retinal pigment epithelial cells, and in the pigmented and nonpigmented layers of the ciliary body epithelium in diabetic and nondiabetic retinas, confirming previous reports.[31,34–36] The expression of the

antiapoptotic molecules Akt, Cox-2, and Mcl-1 in retinal ganglion cells may reflect the fact that neurons tend to be maintained for the entire life span of an individual.

In the present study, Bad was expressed in ganglion cells and in few cells in the inner nuclear layer in diabetic retinas. These observations suggest that retinal ganglion cells upregulate the expression of Bad in response to diabetes-induced neuronal injury. Similar observations were reported by Chen *et al*[37] who demonstrated that neurotoxin-induced retinal neuronal degeneration induced a *de novo* expression of Bad in the retinal ganglion cells. In addition, transient retinal ischaemia by central retinal artery occlusion-induced upregulation of Bad expression in cells in the ganglion cell layer and inner nuclear layer.[38]

Mitochondria are key regulators in the process of cell death. Early during the apoptotic process, mitochondria can release a number of proapoptotic proteins from their intermembrane space, such as cytochrome *c*, and AIF.[10,27,28] Cytoplasmic cytochrome *c* forms a complex, termed the apoptosome, with procaspase-9, and apoptotic protease activating factor-1 (Apaf-1), which activates caspase-3 and results in DNA fragmentation.[10,27] AIF has been shown to cause high molecular weight DNA fragmentation and chromatin condensation in cells and isolated nuclei in a caspase-independent manner.[39,40] The distribution of cytochrome *c* immunoreactivity observed in nondiabetic retinas is in agreement with previous human,[41] and animal studies.[42,43] In addition, the distribution of AIF expression in nondiabetic retinas observed in the present study is consistent with a previous animal study.[44] Diabetic retinas showed upregulation of cytochrome *c* and AIF immunoreactivity. Our data for cytochrome *c* in diabetic retinas are in agreement with several studies reporting increased cytochrome *c* immunoreactivity and activity in the retina postaxotomy and following optic nerve crush[43,45] suggesting that one of the early responses in the retina after optic nerve injury is to scale up the energy production. The study of Muranyi *et al*[46] demonstrating that mitochondria dysfunction and mitochondria-initiated cell death pathway, which involves cytochrome *c* release, may play a key role in mediating diabetes-enhanced ischaemic brain damage is also in agreement with our findings. Several studies demonstrated that AIF is an important player in the regulation of caspase-independent neuronal cell death after cerebral hypoxia-ischaemia.[47,48] Furthermore, enforced expression of AIF can induce neuronal cell death in a caspase-independent manner, and blocking AIF function with neutralizing antibodies provides significant protection against cell death, suggesting that AIF may represent an important therapeutic target for neuroprotection after acute injury.[47]

Several *in vitro* and *in vivo* studies demonstrated that oxidative stress induced by hyperglycaemia is closely linked to apoptosis in a variety of cell types. Oxidative stress has been implicated in impaired mitochondrial function and activation of programmed cell death caspase pathway in diabetic neurons.[1–3] There is overwhelming evidence for an involvement of reactive oxygen species in triggering mitochondria to release several essential players of apoptosis, such as cytochrome *c*, and AIF, into cytosol.[28,49] Several studies demonstrated that oxidative stress is increased in the retina with diabetes and is believed to play a significant role in the development of diabetic retinopathy.[4–6] Administration of antioxidants inhibited the apoptosis-executer enzyme caspse-3 activation and inhibited the development of retinopathy.[5,6] Recently, it was demonstrated that diabetes-induced dysfunction of retinal mitochondria and increased the release of cytochrome *c* into the cytosolic fraction prepared from retina of rats.[50]

Although these observations, by their descriptive nature, do not allow a precise understanding of the function of these molecules, they do suggest that retinal ganglion cells possess protective mechanisms to guard against apoptosis, that retinal ganglion cells express Bad in response to diabetes-induced neuronal injury, and that diabetic retinas show upregulation of the mitochondrial proteins such as cytochrome *c*, and AIF.

Acknowledgements

This work was supported in part by the College of Medicine Research Centre, King Saud University. The authors thank Ms Christel van den Broeck for technical assistance, and Ms Connie B Unisa-Marfil for secretarial work.

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