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Diabetes-induced Activation of Nuclear Transcriptional Factor in the Retina, and its Inhibition by Antioxidants

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Oxidative stress is increased in the retina in diabetes, and long-term administration of antioxidants inhibits the development of retinopathy in diabetic rats. The purpose of this study is to determine how diabetes affects the activation of a redox-sensitive nuclear transcriptional factor in the retina, NF-κB, and its inhibition by antioxidants. Alloxan diabetic rats were assigned to receive standard diet or the diet supplemented with multiple antioxidants, including ascorbic acid, Trolox, *dl* α-tocopherol acetate, N-acetyl cysteine, β-carotene, and selenium for up to 14 months. NF-κB activation, oxidative stress and nitric oxides were measured in the retina at 2, 8 and 14 months of diabetes. Retinal NF-κB was activated by about 60% at two months after induction of diabetes, remained activated for up to 14 months of diabetes, and the duration of diabetes had no effect on the intensity of NF-κB activation. Similarly, oxidative stress and nitric oxides were elevated by over 50% in the retina of rats diabetic for 14 months, and nitrotyrosine levels were elevated by over two folds. Administration of the antioxidants to the rats for the entire duration of diabetes inhibited activation of NF-κB and elevations in oxidative stress, nitric oxides and nitrotyrosine formation without ameliorating the severity of hyperglycemia. These *in vivo* results were confirmed by *in vitro* studies showing that high glucose activates NF-κB and elevates NO and lipid peroxides in both retinal endothelial cells and pericytes that can be inhibited by antioxidants.

Thus, the results suggest that the activation of retinal NF-κB in diabetes is an early event in the development of retinopathy, and it remains active when the retinal capillary cell death is accelerating, and histopathology is developing. Beneficial effects of antioxidants on the development of diabetic retinopathy might involve inhibition of NF-κB activation and its downstream pathways in the retina.

Keywords: Antioxidants; Cell death; Diabetic retinopathy; Nuclear transcriptional factor; Oxidative stress

INTRODUCTION

Diabetes is shown to increase oxidative stress, and increased oxidative stress is postulated to play an important role in the development of diabetic complications.[1–4] Retinal oxidative stress is increased, antioxidant defense systems are impaired,[3,5–7] GSH levels are decreased,[8–10] and the expressions of antioxidant defense enzymes are down regulated in diabetes.[11] A correlation is postulated between increased serum lipid hydroperoxides and the prevalence of retinopathy in diabetic patients,[2] and our studies have suggested a strong association between oxidative stress and the development of retinopathy in diabetes.[4]

In the pathogenesis of diabetic retinopathy, pericytes and endothelial cells are lost selectively before other histopathology is detectable.[12,13] How these capillary cells die is unclear, but apoptosis is considered as one of the possible mechanisms in their death.[12] Retinal capillary cell death precedes the development of other lesions characteristic of retinopathy in diabetes, and the frequency of early death of retinal capillaries can predict the development of histological lesions of retinopathy.[13] Recent studies from our laboratory have shown that the same antioxidants that inhibit the development of retinal pathology in two animal models of diabetic retinopathy, diabetic rats and galactose-fed rats,[4] also inhibit the activation of retinal caspase-3, an enzyme involved in the execution of apoptosis,[14] further strengthening the role of oxidative stress in retinal apoptosis and the development of diabetic retinopathy.

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A redox-sensitive nuclear transcriptional factor, NF-κB, is an important regulator of antioxidant enzymes.[15–18] Activation of NF-κB is considered a key-signaling pathway by which high glucose induces apoptosis in endothelial cells.[19] In the retina, NF-κB is localized in sub-retinal membranes and in microvessels,[20] and is activated very early in the course of development of retinopathy in diabetes.[21] Activated NF-κB binds to nuclear DNA and modulates the expression of several genes,[22] and this amplification cascade in turn, results in increased free radical production eventually leading to the cell death. Recent studies have shown that NF-κB activation in retinal pericytes is responsible for the hyperglycemia-induced accelerated loss of pericytes observed in diabetic retinopathy.[23]

In the present study, we have examined the activation of NF-κB in the retina and its isolated capillary cells in high glucose conditions, and the effect of antioxidants on glucose-induced NF-κB activation.

METHODS

Animals

Sprague Dawley rats (male, 200 g) were randomly assigned to normal or diabetic groups. Diabetes was induced with alloxan monohydrate (45 mg/kg BW), and insulin was given as needed to allow slow weight gain while maintaining hyperglycemia (blood glucose levels of 20–25 mM). A group of diabetic rats was fed a diet supplemented with multiple antioxidants (ascorbic acid, 1 g/kg; Trolox 500 mg/kg; *dl* α-tocopherol acetate, 250 mg/kg; N-acetyl cysteine, 200 mg/kg; β-carotene, 45 mg/kg; and selenium, 0.1 mg/kg of diet). The antioxidant diet was replaced weekly, and the food consumption was measured to calculate the amount of antioxidants consumed. The choice and concentrations of antioxidants used in the present study is based on our previous reports showing that the long-term administration of a mixture of these antioxidants inhibits diabetes-induced activation of retinal caspase-3 and the development of retinopathy in rats.[4,14] These experiments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. Nonenzymatically glycated hemoglobin (GHb) was measured at 2 months of diabetes, and every 3–4 months thereafter, using affinity columns (Glyc-Affin; Pierce, Rockford, IL). Animals were sacrificed at 2, 8 or 14 months of diabetes after an overnight fast. The retina was immediately removed, and frozen at −70°C for further analysis. Age-matched normal animals served as controls. We have described these methods previously.[4]

NF-κB Activation

Nuclear extracts were prepared from retina as described by others[24] and recently used by us.[25] The tissue was solubilized in 10% IGEPAL CA-630 (Sigma Chemicals, MO), and centrifuged at 10,000*g* for 30 s. The nuclear pellet was resuspended in ice-cold buffer containing 20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF, and the tube was vigorously rocked at 4°C for 15 min. The nuclear extract was centrifuged at 15,000*g* for 5 min, and the supernatant was frozen at −70°C until further use. NF-κB consensus oligonucleotide (Promega, WI, USA) DNA probes (see below) were prepared by end labeling with (γ-^{32}P) ATP (Amersham, Quebec, Canada) using T4 polynucleotide kinase. The probes were purified by ethanol precipitation and resuspended in 10 mM Tris-HCl and 1 mM EDTA (pH 7.6). Nuclear proteins (5 μg) were incubated with 100,000 cpm of ^{32}P-labeled consensus oligonucleotides (5-AGT TGA GGG GAC TTT CCC AGG C-3, 3-TCA ACT CCC CTG AAA GGG TCC G-5) for 30 min at room temperature in a buffer containing 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 5% glycerol, 0.05% NP-40, 0.5 mM EDTA, 0.5 mM DTT and 0.5 mg poly (dI-dC). Protein-DNA complexes were resolved on a standard 6% nondenaturing polyacrylamide gel. The gels were dried onto Whatman paper and subjected to autoradiography. The specificity of binding was further confirmed by incubation with 100-fold excess of unlabelled oligonucleotides.

NF-κB activation was also determined by performing western blots to measure the protein expression of the p65 subunit. Forty μgrams protein (retinal homogenate) was boiled in Laemmli buffer, separated on 10% denaturing polyacrylamide gels, and blotted onto nitrocellulose membrane. The membranes were blocked in 5% milk in wash buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and incubated overnight with antibody against NF-κB (p65; 1:500 dilution; Santa Cruz Biotechnology, CA). The membranes were washed (3 × 10 min), incubated with anti rabbit IgG, horseradish peroxidase-conjugated antibody in blocking buffer for 1 h, washed again, and developed using ECL-Plus western blotting detection kit from Amersham Pharmacia Biotech.[14,26] Kaleidoscope pre-stained molecular weight markers (Bio-Rad, CA) were run simultaneously on each gel. To ensure equal loading among the lanes, the expression of housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin was determined. After blotting the membranes for NF-κB, the membranes were incubated with stripping buffer (62.5 mM Tris-HCl pH 6.8, 100 mM mercaptoethanol, 2% sodium dodecyl sulfate) at 50°C for 30 min, washed, and incubated with anti-GAPDH

(anti-GAPDH monoclonal antibody, Biodesign International, ME) or anti-β-actin (monoclonal antibody, Sigma Chemicals). The membranes were washed, incubated with horseradish peroxidase-conjugated secondary antibody, and developed using ECL-Plus western blotting detection kit.

Lipid Peroxides

Lipid peroxides were estimated in retina samples by measuring the levels of thiobarbituric acid reactive substances (TBARS).[3,4,27] The fluorescence of malonaldialdehyde-thiobarbituric acid adducts formed by acid hydrolysis at 100°C was measured at excitation and emission wavelengths of 535 and 553 nm, respectively. 1,1,3,3-Tetramethoxy propane was used as a standard, and the assay is sensitive up to 0.3 mM.

Lipid peroxides in isolated cells were measured using an assay kit from Cayman Chemical (Ann Arbor, MI). Hydroperoxides were measured directly by the redox reactions with ferrous ions, and the resulting ferric ions were detected using thiocynate ion as the chromogen.[4,14,28] In a final assay volume of 100 μl, the assay was sensitive as low as 5 pmoles of LPO.

Nitric Oxides

Nitric oxides were determined using the established methods.[4,28,29] The stable metabolites of NO (nitrate + nitrite) were measured using a fluorometric assay kit (Cayman Chemical Company, Ann Arbor, MI). Retinal homogenate was passed through a 10-kDa filter to remove interfering substances in the sample, and conversion of nitrate to nitrite was achieved by incubating the samples with nitrate reductase for 2 h. Fluorescence generated by nitrite reaction with 2,3-diaminonaphthalene was measured at excitation and emission wavelengths of 365 and 450 nm, respectively. The sensitivity of the assay in our laboratory is up to 0.5 nmoles in 100 μl assay volume.

Expression of iNOS

iNOS expression was determined in the retinal homogenate by western blot analysis as previously used by us.[26] Proteins were separated on 8% denaturing polyacrylamide gels, blotted onto nitrocellulose membrane and reacted with the antibody against iNOS (1:500 dilution, Transduction Laboratory, KY). GAPDH was used as an intrinsic protein to ensure equal loading among lanes.

Nitrotyrosine

Nitrotyrosine, a biomarker of peroxynitrite formation, was measured by immunochemical methods described by others,[29] and previously used by us.[26] Existing antibodies were removed from retinal homogenates by incubating first with protein A-sepharose, followed by overnight incubation with rabbit anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), and then protein A-sepharose to precipitate nitrotyrosine complexed with antibody. Proteins were separated on 10% denaturing polyacrylamide gels, followed by incubation with mouse anti-nitrotyrosine (Upstate Biotechnology). Nitrotyrosine was detected using peroxidase conjugated secondary antibody, and the membranes were developed using the standard ECL-Plus western blotting detection kit.

Endothelial Cells and Pericytes

To confirm that retinal capillary cells are involved in hyperglycemia-induced activation of NF-κB in the retina, isolated retinal endothelial cells and pericytes were used. Endothelial cells and pericytes were isolated from bovine eyes,[14,28,30] and endothelial cells were grown to 80% confluence in petridishes coated with 0.1% gelatin in Dulbecco's Modified Eagle Medium (DMEM) containing heparin, 10% fetal calf serum (heat inactivated), 10% Nu-serum, endothelial growth supplement (25 μg/ml) and antibiotic/antimycotic in an environment of 95% air and 5% CO_2. The cells exhibited their typical 'cobblestone = morphology, and positive staining for factor VIII.[14,30] The cells from 4th to 8th passage were incubated in DMEM supplemented with 1% fetal calf serum (heat inactivated), 2.5% Nu-serum, 2.5 μg/ml endothelial cell growth supplement and heparin in 5 mM glucose or 20 mM glucose for 10 days[14,28] in the presence or absence of 0.25 mM N-acetyl cysteine, 0.25 mM α-lipoic acid, 0.2 mM *dl* α-tocopherol acetate or 0.2 mM Trolox.

Pericytes were grown in DMEM supplemented with 10% fetal calf serum, antibiotics and antimycotics, as described by us previously.[14,28,30] Pericytes (4–6th passage) were incubated in DMEM containing 2.5% fetal bovine serum in 5 mM glucose or 20 mM glucose in the presence and absence of antioxidants.

To confirm the role of NF-κB in the endothelial cell death in diabetes, the cells were incubated with SN 50, an inhibitor of NF-κB activation[31] or its inactive peptide SN50 M (each 50 μg/ml, Biomol Research Laboratories, PA) in 5 mM and 20 mM glucose medium. At the end of the incubation, apoptosis was detected by performing ELISA for cytoplasmic histone-associated-DNA-fragments, and by detecting DNA-laddering, using ELISA^{PLUS} and DNA-Ladder kits, respectively (Roche Laboratories). Manufacturer's instructions were followed for both of these kits. NO levels were also measured in these samples.

Tissue protein was measured by the Bradford method[32] using bovine serum albumin as a standard.

Data are expressed as mean ± SD and analyzed statistically using the nonparametric Kruskal–Wallis test followed by the Mann–Whitney U test for multiple-group comparisons. Also, similar conclusions were reached by using analysis of variance (ANOVA) followed by Tukey's test.

RESULTS

Glycated hemoglobin (an index of severity of hyperglycemia) and body weights of the rats remained significantly higher and lower, respectively ($P < 0.05$) in the diabetic rats (with or without antioxidants) at 2, 8 and 14 months duration as compared with their age-matched normal rats (Table I).

Two months after induction of diabetes in rats NF-κB was activated in the retina, and NF-κB remained active at 8 months of diabetes (a duration of diabetes when retinal capillary cell apoptosis is demonstrable[12,13]), and at 14 months of diabetes (when both capillary cell apoptosis and histopathology can be seen in the retinal microvessels[4,12]). The diabetes-induced activation of NF-κB was confirmed by two independent methods: electrophoretic mobility shift assay (Fig. 1a) and by western blot analysis of p65 subunit protein expression (Fig. 1b). Despite the difference in the expression of 65 kD band, as shown in Fig. 1b, the expression of housekeeping protein, GADPH, remained similar among various lanes.

Diabetes of 2 months duration, as reported previously,[4,5] resulted in elevated TBARS in the retina ($P < 0.05$ compared to normal), and TBARS remained elevated at 8 and 14 months of diabetes. The duration of diabetes had no effect on the severity

TABLE I Administration of antioxidants has no effect on the severity of diabetes

	Glyc hemoglobin (%)	Urine volume (ml/24 h)	Body weight (g)
2 Months			
Normal (7)	4.4 ± 0.7	5 ± 2	397 ± 13
Diabetes (9)	13.1 ± 1.6*	115 ± 35*	254 ± 30*
Diab+Antiox (7)	12.9 ± 0.9*	109 ± 19*	263 ± 21*
8 Months			
Normal (9)	4.7 ± 0.9	8 ± 1.5	415 ± 25
Diabetes (6)	12.9 ± 2.1*	128 ± 19*	270 ± 37*
Diab+Antiox (6)	13.4 ± 2.5*	114 ± 31*	291 ± 24*
14 Months			
Normal (8)	4.0 ± 0.6	5 ± 3	576 ± 76
Diabetes (7)	12.5 ± 0.8*	126 ± 21*	304 ± 49*
Diab+Antiox (8)	13.5 ± 2.2*	112 ± 20*	280 ± 50*

24-h Urine was measured over 2–3 consecutive days. The values are mean ± SD, and the numbers in the parentheses represent the number of rats in each group. *$P < 0.05$ compared to normal.

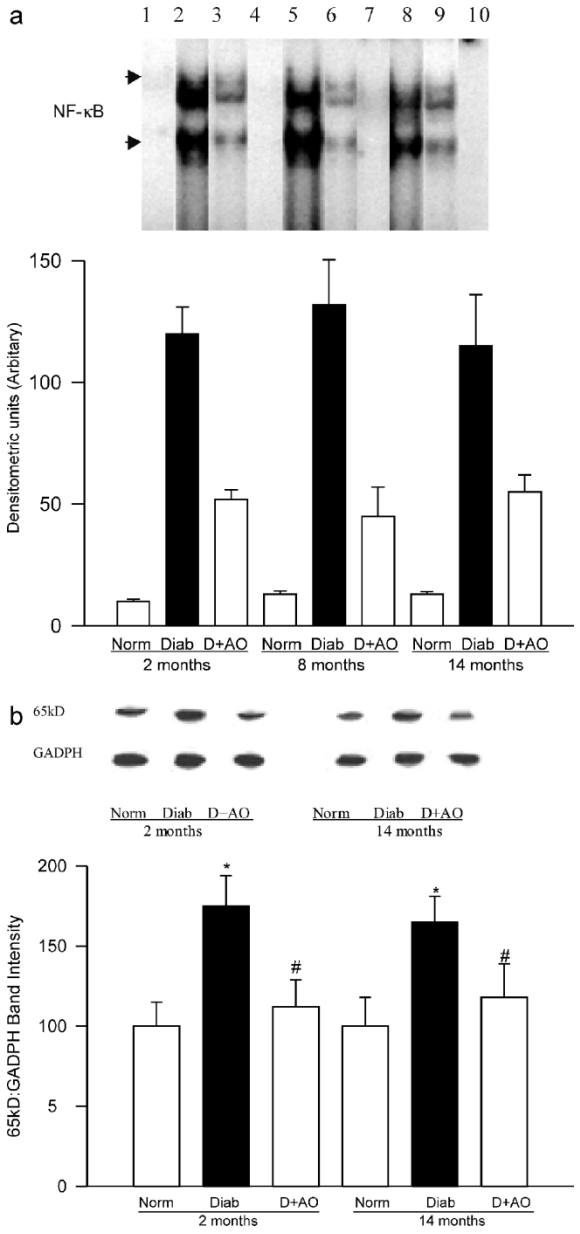

FIGURE 1 Effect of diabetes and antioxidants on NF-κB activation in rat retina. (a) Electrophoretic mobility shift assay: electrophoretic mobility shift assay was performed in the nuclear fraction of the retina obtained from the rats diabetic or diabetic + antioxidants for 2, 8 and 14 months, and their age-matched normal rats. The activation of NF-κB was not affected by the age of the rat. 1, 4, 7 = normal, 2, 5, 8 = diabetes, and 3, 6, 9 = diabetes + antioxidants at 2 months, 8 months and 14 months, respectively. 10 = non-competitive binding (this confirms the specificity of the binding). P65 containing bands were quantitated by densitometry, the bars represent mean ± SD of the results obtained from 4 rats (assayed in duplicate) in each group, and the values obtained from the age-matched normal rats are considered 100%. The lane intensities shown in this figure reflect the actual differences between the samples. Norm = Normal, Diab = Diabetes and D + AO = Diabetes + antioxidants. (b) Expression of P65 subunit of NF-κB: western blots were performed for p65 subunit of NF-κB in the retinal homogenates. The band intensities were adjusted to the expression of the intrinsic protein, GADPH in each sample. The data are representative of 4–5 rats/group, and each retina was analyzed in duplicate. The rats at 8 months duration in all of the three groups showed similar results as obtained at 2 or 14 months durations. *$P < 0.05$ compared to normal, and #$P < 0.05$ compared to diabetes.

TABLE II Retinal TBARS and NO are elevated at 2 months of diabetes, and remain activated at 8 and 14 months of diabetes: effect of antioxidants

	TBARS (pmols/mg protein)	NO (nmols/mg protein)
2 Months		
Normal (6)	142 ± 8	10.3 ± 1.5
Diabetes (8)	265 ± 35*	17.1 ± 1.3*
Diab+Antiox (6)	147 ± 21#	11.8 ± 0.9*
8 Months		
Normal (6)	132 ± 11	11.4 ± 1.7
Diabetes (6)	297 ± 48	17.9 ± 1.4*
Diab+Antiox (6)	173 ± 33#	12.7 ± 1.2#
14 Months		
Normal (5)	138 ± 12	11.1 ± 1.5
Diabetes (8)	288 ± 39	16.3 ± 0.9*
Diab+Antiox (6)	155 ± 29#	13.1 ± 1.3#

The numbers in the parentheses represent the number of rats in each group, and the values are reported as mean ± SD. *$P < 0.05$ compared to normal and #$P < 0.05$ compared to diabetes.

of increased oxidative stress (Table II). Likewise, NO levels remained elevated by more than 50% in the retina of rats at 2, 8 and 14 months of diabetes compared with their age-matched normal control rats ($P < 0.05$; Table II).

As shown in Fig. 2, the expression of iNOS was significantly increased in the retina obtained from diabetic rats as compared with their age-matched normal rats ($P < 0.05$). The increases in retinal iNOS expression at 2, 8 and 14 months of diabetes were not statistically different from each other ($P > 0.05$).

Peroxynitrite formation, as detected by measuring the major nitrosylated protein of 80 kD,[26,29] was elevated in the retina obtained from diabetic rats. Although several proteins were nitrosylated in the retinal homogenate, the band around 80 K was the most prominent band. The pattern of nitrotyrosine-containing proteins was similar in the retina obtained from 2, 8 and 14 months diabetic rats, and the extent of nitrosylation of 80 kD protein was not influenced by the duration of diabetes.

Administration of antioxidants for the entire duration of diabetes inhibited activation of NF-κB, which was demonstrated by both the electrophoretic mobility shift assay and by increased expression of the p65 subunit of NF-κB (Fig. 1a,b). Effect of antioxidants on NF-κB activation in the retina was similar at 2, 8 and 14 months of diabetes. In the same diabetic rats, administration of antioxidants normalized increases in retinal oxidative stress and NO (Table II). The methods used to measure TBARS and NO were not influenced by the high glucose levels present in the retina of diabetic rats since the values for both TBARS and NO were significantly lower in the diabetes + antioxidant group as compared to the diabetes group despite similar degree of hyperglycemia experienced by the rats in these two diabetic groups (Table I).

Administration of antioxidants inhibited diabetes-induced increases in iNOS expression, and nitrosylation of proteins in the retina at 2, 8 and 14 months of diabetes (Figs. 2 and 3). These beneficial effects of antioxidants were not mediated by the reduction in the severity of hyperglycemia, GHb

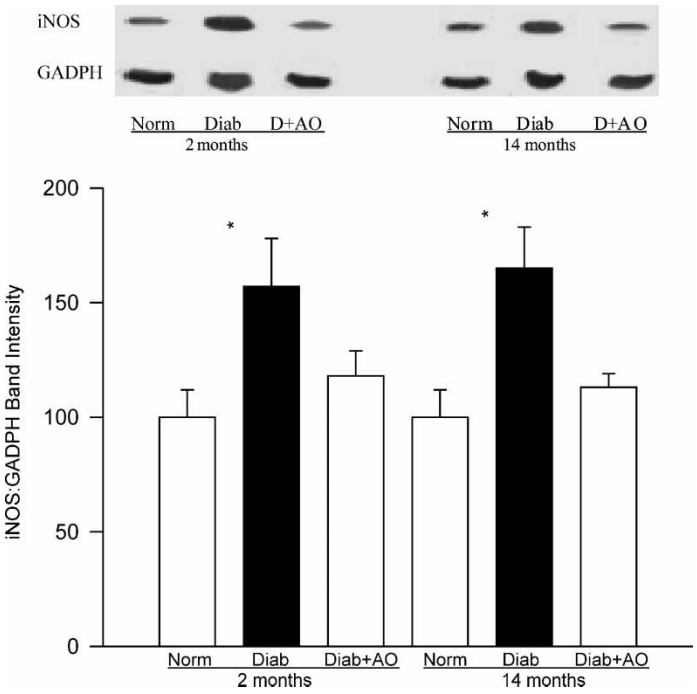

FIGURE 2 Effect of diabetes on the expression of retinal iNOS: iNOS expression was determined in the retinal homogenates by western blot technique. The proteins were separated on 8% SDS gel, and the band intensity was adjusted to the GADPH expression. The results are representative of 2–3 separate experiments using at least 5 rats in each group.

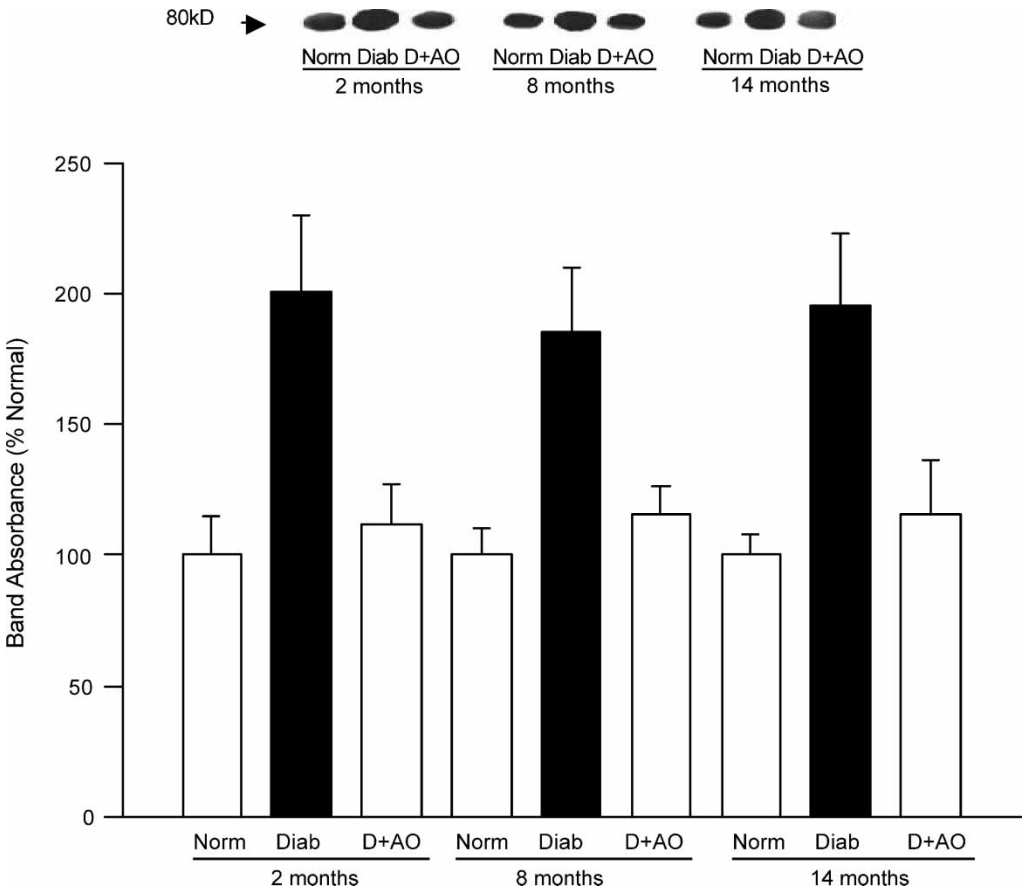

FIGURE 3 Effect of diabetes on nitrosylation of retinal proteins. Nitrosylated proteins were detected in the retina by immunochemical methods using rabbit anti-nitrotyrosine antibody, as described in the method section. The figure is the representative of five or more rats in each group, and each retina being analyzed in duplicate. The histogram represents the absorbance of 80 kD band. *$P < 0.05$ compared to normal.

and 24 h urine volumes were similar in diabetic and diabetic + antioxidant groups (Table I).

In isolated retinal capillary cells incubation of the cells in 20 mM glucose medium for 10 days resulted in activation of NF-κB in both endothelial cells and pericytes, as determined by the increased expression of the p65 subunit of NF-κB (Fig. 4a,b), and in elevation of lipid hydroperoxides and NO levels (Table III) as compared to the cells incubated in 5 mM glucose medium for the entire duration ($P < 0.02$). The glucose-induced activation of NF-κB was not due to an increase in osmolarity, since addition of 20 mM mannitol, instead of glucose, failed to activate NF-κB or increase lipid peroxides and NO (data not shown). A similar effect of glucose on the activation in NF-κB in the human umbilical cord vein endothelial cells, determined by electrophoretic mobility shift assay, has been reported by us.[25] Addition of antioxidants (N-acetyl cysteine, α-lipoic acid, α-tocopherol acetate or Trolox) inhibited glucose-induced activation of NF-κB (Fig. 4a,b) and glucose-induced increased lipid peroxides and NO (Table III), and when the cells were incubated with a mixture of N-acetyl cysteine and Trolox, no statistically significant additional beneficial effect on lipid peroxides and NO was observed (data not shown).

As shown in Fig. 5, in endothelial cells SN50 inhibited glucose-induced formation of histone-associated DNA fragments, as determined by ELISA^{plus} method. Similar beneficial effects of SN50 were observed on increased DNA-laddering in these cells (data not shown). The inactive peptide, SN50M, had no effect on endothelial cell apoptosis.

DISCUSSION

The data presented here show that NF-κB is activated in the retina two months after induction of diabetes in rats, and remains activated for the entire duration of diabetes when both capillary cell death and histopathology are developing.[12,13] This suggests that the NF-κB is activated in the retina early in the course of development of retinopathy in diabetes, and activated NF-κB initiates a cascade of signaling pathways, culminating in cell death. Our data is supported by the results reported by others showing that leukocyte adhesion to the diabetic retinal vasculature is an early event in the pathogenesis of

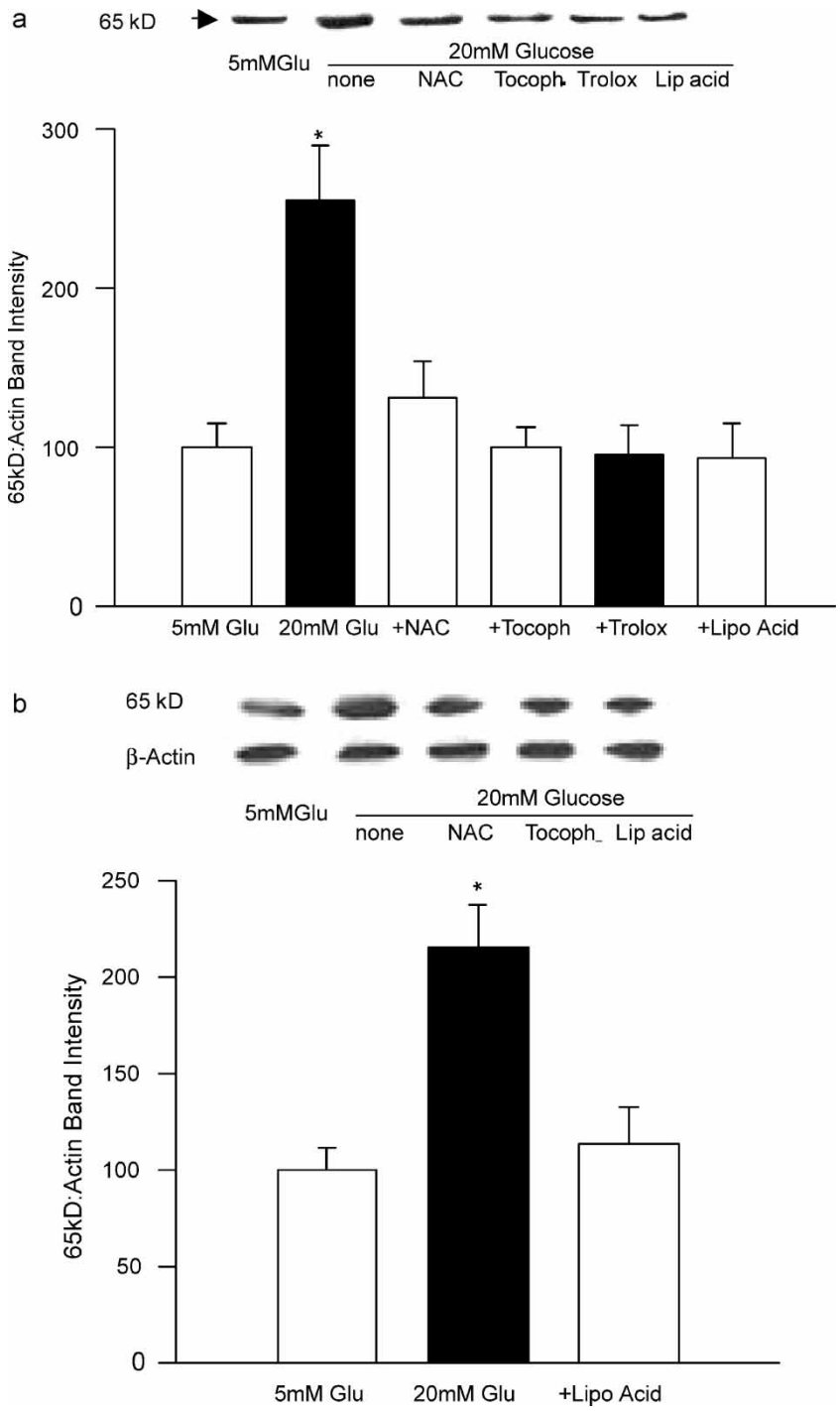

FIGURE 4 NF-κB activation in (a) endothelial cells and (b) pericytes incubated in high glucose medium: NF-κB activation was measured by performing western blots for p65 subunit of NF-κB in bovine retinal endothelial cells and pericytes incubated in 5 mM and 20 mM glucose medium for 10 days. The graphs represent absorbance of the p65 band adjusted to the expression of housekeeping protein, β-actin. Each measurement was performed in duplicate using three separate cell preparations. The values obtained at 5 mM glucose are considered 100%. 5 mM Glu = 5 mM glucose; 20 mM Glu = 20 mM glucose; +NAC = 20 mM glucose + 0.25 mM N-acetyl cysteine; +Tocoph = 20 mM glucose + 0.2 mM *dl* α-tocopherol; +Trolox = 20 mM glucose +0.2 mMTrolox; +Lipo acid = 20 mM glucose + 0.25 mM α-lipoic acid. *$P < 0.05$ compared to 5 mM glucose.

diabetic retinopathy and NF-κB is important in leukocyte activation.[21] The activation of NF-κB in retina during the course of development of diabetic retinopathy in rats is similar to that of NO accumulation and lipid peroxidation.

We have shown that at 12–14 months of diabetes in rats, histopathology is present in the retina and caspase-3 is active, and administration of antioxidants inhibit both histopathology and caspase-3 activation.[4,14] Here we provide data that NF-kB

TABLE III Antioxidants inhibit glucose-induced increase in lipid peroxides and NO in retinal endothelial cells and pericytes

	LPO (pmols/mg protein)		NO (nmols/mg protein)	
	Endo cells	Pericytes	Endo cells	Pericytes
5 mM Glucose	29 ± 3.1	20 ± 2.9	15 ± 2.9	24 ± 4.2
20 mM Glucose	53 ± 2.5*	35 ± 4.5*	32 ± 2.3*	37 ± 5.6*
20 mM Gluc+N-Acetyl Cyst	25 ± 1.1	24 ± 2.1	21 ± 3.1	26 ± 1.7
20 mM Gluc+Tocopherol	20 ± 5.4	21 ± 2.7	19 ± 1.5	ND
20 mM Gluc+Trolox	28 ± 1.9	23 ± 1.9	19 ± 2.4	22 ± 2.5
20 mM Gluc+α-Lipoic acid	19 ± 4.3	23 ± 3.2	22 ± 2.7	25 ± 3.4

Endothelial cells and pericytes isolated from bovine retina were incubated in 5 or 20 mM glucose for 10 days in the presence or absence of antioxidants. The cells were washed and scraped, and lipid peroxides were measured using thiocynate ion as the chromogen, and NO using 2,3 diaminonaphthalene. Each measurement was performed in duplicate, the experiments were repeated with at least three separate cell preparations, and the results are represented as mean ± SD. *$P < 0.05$ compared to 5 mM glucose.

remains active for 12–14 months of diabetes, and the same antioxidants that are able to inhibit histopathology and caspase-3 activation[4,14] are able to inhibit retinal NF-κB activation. Thus, the combined results from this study and from our published data demonstrate in "principle" a strong association between activation of NF-κB and caspase-3, oxidative stress and the development of diabetic retinopathy. Beneficial effects of antioxidants on retinal metabolic abnormalities, postulated to be involved in the development of retinopathy, have been reported previously by others: α-lipoic acid inhibits diabetes-induced up-regulation of retinal vascular endothelial growth factor in rats,[7] vitamin E supplementation reduces retinal hemodynamics abnormalities seen in diabetic patients,[33] and administration of vitamin E, selenium and taurine ameliorates diabetes-induced metabolic abnormalities in rat retina.[34] The recent study by Stitt *et al.* has shown that Pyridoxamine, a potent scavenger of reactive carbonyls, inhibits the formation of diabetes-induced acellular strands in rats.[35] However, by contrast, some studies failed to show any effects of antioxidants on retinal vascular lesions,[36] and the differences for such discrepancies are not clear. In order to reduce the risk of only single or a few antioxidants not being adequate to inhibit oxidative stress completely, in the present study, we have used multiple antioxidants consisting of compounds with a wide range of antioxidant properties: e.g. α-lipoic acid directly scavenge reactive oxygen species, Trolox protects the polyunsaturated membrane lipids against free radical attack,[37] N-acetylcysteine inhibits the genotoxicity of reactive oxygen species, protects DNA and prevents activation of NF-κB,[38] selenium protects oxidation and nitration reactions caused by peroxynitrite,[39] and β carotene efficiently quenches singlet oxygen.[40] Further, increasing the diversity of antioxidants in the diet has been shown to provide significantly more protection against oxidative stress in various organs than any individual antioxidant.[41] Our results strongly suggest that the beneficial effects of antioxidants seen in the retina were not ameliorated by the severity of hyperglycemia; glycated hemoglobin and body weights were similar in diabetes and diabetes + antioxidant groups. However, there remains a possibility that these antioxidants administered to diabetic rats might have been acting via mechanisms in addition to those related to correction of oxidative stress, for example, α-tocopherol has been found also to normalize diabetes-induced increases in PKC activity via its effect on diacylglycerol accumulation.[42]

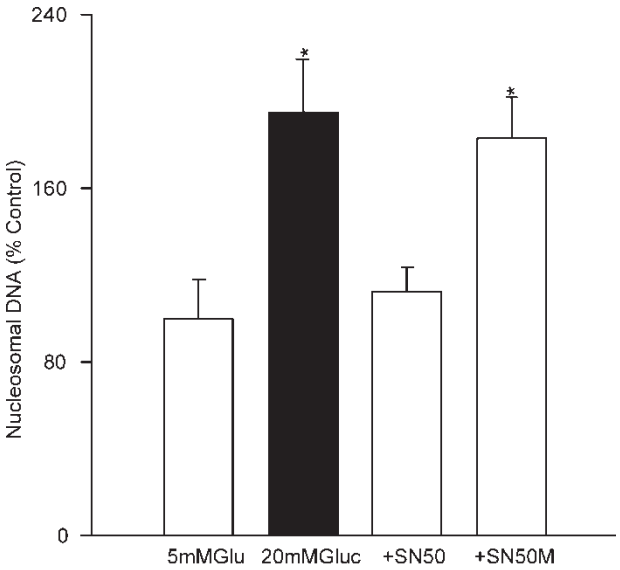

FIGURE 5 Glucose-induced apoptosis of retinal endothelial cells, and effect of an inhibitor of NF-κB activation: endothelial cells were incubated in 5 or 20 mM glucose in the presence of SN 50 or inactive peptide SN 50M, and apoptosis was measured by performing ELISA for cytoplasmic histone-associated-DNA-fragments. The values in each sample were adjusted to the total DNA, and the values obtained with 5 mmols/l glucose were considered 100%. 5 mM Glu = 5 mM glucose; 20 mM Glu = 20 mM glucose; +SN50 = 20 mM glucose + 50 μg/ml SN 50; +SN50M = 20 mM glucose + 50 μg/ml SN 50M. *$P < 0.05$ compared to 5 mM glucose.

NF-κB is considered a key regulator of antioxidant enzymes,[18] and decrease in intracellular GSH redox status is shown to activate NF-κB.[43] We and others have shown previously that GSH levels are decreased, and the glutathione redox system is impaired in the retina in diabetes, and

administration of antioxidants inhibits those abnormalities.[3,5,10] Activation of NF-κB modulates the expression of several genes, and upregulates the production of various proinflammatory cytokines, including tumor necrosis factor, and iNOS,[44] and antioxidants inhibit such activation. Further, previous studies have shown that Aminoguanidine treatment in diabetic rats inhibits the development of retinopathy, and NO, nitrotyrosine and the expression of the iNOS. Thus, there remains a strong possibility that the beneficial effects of antioxidants on NF-κB activation might be due to normalization of the intracellular redox state of the retina.

NF-κB sites in the promoter region of the gene encoding NOS are considered to be essential,[44] and here we show that the increase in retinal NO, iNOS expression and NF-κB activation follow similar patterns during the course of development of retinopathy in diabetes, all being active at very early stages of diabetes in rats, remain fully activated when the histopathology can be seen in the retina, and can be inhibited by antioxidants. Increase in the expression of iNOS in the retina is reported as early as 8–10 weeks of diabetes in rats,[29] and our data clearly show that iNOS remains elevated at duration of diabetes in rats when capillary cell apoptosis and pathology are reported.[12,13] However, it needs to be acknowledged that iNOS may not be the only enzyme responsible for increased NO in the retina in diabetes. In early stages of the development of diabetic retinopathy, increases in both constitutive nitric oxide synthases (nNOS and eNOS) are postulated to be associated with retinal vascular permeability and clinical vascular dysfunction.[45]

Peroxynitrite formed by the reaction between superoxides and NO is elevated in the retina early in diabetes, and the distribution of nitrotyrosine is detected only in the vasculature of the retina,[29] the site where histopathology is present in long-term diabetic rats and humans. Here we show that nitrosylated proteins remain elevated at 14 months of diabetes in rats. Nitrosylation of proteins can disrupt protein assembly and functions, which can lead to pathological consequences, and peroxynitrite itself can increase iNOS through NF-κB.[46] Several retinal proteins are nitrosylated before pathology is detectable, and they remain nitrosylated during the development of retinal pathology, but the one with a molecular weight of about 80 kD is the most prominent. This is consistent with the recent reports showing that the most extensively nitrated protein in the retina of 8–10 weeks diabetic rats is about 80 kD, and diabetes-induced increase in its expression is inhibited by a therapy that can inhibit the development of retinopathy.[29] The data presented here show that antioxidants that inhibit the development of retinopathy in rats[4] inhibit formation of this nitrosylated protein. Further, we have recently reported that the expressions of nitrosylated 80 kD protein and iNOS in rat retina remain elevated for at least 7 months after re-institution of normal glycemic control in the rats hyperglycemic for 6 months,[26] suggesting that nitrosylation of this 80 kD protein may also be playing an important role in the resistance of retinopathy to reverse after initiation of good glycemic control in diabetic patients and animals.[47] However, at this point the identification of this protein, effect of nitrosylation on its function, and its role in the development of diabetic retinopathy remains to be explored.

NF-κB activation is known to be a key step in the signaling pathway by which high glucose induces apoptosis.[19] We have shown that retinal caspase-3 is activated in diabetes and in the isolated retinal capillary cells incubated in high glucose medium, and administration of the same multiple antioxidants, as used in the present study, inhibits caspase-3 activation in diabetic rats.[14] Further, we have recently shown that the expression of Bax, a NF-κB-dependent proapoptotic gene, is increased in the mitochondria obtained from the retina of diabetic rats and from the retinal endothelial cells and pericytes incubated in high glucose.[48] This suggests that this NF-κB target gene is involved in the apoptosis of retinal capillary cells, and supports a possible role of NF-κB in the apoptosis of retinal endothelial cells and pericytes. This is further strengthened by the reports of others showing that in diabetic patients, NF-κB activation is seen in retinal pericytes, and is postulated to trigger a proapoptotic program resulting, possibly, in the formation of pericyte ghosts seen in the pathogenesis of diabetic retinopathy.[23] Although the effects of NF-κB activation can be both, inhibitory or proapoptotic depending on the cell system,[19,23,49] this study and that of others[23] strongly suggest that in diabetes NF-κB activation is proapototic in the retina, and might be playing a crucial role in the development of histopathology.

Our results show that NF-κB remains active in the retina when the retinopathy is developing in diabetic rats, and the administration of antioxidants that inhibit the development of retinopathy and retinal caspase-3 activation in diabetic rats inhibits NF-κB activation. This suggests that increased oxidative stress is one of the factors leading to NF-κB activation, but the exact mechanism involved in its activation remains to be explored. Glucose-induced activation of NF-κB is reported to involve protein kinase C (PKC) and mitogen-activated kinase (MAPK),[50] and both PKC and the MAPK pathways have been implicated in diabetic retinopathy.[51] Our previous studies have shown that activation of PKC in retina of diabetic rats follows a similar pattern as by NF-κB activation,[4] and others have shown that

retinal MAPK and NF-κB are activated at very early stages in the course of development of retinopathy.[21] In addition, diabetes-induced metabolic abnormalities in the retina appear to be interrelated,[28] and as proposed by others in the development of diabetic neuropathy,[50] NF-κB activation in retina might also represent a major site of interaction between hemodyanamics and glucose-dependent pathways. Further, the results show that the kinetics of NF-κB activation may be different in chronic diseases as compared to acute insults. In diabetes, sustained hyperglycemia is present throughout the development of retinopathy and that could account for the sustained activation of NF-κB observed in the present study. In support of this, our previous results have shown that NF-κB activation can be seen in various organs, including retina and kidney three months after induction of diabetes in rats.[25]

The results presented here show that incubation of isolated retinal endothelial cells and pericytes with high glucose activates NF-κB, and increase their apoptosis, and addition of antioxidants or SN50 (a blocker of translocation of the NF-κB complex into the nucleus) greatly diminishes glucose-induced NF-κB activation and apoptosis. However, this increase in NF-κB activation in endothelial cells is in contrast to the results reported by Romeo *et al.*, they observed glucose-induced activation of NF-κB in retinal pericytes, but failed to see NF-κB activation and apoptosis in isolated endothelial cells.[24] The reasons for such discrepancies are not clear. In diabetes apoptosis of both retinal endothelial cells and pericytes is well-documented,[12,13] and here we present data showing that incubation of both retinal endothelial cells and pericytes under high glucose conditions increases their apoptosis that can be inhibited by inhibition of NF-κB activation.

The effect of NF-κB activation on gene expression (e.g. adhesion molecules etc.) can be demonstrated very early in the course of development of retinopathy in diabetes.[21] Retinal oxidative stress continues to be elevated for a long time[4] that may lead to sustained NF-κB activation and continued increased gene expression of several cellular macromolecules. Recognizable retinal structural lesions are detectable only after 14–18 months of diabetes in rats,[4,13] although the surrogate biochemical changes can be demonstrated within 2 months.[3–6] Diabetic retinopathy is a progressive disease, and our data show that although NF-κB is activated early during the course of the development of retinopathy in diabetes, it remains active when the histopathology can be seen. This suggests that the kinetics of NF-κB activation may be different in progressive diseases. There is sustained hyperglycemia in diabetes, and such stimulus could result in the sustained activation of NF-κB. In support of this, our previous studies have shown that NF-κB is activated in the retina and other tissues that are associated with the complication, including kidney and heart, at three months of diabetes.[25]

In summary, the results presented here show that NF-κB is activated in the retina at early stages of the pathogenesis of retinopathy in diabetes, and remains active when the histological lesions are developing. Activation of NF-κB in the retina can be inhibited by the administration of the same antioxidants that are shown to inhibit caspase-3 activation and histopathology. This suggests that activation of NF-κB triggers a signaling pathway that could play an important role in the development of retinopathy, and one of the possible mechanism by which antioxidants inhibit the development of retinopathy could involve activation of NF-κB and its related pathways.

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References

[1] Baynes, J.W. (1991) "Role of oxidative stress in development of complications in diabetes", *Diabetes* **40**, 405–412.
[2] Armstrong, D., Abdella, N., Salman, A., Miller, N., Rahman, E.A. and Bojancyzk, M. (1992) "Relationship of lipid peroxides to diabetic complications", *J. Diabetes Complications* **6**, 116–122.
[3] Kowluru, R.A., Kern, T.S. and Engerman, R.L. (1996) "Abnormalities of retinal metabolism in diabetes or experimental galactosemia. IV. Antioxidant defense system", *Free Radic. Biol. Med.* **22**, 587–592.
[4] Kowluru, R.A., Tang, J. and Kern, T.S. (2001) "Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy", *Diabetes* **50**, 1938–1942.
[5] Kowluru, R.A., Kern, T.S., Engerman, R.L. and Armstrong, D. (1996) "Abnormalities of retinal metabolism in diabetes or experimental galactosemia. III. Effects of antioxidants", *Diabetes* **45**, 1233–1237.
[6] Kowluru, R.A., Engerman, R.L. and Kern, T.S. (1999) "Abnormalities of retinal metabolism in diabetes or experimental galactosemia VI. Comparison of retinal and cerebral cortex metabolism, and effects of antioxidant therapy", *Free Radic. Biol. Med.* **26**, 371–378.
[7] Obrosova, I.G., Fathallah, L. and Greene, D.A. (2000) "Early changes in lipid peroxidation and antioxidative defense in diabetic rat retina: effect of DL-alpha-lipoic acid", *Eur. J. Pharmacol.* **398**, 139–146.
[8] Heath, H., Rutter, A.C. and Beck, T.C. (1962) "Changes in the ascorbic acid and glutathione content of the retinae and adrenals from alloxan-diabetic rats", *Vision Res.* **2**, 431–437.
[9] Kern, T.S., Kowluru, R. and Engerman, R.L. (1994) "Abnormalities of retinal metabolism in diabetes or galactosemia, ATPases and glutathione", *Invest. Ophthalmol. Vis. Sci.* **35**, 2962–2967.

[10] Szabo, M.E., Haines, D., Garay, E., Chiavaroli, C., Farine, J.C., Hannaert, P., Berta, A. and Garay, R.P. (2001) "Antioxidant properties of calcium dobesilate in ischemic/reperfused diabetic rat retina", *Eur. J. Pharmacol.* **428**, 277–286.
[11] Li, W., Yanoff, M., Jian, B. and He, Z. (1999) "Altered mRNA levels of antioxidant enzymes in pre-apoptotic pericytes from human diabetic retinas", *Cell. Mol. Biol.* **45**, 59–66.
[12] Mizutani, M., Kern, T.S. and Lorenzi, M. (1996) "Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy", *J. Clin. Investig.* **97**, 2883–2890.
[13] Kern, T.S., Tang, J., Mizutani, M., Kowluru, R., Nagraj, R. and Lorenzi, M. (2000) "Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia", *Invest. Ophthal. Vis. Sci.* **41**, 3972–3978.
[14] Kowluru, R.A. and Koppolu, P. (2002) "Diabetes-induced activation of Caspase-3 in retina: Effect of antioxidant therapy", *Free Radic. Res.* **36**, 933–999.
[15] Lenardo, M.J. and Baltimore, D. (1998) "NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control", *Cell* **58**, 227–229.
[16] Baeuerle, P.A. (1991) "The inducible transcription activator NF-kappa B: regulation by distinct protein subunits", *Biochim. Biophys. Acta* **1072**, 63–80.
[17] Rahman, I. and MacNee, W. (2000) "Regulation of redox glutathione levels and genes transcription in lung inflammation: therpeutic approaches", *Free Radic. Biol. Med.* **28**, 1405–1420.
[18] Anderson, M.T., Staal, F.J., Gitler, C. and Herzenberg, L.A. (1994) "Separation of oxidant-initiated and redox-regulated steps in the NF-kappa B signal transduction pathway", *Proc. Natl Acad. Sci. USA* **22**, 11527–11531.
[19] Du, X., Stocklauser-Farber, K. and Rosen, P. (1999) "Generation of reactive oxygen intermediates, activation of NF-kappaB, and induction of apoptosis in human endothelial cells by glucose: role of nitric oxide synthase?", *Free Radic. Biol. Med.* **27**, 752–763.
[20] Hammes, H.P., Hoerauf, H., Alt, A., Schleicher, E., Clausen, J.T., Bretzel, R.G. and Laqua, H. (1999) "N(epsilon) (carboxymethyl) lysin and the AGE receptor RAGE colocalize in age-related macular degeneration", *Invest. Ophthalmol. Vis. Sci.* **40**, 1855–1859.
[21] Joussen, A.M., Huang, S., Poulaki, V., Camphausen, K., Beecken, W.D., Kirchhof, B. and Adamis, A.P. (2001) "*In vivo* retinal gene expression in early diabetes", *Invest. Ophthalmol. Vis. Sci.* **42**, 3047–3057.
[22] Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) "Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1", *EMBO J.* **10**, 2247–2258.
[23] Romeo, G., Liu, W., Asnaghi, V., Kern, T.S. and Lorenzi, M. (2002) "Activation of nuclear factor-kappa B induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes", *Diabetes* **51**, 2241–2248.
[24] Zhou, D., Brown, S.A., Yu, T., Chen, G., Barve, S., Kang, B.C. and Thompson, J.S. (1999) "A high dose of ionizing radiation induces tissue-specific activation of nuclear factor-kappaB *in vivo*", *Radiat. Res.* **151**, 703–709.
[25] Chen, S., Khan, Z.A., Cukiernik, M. and Chakrabarti, S. (2003) "Differential activation of NF-kappa B and AP-1 in increased fibronectin synthesis in target organs of diabetic complications", *Am. J. Physiol. Endocrinol. Metab.* **284**, E1089–E1097.
[26] Kowluru, R.A. (2003) "Effect of re-institution of good metabolic control on retinal oxidative stress and nitrative stress in diabetic rats", *Diabetes* **52**, 818–823.
[27] Shibuki, H., Katai, N., Yodoi, J., Uchida, K. and Yoshimura, N. (2000) "Lipid peroxidation and peroxynitrite in retinal ischemia-reperfusion injury", *Invest. Ophthalmol. Vis. Sci.* **41**, 3607–3614.
[28] Kowluru, R.A. (2001) "Diabetes-induced elevations in retinal oxidative stress, protein kinase C and nitric oxide are interrelated", *Acta Diabetologica* **38**, 179–185.
[29] Du, Y., Smith, M.A., Miller, C.M. and Kern, T.S. (2002) "Diabetes-induced nitrative stress in the retina, and correction by aminoguanidine", *J. Neurochem.* **80**, 771–779.
[30] Kennedy, A., Frank, R.N., Sotolongo, L.B., Das, A. and Zhang, N.L. (1990) "Proliferative response and macromolecular synthesis by ocular cells cultured on extracellular matrix materials", *Curr. Eye Res.*, 307–322.
[31] Lin, Y.Z., Yao, S.Y., Veach, R.A., Torgerson, T.R. and Hawiger, J. (1995) "Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence", *J. Biol. Chem.* **270**, 14225–14228.
[32] Bradford, M.M. (1974) "A rapid and sensitive method for the quantitation of microgram quantities of protein", *Anal. Biochem.* **72**, 248–258.
[33] Bursell, S.E., Clermont, A.C., Aiello, L.P., Aiello, L.M., Schlossman, D.K., Feener, E.P., Laffel, L. and King, G.L. (1999) "High-dose vitamin E supplementation normalizes retinal blood flow and creatinine clearance in patients with type 1 diabetes", *Diabetes Care* **22**, 1245–1251.
[34] Di Leo, M.A., Ghirlanda, G., Gentiloni Silveri, N., Giardina, B., Franconi, F. and Santini, S.A. (2003) "Potential therapeutic effect of antioxidants in experimental diabetic retina: a comparison between chronic taurine and vitamin E plus selenium supplementations", *Free Radic. Res.* **37**, 323–330.
[35] Stitt, A., Gardiner, T.A., Anderson, N.L., Canning, P., Frizzell, N., Duffy, N., Boyle, C., Januszewski, A.S., Chachich, M., Baynes, J.W. and Thorpe, S.R. (2002) "The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes", *Diabetes* **51**, 2826–2832.
[36] Robison, W.G., Jacot, J.L., Katz, M.L. and Glover, J.P. (2000) "Retinal vascular changes induced by the oxidative stress of alpha-tocopherol deficiency contrasted with diabetic microangiopathy", *J. Ocul. Pharmacol. Ther.* **16**, 109–120.
[37] Wang, S.Y. and Lin, H.S. (2000) "Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage", *J. Agric. Food Chem.* **48**, 140–146.
[38] Van Zandwijk, N. (1995) "N-acetylcysteine for lung cancer prevention", *Chest* **10**, 1437–1441.
[39] Sies, H. and Arteel, G.E. (2000) "Interaction of peroxynitrite with selenoproteins and glutathione peroxidase mimics", *Free Radic. Biol. Med.* **8**, 1451–1455.
[40] Panasenko, O.M., Sharov, V.S., Briviba, K. and Sies, H. (2000) "Interaction of peroxynitrite with carotenoids in human low density lipoproteins", *Arch. Biochem. Biophys.* **373**, 302–305.
[41] Knudsen, C.A., Tappel, A.L. and North, J.A. (1996) "Multiple antioxidants protect against heme protein and lipid oxidation in kidney tissue", *Free Radic. Biol. Med.* **20**, 165–173.
[42] Koya, D., Li, K., Ishii, H., Kanoh, H. and King, G.L. (1997) "Prevention of glomerular dysfunction in diabetic rats by treatment with d-alpha tocopherol", *J. Am. Soc. Nephrol.* **8**, 426–435.
[43] Yanagihara, Y., Basaki, Y., Kajiwara, K. and Ikizawa, K. (1997) "A thiol antioxidant regulates IgE isotype switching by inhibiting activation of nuclear factor-kappaB", *J. Allergy Clin. Immunol.* **100**, S33–S38.
[44] Griscavage, J.M., Wilk, S. and Ignarro, L.J. (1996) "Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-kappa B", *Proc. Natl Acad. Sci. USA* **93**, 3308–3312.
[45] Takeda, M., Mori, F., Yoshida, A., Takamiya, A., Nakagomi, S., Sato, E. and Kiyama, H. (2001) "Constitutive nitric oxide synthase is associated with retinal vascular permeability in early diabetic rats", *Diabetologia* **44**, 1043–1050.
[46] Beckman, J.S. and Koppenol, W.H. (1996) "Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly", *Am. J. Physiol.* **271**, C1424–C1437.
[47] Diabetes Control and Complications Trial Research Group(1998) "Early worsening of diabetic retinopathy in the diabetes control and complication trial", *Arch. Ophthalm.* **116**, 874–886.

[48] Kowluru, R.A. and Abbas, S.N. (2003) "Diabetes-induced mitochondrial dysfunction in the retina", *Invest. Ophthalmol. Vis. Sci.*, in press.
[49] Kuhnel, F., Zender, L., Paul, Y., Tietze, M.K., Trautwein, C., Manns, M. and Kubicka, S. (2000) "NFkappaB mediates apoptosis through transcriptional activation of Fas (CD95) in adenoviral hepatitis", *J. Biol. Chem.* **275**, 6421–6427.
[50] Cooper, M.E. (2001) "Interaction of metabolic and haemodynamic factors in mediating experimental diabetic nephropathy", *Diabetologia* **44**, 1957–1972.
[51] Awazu, M., Ishikura, K., Hida, M. and Hoshiya, M. (1999) "Mechanisms of mitogen-activated protein kinase activation in experimental diabetes", *J. Am. Soc. Nephrol.* **10**, 738–745.