Original Article

Salicylate-Based Anti-Inflammatory Drugs Inhibit the Early Lesion of Diabetic Retinopathy

Ling Zheng,[1] Scott J. Howell,[2] Denise A. Hatala,[2] Kun Huang,[3] and Timothy S. Kern[1,2]

It has been previously reported that aspirin inhibited the development of diabetic retinopathy in diabetic animals, raising the possibility that anti-inflammatory drugs may have beneficial effects on diabetic retinopathy. To further explore this, we compared effects of oral consumption of three different salicylate-based drugs (aspirin, sodium salicylate, and sulfasalazine) on the development of early stages of diabetic retinopathy in rats. These three drugs differ in their ability to inhibit cyclooxygenase but share an ability to inhibit nuclear factor-κB (NF-κB). Diabetes of 9–10 months duration significantly increased the number of TUNEL (transferase-mediated dUTP nick-end labeling)-positive capillary cells and acellular (degenerate) capillaries in the retinal vasculature, and all three salicylate-based drugs inhibited this cell death and formation of acellular capillaries without altering the severity of hyperglycemia. In short-term diabetes (2–4 months), all three salicylates inhibited the diabetes-induced loss of neuronal cells from the ganglion cell layer. Oral aspirin (as a representative of the salicylate family) inhibited diabetes-induced increase in NF-κB DNA-binding affinity in electrophoretic mobility shift assay and transcription factor array in nuclear extract isolated from whole retina. All three salicylates inhibited the diabetes-induced translocation of p50 (a subunit of NF-κB) into nuclei of retinal vascular endothelial cells of the isolated retinal vasculature, as well as of p50 and p65 into nuclei of cells in the ganglion cell layer and inner nuclear layer on whole-retinal sections. Sulfasalazine (also as a representative of the salicylates) inhibited the diabetes-induced upregulation of several inflammatory gene products, which are regulated by NF-κB, including vascular cell adhesion molecule, intracellular adhesion molecule-1, inducible nitric oxide synthase, and cyclooxygenase-2 in whole-retinal lysate. Salicylates, in doses administrated in our experiments, inhibited NF-κB and perhaps other transcription factors in the retina, were well tolerated, and offered new tools to investigate and inhibit the development of diabetic retinopathy. *Diabetes* 56:337–345, 2007

Degeneration of retinal capillaries is believed to be an important contributor to the development of clinically significant diabetic retinopathy. We and others previously demonstrated that retinal capillary cells die by an apoptotic-like process (1) in diabetes and that the ability of a therapy to inhibit apoptosis in retinal microvascular cells predicts development of the degenerate acellular capillaries in retinas of diabetic animals (2).

An observation that aspirin consumption was associated with a lower-than-expected severity of diabetic retinopathy in patients with arthritis led to a suggestion many years ago that aspirin may be a potentially effective therapy against diabetic retinopathy (3,4). Inhibition of diabetes-induced degeneration of retinal capillaries in a 5-year study of diabetic dogs given aspirin (5) confirmed this effect, but prospective clinical trials yielded contradictory conclusions (6,7). Aspirin is a potent anti-inflammatory agent that acts not only by inhibiting the enzymatic activity of cyclooxygenases (COXs) by acetylation (8,9), but also by inhibiting the activation of some transcription factors, including nuclear factor-κB (NF-κB) (10) and CCAAT enhancer binding protein (C/EBP) (11).

NF-κB is a transcription factor that plays an important role in transcriptional regulation of inflammatory proteins. The NF-κB complex consists of a number of structurally related subunits (p50, p52, RelA [also known as p65], RelB, and RelC), which can form heterodimers or homodimers. The best characterized member of the NF-κB-family is the p50/p65 heterodimer, which consists of a p65 transactivation domain and a p50 DNA-binding domain.

Increased NF-κB DNA-binding affinity has been detected both in the retinas of diabetic animals and in retinal cells cultured in elevated levels of glucose (12–16). Prior studies (12,15) have suggested that NF-κB plays an important role in the development of diabetic retinopathy via its ability to induce an inflammatory condition. NF-κB–regulated inflammatory gene products reported to be upregulated in retinas during diabetes by several (but not all) investigators include COX-2 (12,17,18), inducible nitric oxide synthase (iNOS) (17,19,20), adhesion molecules such as intracellular adhesion molecule (ICAM)-1 (15,21,22), and proinflammatory cytokines such as tumor necrosis factor-α (12) and interleukin-1β (23–26). These changes likely contribute to diabetes-induced increases of prostaglandin

From the [1]Department of Medicine, Case Western Reserve University, Cleveland, Ohio; the [2]Department of Ophthalmology, Case Western Reserve University, Cleveland, Ohio; and the [3]Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio.

Address correspondence and reprint requests to Timothy S. Kern, PhD, Department of Medicine and Ophthalmology, 434 Biomedical Research Building, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106. E-mail: tsk@case.edu.

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C/EBP, CCAAT enhancer binding protein; COX, cyclooxygenase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GCL, ganglion cell layer; ICAM, intracellular adhesion molecule; HPLC, high-performance liquid chromatography; INL, inner nuclear layer; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; PMSF, phenylmethylsulfonyl fluoride; TUNEL, transferase-mediated dUTP nick-end labeling; VCAM, vascular cell adhesion molecule.

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E2 (17), nitrotyrosine (a marker of peroxynitrite) (20), and leukostasis (15,21,22) in retinas.

To further explore the mechanism by which aspirin was able to inhibit diabetic retinopathy in animals, we assessed the ability of three different salicylates (aspirin, sodium salicylate, and sulfasalazine) to inhibit diabetes-induced degeneration of retinal capillaries in rats. All three drugs can inhibit NF-κB (27–30), but, unlike aspirin, neither sodium salicylate nor sulfasalazine can directly inhibit COX at therapeutic doses. Others have speculated that serum levels of salicylate measured in aspirin-fed rats (30 mg · [kg body wt · day]$^{-1}$) were not sufficient to inhibit NF-κB (31). The aims of this study were to compare salicylates with respect to their abilities to inhibit diabetes-induced *1*) degeneration of retinal capillaries and neurons, *2*) NF-κB activation in retinal cells, and *3*) expression of inflammatory markers in retinas.

RESEARCH DESIGN AND METHODS

Streptozotocin-induced diabetic rats. Diabetes was induced in fasted male rats (Lewis) with streptozotocin (60 mg/kg body wt), and animals were housed in ventilated microisolator cages. All experiments followed the guidelines set forth by the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research. Diabetic rats were treated with insulin (0–3 units) as needed to maintain body weight while still allowing chronic hyperglycemia. This amount of insulin was adjusted as necessary to keep the same glycemia level among diabetic groups. The extent of blood glucose elevation was estimated every 2–3 months by measuring glycated hemoglobin level and by measuring blood glucose concentration as previously described (2,15). One week after the injection of streptozotocin, diabetic rats were randomly assigned to receive commercially available buffered aspirin (pills were crushed into powder using an electrical blender), sodium salicylate (Sigma, St. Louis, MO), or sulfasalazine (Sigma) or to remain as diabetic controls. Aspirin and sodium salicylate were given in powered food, and sulfasalazine was given in drinking water. Rats were killed at two durations of diabetes: 2–4 months after the onset of diabetes (to determine what biochemical effects salicylates had) and after 9–10 months of diabetes (to determine the effects of salicylates on retinal histopathology). Actual consumption of aspirin, sodium salicylate, and sulfasalazine during the long-term experiment amounted to 26.6 ± 1.5, 26.9 ± 2.5, and 102.5 ± 10.1 mg · (kg body wt · day)$^{-1}$, respectively. Water was changed and measured every other day. Food was changed and measured weekly. For long-term (9–10 months) studies, diabetic rats in one experiment (purchased at the same time) were randomly assigned into three diabetic groups (control, treated with aspirin, or treated with sodium salicylate), while for another experiment (purchased at a different time), diabetic rats were randomized into control and sulfasalazine treatment groups. Age-matched nondiabetic rats were set up each time as normal controls. The doses of aspirin and sodium salicylate were chosen according to the dose of aspirin previously reported by us, which has beneficial effects on diabetic retinopathy (5), while the dose of sulfasalazine was selected based on the dose used in studies of inflammation (32,33).

Isolation of retinal vasculature. Retinal vasculature was isolated as previously described by us (2,15). The isolated vasculature was mounted on slides and used for p50 and p65 immunostaining, transferase-mediated dUTP nick-end labeling (TUNEL) assay, and acellular capillary quantitation.

Nuclear extract and electrophoretic mobility shift assay. Nuclear proteins from retinas were isolated as described by others (34) with some modifications. All steps were carried out on ice or at 4°C. Freshly isolated retinas were rinsed in PBS buffer and spun at 1,500*g* for 5 min. Buffer A (10 mmol/l HEPES, pH 7.9, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 0.5 mmol/l dithiothreitol [DTT], and 0.5 mmol/l phenylmethylsulfonyl fluoride [PMSF]) was added to the tissue (2.5 ml/g wet weight). Five strokes on a hand-held tissue grind tube were used to homogenize tissue. After the addition of NP-40 (0.5%), five additional strokes were performed. The suspension was incubated for 10 min to lyse the cells with detergent, and the homogenate was centrifuged for 10 min at 2,000*g*. The pelleted nuclei were resuspended in cold buffer B (25% glycerol, 20 mmol/l HEPES, pH 7.9, 1.5 mmol/l $MgCl_2$, 0.42 mol/l NaCl, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 0.5 mmol/l PMSF, and 10 μg/ml pepstain, leupeptin, and aprotinin) and shaken at 4°C for 30 min for high-salt extraction. The lysed nuclei were centrifuged for 10 min at 12,000*g*. The supernatants were transferred to a microcentrifuge tube, buffer D (20% glycerol, 20 mmol/l HEPES, pH 7.9, 0.1 mmol/l KCl, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 0.5 mmol/l PMSF, and 10 μg/ml pepstain, leupeptin, and aprotinin) was added at a ratio of 1:1 (vol:vol), and the supernatants were centrifuged for 10 min at 12,000*g*. The amount of nuclear protein in the supernatant was determined by protein assay (Bio-Rad, Hercules, CA) and stored at −80°C until use. Nuclear proteins (5 μg) were incubated with a ^{32}P-labeled double-strand NF-κB consensus sequence (Promega, Madison, WI), and the electrophoretic mobility shift assay (EMSA) was performed according to instructions provided by the manufacturer. DNA protein complexes were resolved on a 4% nondenaturing polyacrylamide gel, and the bands were examined by autoradiography. The specificity of binding was examined by competition with a 50-fold nonlabeled NF-κB or AP-1 consensus sequence (Promega), preincubated with nuclear proteins and labeled NF-κB oligouncleotide. A supershift assay was also performed to determine the specificity of NF-κB binding to its specific consensus sequence by using anti-p65 and anti-p50 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Transcription factor array. Two array systems were purchased from Panomics (Fremont, CA). Array I includes oligouncleotides known to bind to C/EBP (35) and the classic p50/p65 heterodimer of NF-κB (36), while array V includes a oligonucleotide known to bind to the p65/p65 homodimer (37) of NF-κB. Array V also includes a oligonucleotide that has been shown bind to p50/p75 heterodimers better than p50/p65 heterodimers (38). (p75 is a p65-related protein.) Array I was performed first with one sample from each experimental group and another time with three different (pooled) samples from each group. Both experiments yielded similar results. Array V was performed once with pooled samples. Results shown here are of the pooled samples.

Nuclear extract from retina was isolated as above. For pooled samples, biotin-labeled DNA-binding oligonucleotides were incubated with 9 μg nuclear extract (3 μg nuclear extract from three different rats in one group) for 30 min to allow the formation of protein/DNA complexes. The protein/DNA complexes were then separated from the free probes on the spin columns provided by the manufacturer and hybridized to the TranSignal Array membrane overnight at 42°C. Hybridized membranes were incubated with horseradish peroxidase–labeled streptavidin. Signals were detected, and resulting images were analyzed using Metamorph imaging software (Universal Imaging, Downington, PA). Background values were generated by obtaining intensity values from blank areas on each blot. These background intensity values were averaged, and the resulting average background was adjusted to correspond to each samples' given area and then subtracted from each sample. The resulting values are displayed as total intensity, which represents the collective values for each pixel in a given samples' area. A housekeeping gene (*TFIID* for array I and *NF-1/L* for array V) was used, and the values of NF-κB and C/EBP on the arrays were normalized to it. Spots that increased or decreased by at least twofold were considered significant.

Immunohistochemical detection of NF-κB subunits (p50 and p65)

Retinal blood vessels. Retinal vessels were isolated by the trypsin digest method. p50 was stained as previously described (15). Three different p65 antibodies (p65 [MAB3026], Chemicon International, Temecula, CA; p65 [sc-109], Santa Cruz Biotechnology, Santa Cruz, CA; p65 [RB-9034], Lab Vision, Fremont, CA) were also applied to the retinal blood vessels in order to study the nuclear translocation of p65. Four different fields containing ~750–850 capillary cells (endothelial cells and pericytes) of each sample were evaluated in a masked manner. The capillary endothelial cells (elongated nuclear shape) or pericytes (round nuclei on outside of vessel wall) having positive immunostaining in nuclei were quantified separately.

Retinal sections. Formalin-fixed paraffin sections (5 μm) were immunostained with the p50 (Santa Cruz Biotechnology) and p65 (Lab Vision) antibodies applied at a dilution of 1:500 overnight at 4°C.

TUNEL assay

The retinal vasculature from rats 9–10 months post-onset of diabetes was isolated by the trypsin digest method. TUNEL reaction (In Situ Cell Death Detection kit, fluorescein; Roche, Mannheim, Germany) was performed as previously described (15). The number of TUNEL-positive nuclei in retinal capillaries of different groups was counted in the entire retinal vasculature and reported relative to that in nondiabetic controls.

Quantitation of acellular capillaries

After quantifying TUNEL-positive cells, the coverslips were gently soaked from the slides. Retinal vessels were then stained with periodic acid—Schiff and hematoxylin. Acellular capillaries were quantified in five to seven field areas in the mid-retina (200× magnification) in a masked manner. Acellular capillaries were identified as capillary-sized vessel tubes having no nuclei anywhere along their length and were reported per square millimeter of retinal area.

Ganglion cell count and retinal thickness measurement in whole-retina sections. Formalin-fixed paraffin retinal sections were stained with hematoxylin and eosin for light microscopy and morphometry of retinal thickness. To control the sectioning angle, all eyes were cut through the pupil and optic nerve area, thus insuring that sections were essentially tangential through the

TABLE 1
Glycemia and body weight of experimental groups

Group	*n*	Duration (months)	Body wt (g)	Nonfasting blood glucose (mg/dl)	GHb (%)
Nondiabetes	11	10	589 ± 55	73 ± 28	4.0 ± 0.2
Diabetes	9	10	302 ± 44*	302 ± 95*	8.0 ± 2.1*
Diabetes + SUL	8	10	344 ± 50*	300 ± 36*	9.9 ± 0.7*
Nondiabetes	15	9	583 ± 60	86 ± 15	4.3 ± 1.0
Diabetes	4	9	278 ± 37*	332 ± 110*	10.2 ± 2.5*
Diabetes + ASP	5	9	257 ± 8*	316 ± 44*	9.8 ± 0.7*
Diabetes + SAL	8	9	261 ± 19*	376 ± 58*	9.8 ± 1.3*

Data are means ± SD. *$P < 0.01$ compared with nondiabetic rats. ASP, aspirin; SAL, salicylate; SUL, sulfasalazine.

retina. Pictures were taken at four locations in the retina (both sides of the optic nerve [posterior] and mid-retina [central]) at 400× magnification. The nuclei in the ganglion cell layer (GCL) (not including nuclei of blood vessels in that layer) were counted, and the thicknesses of retinal layers were measured using a Image-Pro Plus software as follows: GCL, inner plexiform layer, inner nuclear layer (INL), outer plexiform layer, and outer nuclear layer.

Serum salicylate measurement. Blood was withdrawn from anesthetized rats (not fasted) at 10:00–11:00 A.M. Sample preparation and reverse-phase high-performance liquid chromatography (HPLC) analysis were performed as previously described (39,40) with modifications. Briefly, 200 μl methanol (HPLC grade; Fisher Scientific, Fair Lawn, NJ) was added to 100 μl serum. Samples were then vigorously vortexed for 1 min and centrifuged at 15,000*g* for 25 min to precipitate proteins. Next, 250 μl supernatant was speed-vac dried at −40°C, redissolved in water, and then injected into an analytical reverse-phase HPLC (C8 column; Vydac, Hesperia, CA). Samples were eluted with an isocratic gradient (20% methanol, 0.1% trifluoroacetic acid) over 35 min and monitored by an ultraviolet detector at 310 nm. Salicylate constantly eluted at ~25 min. Serum salicytate concentrations were calculated by integrating the peak areas of salicyate and then normalized to a HPLC standard curve prepared with salicylic acid standard (Sigma).

Western blot analysis. Rat retinas were isolated and sonicated in radioimmunoprecipitation assay buffer as previously described (15). Total amount of protein was determined by the Bio-Rad Protein Assay. Samples (50 μg) were fractionated by SDS-PAGE and electroblotted onto nitrocellucose membrane, and the membranes were blocked with 5% nonfat milk. Antibodies for ICAM-1 (Santa Cruz Biotechnology, 1:200 dilution), vascular cell adhesion molecule (VCAM) (Santa Cruz Biotechnology, 1:500 dilution), iNOS (Sigma, 1:1,000 dilution), and COX-2 (Cayman Chemical, 1:1,000 dilution) were applied overnight at 4°C. Membranes then were stripped and reprobed with β-actin (Sigma) to confirm equal protein loading. The films were subsequently scanned, and band intensity was quantified using Quantity One 1-D Analysis Software (Bio-Rad).

Statistical analysis. All results are expressed as means ± SD. The data were analyzed by Kruskal-Wallis, followed by the Mann-Whitney test. Differences were considered statistically significant with P values <0.05.

RESULTS

Salicylates did not alter glycemia in diabetic animals. Because the severity of hyperglycemia greatly influences the development of diabetic retinopathy, considerable effort was devoted to maintaining glycemia comparable in the experimental and the control diabetic groups. After 9–10 months' onset of diabetes (Table 1), GHb values in diabetic rats treated with the three salicylates were not significantly different from those of control diabetic rats, and all were significantly higher than corresponding values in nondiabetic rats. Likewise, blood glucose values for diabetic rats and diabetic rats treated with all three salicylates remained significantly higher than values in nondiabetic rats. Body weights of diabetic rats with different treatments were significantly less than those of nondiabetic rats but were not different among diabetic groups. GHb value, blood glucose, and body weight were also measured in the shorter duration study and showed similar results. Administration of the three salicylates did not adversely effect the health or life span of diabetic rats.

Serum was collected at 10:00–11:00 A.M. (4–5 h after lights were turned on). Serum salicylate levels of aspirin-, sodium salicylate–, and sulfasalazine-treated rats were 0.037 ± 0.023, 0.113 ± 0.05, and 0.002 ± 0.002 mmol/l, respectively. The very low serum salicylate concentration of sulfasalazine-treated rats may be due to poor absorption of sulfasalazine and a shorter half-life of its active metabolite (41).

Aspirin altered the DNA-binding affinity of NF-κB in retinas. The ability of aspirin to inhibit NF-κB activation was assessed by EMSA using nuclear extract that was isolated from whole retinas. After 2 months of diabetes, there was increased DNA-binding activity of NF-κB in retinas of diabetic rats compared with nondiabetic rats, and aspirin treatment inhibited this diabetes-induced increase in DNA-binding activity (Fig. 1*A*). Quantitative data of NF-κB DNA-binding affinity is summarized in Fig. 1*B*. Supershift assay (Fig. 1*C*) revealed two protein-DNA complexes with different molecular mass, both of which contained p65 and p50. These two complexes might contain p50 and p65 in different stoichiometric ratios and/or with additional factors such as C/EBPβ or Bcl-3 as suggested by Naschberger et al. (42).

Transcription factor arrays were also used to evaluate the DNA-binding affinity of NF-κB and C/EBP using nuclear extract isolated from whole retinas. In the arrays, diabetes increased NF-κB DNA-binding affinity of the p50/p65 heterodimer by 2.2-fold compared with normal rats, and this diabetes-induced increase in p50/p65-binding affinity was blocked by aspirin treatment (Fig. 1*D*). Moreover, p65/p65 DNA-binding affinity was greatly enhanced in retinas of diabetic rats compared with undetectable levels in normal and aspirin-treated diabetic rats (Fig. 1*E*). DNA-binding affinity of the p50/p65 and p50/p75 heterodimers showed no significant differences among retinas of normal rats, diabetic rats, or diabetic rats treated with aspirin (Fig. 1*F*). Aspirin did inhibit C/EBP DNA-binding affinity in retinas, but diabetes did not increase C/EBP DNA-binding affinity compared with that in nondiabetic rats (Fig. 1*G*).

Aspirin, sodium salicylate, or sulfasalazine inhibited diabetes-induced translocation of NF-κB to the nucleus in retinal cells. Since EMSA and transcription factor array data showed that the oral administration of aspirin to diabetic rats resulted in DNA-binding affinity changes for NF-κB (p50/p65 and p65/p65), we acertained the nuclear location of the NF-κB subunits (p50 and p65) via immunohistochemistry on retinal sections and on isolated retinal blood vessels. Whereas inactivated NF-κB is retained in cytoplasm by binding to inhibitory proteins, activated p50 and p65 are located in nuclei. Increased numbers of p50- and p65-stained nuclei were observed in

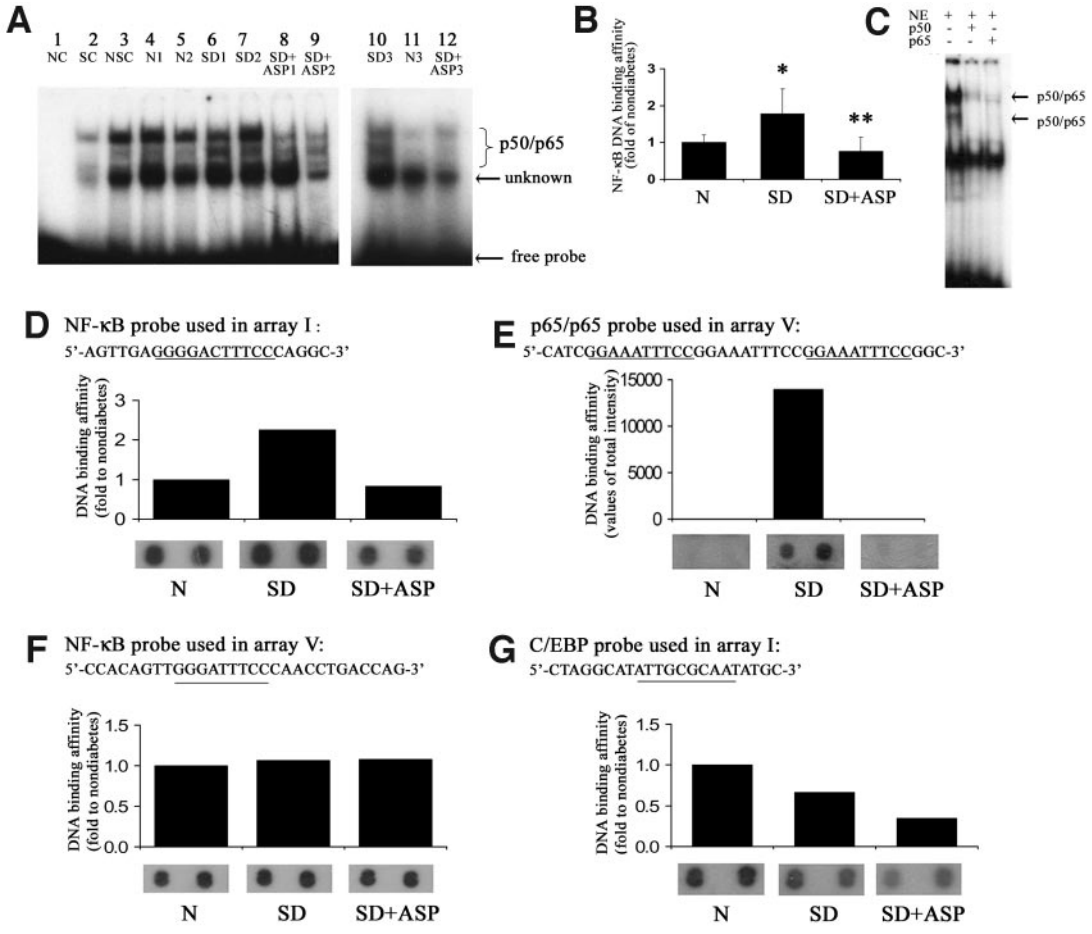

FIG. 1. Aspirin alters NF-κB DNA-binding affinity in retinas of diabetic rats. *A*: Aspirin inhibited the diabetes-induced NF-κB activation in retinas. *Lane 1* is negative control (NC) without nuclear extracts. *Lane 2* is specific competition (SC) with nuclear extracts incubated with labeled and 50-fold nonlabeled NF-κB oligonucleotide. *Lane 3* is nonspecific competition (NSC) with nuclear extracts (NE) incubated with labeled NF-κB oligonucleotide and 50-fold nonlabeled AP-1 oligonucleotide. *Lanes 4*, *5*, and *11* are retina nuclear extracts from nondiabetic rats (N). *Lanes 6*, *7*, and *10* are retina nuclear extracts from diabetic rats (SD). *Lanes 8*, *9*, and *12* are retina nuclear extracts from diabetic rats treated with aspirin (SD+ASP) for 4 months. *B*: Quantitative data of the two NF-κB DNA-binding bands of *A*. *C*: Supershift assay. *D–G*: Results of transcription factor arrays with oligonucleotide sequences at the *top*, quantitative data of total density in the *middle*, and images at the *bottom*. NF-κB (p50/p65) (*D*); NF-κB (p65/p65) (*E*); NF-κB (p50/p65 and p50/p75) (*F*); and C/EBP (*G*). NF-κB and C/EBP binding sites are underlined.

GCL and INL of retinas of diabetic rats, and administration of the three salicylates to the diabetic rats inhibited this diabetes-induced increase of p50 and p65 nuclear staining (Fig. 2*A* and *B*).

We also investigated the localization of p50 and p65 on the isolated retinal vasculature. p50 immunostained endothelial nuclei were found in retinas of both nondiabetic and diabetic rats, but diabetes caused a fourfold increase in the numbers of endothelial nuclei immunostained by p50 antibody compared with the nondiabetic control ($P < 0.01$). Administration of the three salicylates all significantly inhibited the diabetes-induced increase in the number of p50-immunostained endothelial nuclei ($P < 0.05$; Fig. 2*C*). Likewise, diabetes increased p50 nuclear localization in pericytes, and the p50 nuclear localization was significantly inhibited by sulfasalazine ($P < 0.05$) and partially inhibited by aspirin and sodium salicylate (data not shown).

In contrast to the p50 subunit, no p65 immunostaining in either endothelial or pericyte nuclei was observed from isolated retinal vasculatures of diabetic and control rats. However, diabetes appeared to induce an increase in p65 staining in the cytoplasm of endothelial cells (data not shown).

Aspirin, sodium salicylate, or sulfasalazine prevented diabetes-induced retinal ganglion cell loss. Neuron death has been reported in the retinas of diabetic rats (43). When compared with nondiabetic rats, a 4-month duration of diabetes caused a significant loss of cells in the GCL (7.5 ± 0.9 vs. 9.3 ± 1.3 cells/100 μm length of retina; $P < 0.05$) and reduction in retinal thickness (140.7 ± 15.2 vs. 162.7 ± 16.8 μm; $P < 0.05$) (Table 2). Administration of the three salicylates inhibited diabetes-induced cell loss in the GCL but did not prevent the reduction in retinal thickness caused by diabetes.

Aspirin, sodium salicylate, or sulfasalazine prevented diabetes-induced retinal capillary degeneration. Acellular, degenerate, and nonperfused retinal capillaries are believed to be one of the most important early lesions of diabetic retinopathy, and evidence suggests that apoptotic vascular cell death contributes to the formation of acellular capillaries. We used the TUNEL assay to detect DNA damage and apoptosis in vascular cells (endothelial cells and pericytes) on the trypsin-digested retinal vasculature. Using the same samples, we also quantified acellular capillaries.

There was a 3.7-fold increase in the number of TUNEL-positive capillary cells (endothelial cells and

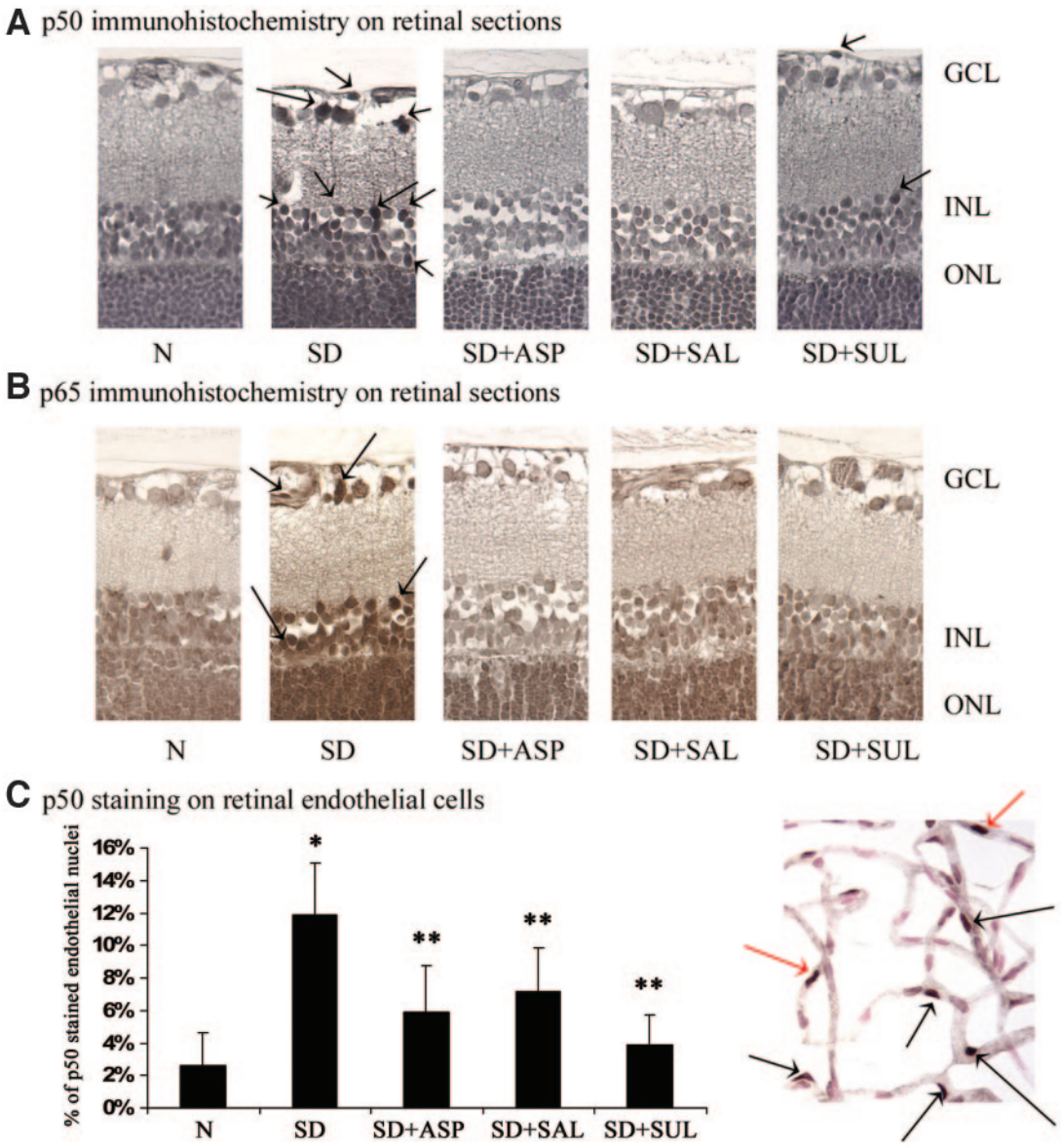

FIG. 2. Salicylates inhibit nuclear translocation of NF-κB subunits (p50 and p65) in retinas of diabetic rats. *A*: Salicylates inhibit increased p50 immunostaining in nuclei of cells in GCL and INL in retinas of diabetic rats. (Black arrows pointing to dark gray indicate positive immunostain.) *B*: Salicylates inhibit increased p65 immunostaining in nuclei of cells in GCL and INL in retinas of diabetic rats. (Black arrows pointing to dark brown indicate positive immunostain.) *C*: Salicylate-based anti-inflammatory drugs inhibited diabetes-induced p50 nucleus translocation in the retinal vasculature. A picture of p50 staining on the retinal blood vessels is shown at the *right*. (Black arrows point to positive-stained pericytes, and red arrows point to positive-stained endothelial cells.) p50-positive stained nuclei of endothelial cells were quantified and reported as percentage of capillary cells (N: nondiabetic rats, $n = 7$; SD: diabetic rats, $n = 6$; SD+ASP: diabetic rats treated with aspirin, $n = 6$; SD+SAL: diabetic rats treated with sodium salicylate, $n = 5$; SD+SUL: diabetic rats treated with sulfasalzine, $n = 7$.) *$P < 0.01$ compared with nondiabetic rats; **$P < 0.05$ compared with control diabetic rats.

pericytes) in the retinas of diabetic rats compared with nondiabetic controls ($P < 0.01$) at 9 months of diabetes, and administration of aspirin or sodium salicylate prevented this increase in cell death ($P < 0.05$; Fig. 3*A*). Consistent with this, the number of acellular capillaries was significantly increased in retinas of diabetic rats compared with age-matched nondiabetic rats ($P < 0.01$), and this increase was significantly inhibited by oral administration of aspirin ($P < 0.01$) or sodium salicylate ($P < 0.05$) (Fig. 3*B*).

In a similar long-term study, sulfasalazine partially inhibited TUNEL-positive capillary cells and significantly inhibited acellular capillary formation caused by diabetes (Fig. 4).

Sulfasalazine inhibited diabetes-induced overexpression of inflammatory markers in the diabetic retinas. To further explore how salicylates exert their beneficial effects on the retinal vasculature in diabetes, we used sulfasalazine as a representative salicylate. After demonstrating that sulfasalazine inhibited NF-κB (p50 and p65 subunits) translocation, we investigated whether it could inhibit gene products that are regulated by NF-κB activation. Inflammatory enzymes such as iNOS and COX-2 are regulated by NF-κB and play roles in the inflammatory process. ICAM-1 and VCAM also are regulated by NF-κB and play a critical role in the adherence of leukocytes to the vessel wall. Diabetes increased expression of iNOS,

TABLE 2
Measurement of retinal thickness and number of ganglion cells in different experimental groups

Group	*n*	Cells in GCL (μm)*	IPL (μm)	ONL (μm)	Whole retina
Nondiabetes	4	9.3 ± 1.3	51.7 ± 5.1	50.0 ± 3.0	162.7 ± 16.8
Diabetes	6	7.5 ± 0.9†	43.8 ± 5.3†	42.5 ± 4.2†	140.7 ± 15.2†
Diabetes + ASP	6	8.7 ± 2.3	46.3 ± 6.4	44.4 ± 2.1	152.1 ± 15.4
Diabetes + SAL	5	9.8 ± 1.2‡	47.7 ± 4.5	43.2 ± 4.0	153.3 ± 17.5
Diabetes + SUL	5	9.7 ± 0.9‡	44.1 ± 6.8	42.1 ± 3.0	137.0 ± 18.6

Data are means ± SD. *Per 100 μm-length retina. †$P < 0.05$ compared with nondiabetic rats. ‡$P < 0.05$ compared with diabetic rats. ASP, aspirin; ONL, outer nuclear layer; SAL, salicylate; SUL, sulfasalazine.

ICAM-1, VCAM, and COX-2 about twofold in the whole-retinal extract of diabetic rats compared with nondiabetic controls ($P < 0.05$), and administration of sulfasalazine significantly inhibited the diabetes-induced upregulation of these proteins in retinas (Fig. 5*A–D*).

DISCUSSION

In the present study, we demonstrate that three different salicylate-based anti-inflammatory drugs were able to significantly inhibit the degeneration of retinal capillaries (one of the key markers of early lesions of diabetic retinopathy) and prevent ganglion cell loss in diabetic rats. We postulate that the salicylate-mediated inhibition of early stages of diabetic retinopathy is due at least in part to inhibition of the diabetes-induced activation of NF-κB and other transcription factors in the retina. Activation of NF-κB has been implicated in the death of cultured retinal endothelial cells and pericytes in high glucose, and specific NF-κB inhibitors tested were able to inhibit the cell death under those circumstances (15,16).

The mechanism of this action of salicylates is of considerable interest. Aspirin effects vary with different doses (44): 80 mg/day has been regarded as a low dose in humans (~1.1 mg · [kg body wt · day]$^{-1}$ based on average human body weight of 70 kg), 2–4 g/day (~28–56 mg · [kg body wt · day]$^{-1}$) as intermediate doses, and 6–8 g/day (~86–114

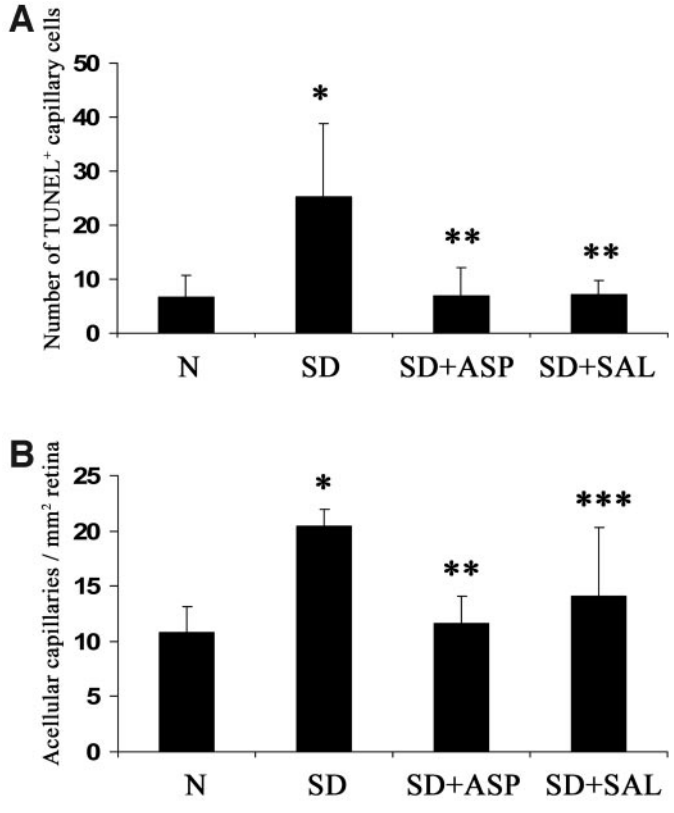

FIG. 3. Aspirin and sodium salicylate inhibit diabetes-induced increased capillary cell death and formation of acellular capillaries (9 months of diabetes). *A*: TUNEL-positive cells were counted on the isolated retinal vasculatures. (N: nondiabetic rats, $n = 8$; SD: diabetic rats, $n = 8$; SD+ASP: diabetic rats treated with aspirin, $n = 5$; SD+SAL: diabetic rats treated with sodium salicylate, $n = 8$.) Average number of TUNEL-positive cells in the nondiabetic group is 6.7 ± 3.9. *$P < 0.01$ compared with nondiabetic rats; **$P < 0.05$ compared with diabetic rats. *B*: Acellular capillaries were counted on the isolated retinal vasculatures of rats. (N: nondiabetic rats, $n = 15$; SD: diabetic rats, $n = 4$; SD+ASP: diabetic rats treated with aspirin, $n = 5$; SD+SAL: diabetic rats treated with sodium salicylate, $n = 8$.) *$P < 0.01$ compared with nondiabetic controls; **$P < 0.01$ compared with diabetic rats; ***$P < 0.02$ compared with diabetic rats.

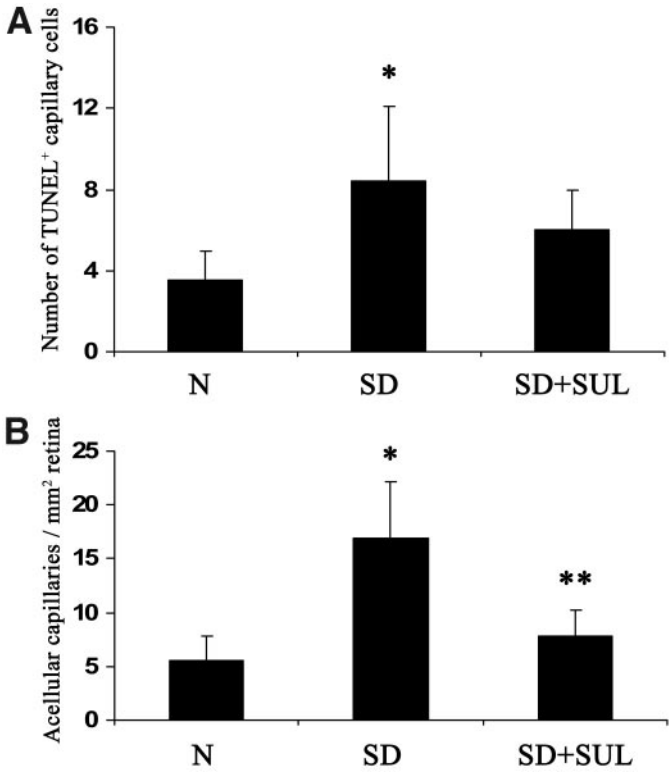

FIG. 4. Sulfasalazine inhibits diabetes-induced increased formation of acellular capillaries (10 months of diabetes). *A*: TUNEL-positive cells were counted on the isolated retinal vasculatures. (N: nondiabetic rats; SD: diabetic rats; SD+SUL: diabetic rats treated with sulfasalazine.) Average number of TUNEL-positive cells in the nondiabetic group is 3.5 ± 1.4. *$P < 0.01$ compared with nondiabetic rats. $n = 8$ in all groups. *B*: Acellular capillaries were counted on the isolated retinal vasculatures of rats. (N: nondiabetic rats; SD: diabetic rats; SD+SUL: diabetic rats treated with sulfasalazine.) *$P < 0.001$ compared with nondiabetic rats; **$P < 0.02$ compared with diabetic rats.

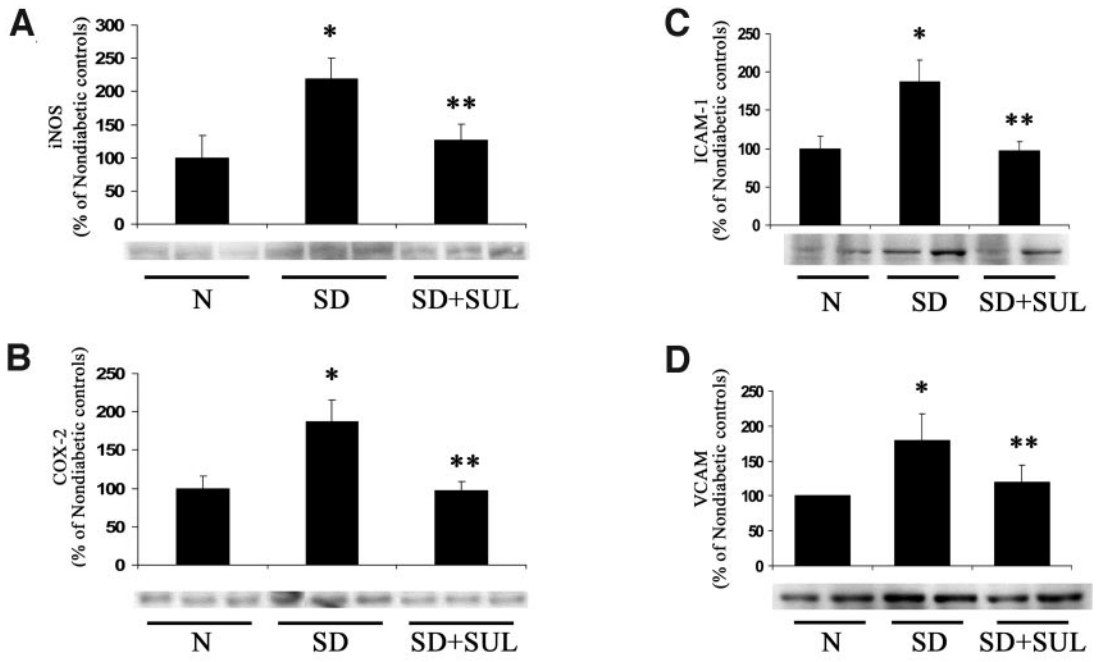

FIG. 5. Sulfasalazine prevents the hyperglycemia-induced induction of inflammatory proteins. Protein expression was measured in retinal homogenates of nondiabetic rats, diabetic rats, and diabetic rats treated with sulfasalazine. *A*: iNOS. *B*: ICAM-1. *C*: VCAM. *D*: COX-2. Expression is reported relative to β-actin in the same sample and normalized to nondiabetic controls, set as 100. (N: nondiabetic rats; SD: diabetic rats; SD+SUL: diabetic rats treated with sulfasalazine.) *$P < 0.05$ compared with nondiabetic rats; **$P < 0.05$ compared with diabetic rats. $n = 3$–5 in all groups.

mg · [kg body wt · day]$^{-1}$) as high doses. Aspirin can irreversibly acetylate serine groups on COX-1 to inhibit platelet thromboxane A2 generation at a low dose, globally inhibit COX-1 and -2 and block prostaglandin production at intermediate doses, and has unknown mechanism(s) at high doses (44). The dose of aspirin (27 mg · [kg body wt · day]$^{-1}$) that we gave the diabetic rats was on the low side of the intermediate range, which should inhibit COXs and prostaglandin production. Sodium salicylate, however, is inactive against COXs in purified enzyme preparations (45), arguing against the idea that the inhibition of retinopathy by salicylates observed by us was secondary to inhibition of COXs. Moreover, it was recently reported that clopidogrel, an inhibitor of ADP-induced platelet aggregation, did not inhibit development of acellular capillaries in retinas of diabetic rats, whereas retinopathy was inhibited by low-intermediate doses of aspirin similar to ours (30 mg · [kg body wt · day]$^{-1}$) (31).

Another known effect of salicylates is their ability to inhibit activation of some transcription factors, including NF-κB. Millimolar concentrations of salicylate are commonly used to inhibit the activation of NF-κB (10,28,29,46), probably mediated predominantly through effects on activities of cellular kinases such as IκB kinase β (30,47). In the present study, aspirin at doses of 27 mg · (kg body wt · day)$^{-1}$ gave morning serum salicylate concentrations of only ~0.037 ± 0.023 mmol/l at this time of the day (similar to that reported by Sun et al. [31]). Despite low blood level of salicylates, however, multiple independent tests (EMSA, transcription factor array, and immunostaining for nuclear p50 and p65) all suggest that the dose of salicylates used by us did indeed result in inhibition of NF-κB nuclear translocation and DNA binding in the retina. Joussen et al. (12) demonstrated by enzyme-linked immunosorbent assay that a somewhat higher dose of aspirin (50 mg · kg [body wt · day]$^{-1}$) likewise inhibited NF-κB activation in retinas of diabetic rats. Whether the inhibition of NF-κB activation in our studies occurs via direct or indirect effects remains to be determined. Blood levels of salicylate might have been considerably higher soon after consuming the drug, thereby inhibiting NF-κB during periods of high drug levels.

As reported by us before (15) and repeated in this study, diabetes results in increased translocation of the NF-κB subunit p50 into nuclei of endothelial cells in retinas of diabetic animals. Neither we nor Romeo et al. (16) found evidence of nuclear location of p65 into retinal endothelial cells in diabetes. In contrast, diabetes increased nuclear translocation of both p50 and p65 and was detected in cells of the INL and GCL. All three salicylates inhibited the diabetes-induced translocation of p50 into nuclei of retinal endothelial nuclei and of p50 and p65 into nuclei in the INL and GCL. Thus, diabetes-induced capillary degeneration is at least closely associated with NF-κB activation in both vascular and neural compartments of retina. It is well-known that p65 is the subunit of NF-κB, which has transcriptional activity, but some recent studies show that p50/p50 homodimers can also be involved in transcription of genes, including P-selectin (48), p53 (49), and Bax (50). p50 does not bind solely to NF-κB subunits and can also synergistically act with C/EBPβ and Bcl-3 to regulate gene transcription (51,52).

Besides inhibition of NF-κB, aspirin can also inhibit other transcription factors, such as AP-1 and C/EBP (10,11). Sun et al. (31) reported that C/EBPβ expression was increased in retinas of diabetic rats, and they speculated that inhibition of retinopathy by aspirin might be mediated via effects on C/EBPβ. C/EBPβ is also of interest because it regulates expression of at least some proinflammatory proteins including COX-2 (53). Further study is needed, but our studies indicate that at 2 months' duration of diabetes, the C/EBP DNA-binding affinity did not signif-

icantly change between nondiabetic and diabetic rats, whereas aspirin did inhibit this binding affinity. Multiple members of the C/EBP family exist; however, whether the binding affinity of some of these members changes in diabetes is not yet clear. Even if C/EBPβ activity is increased in diabetes, several recent studies have reported that p50 regulates its transcriptional activity, especially of C/EBPβ (54,55), thus potentially linking our findings with p50 with those of C/EBPβ reported by Sun et al. (31).

Inhibition of NF-κB activation by salicylates was further suggested by downregulation of COX-2, iNOS, ICAM-1, and VCAM in whole-retinal lysate. The transcription of these proinflammatory molecules is regulated by NF-κB, as well as other transcription factors. Most of these inflammatory proteins have been reported to be expressed in vascular and nonvascular cells of the retina (17,19,31,56,57), and the possible contribution of each of these cell types to the neurodegeneration and vascular degeneration in diabetic retinopathy remains to be clarified. Increasing evidence suggests that inflammation plays a critical role in the development of diabetic retinopathy, and present findings are consistent with this postulate. Further studies will be needed to assess whether the salicylates can inhibit other parameters of inflammation, including vascular permeability and inflammatory cell infiltration, and whether the beneficial effects of salicylates are selectively mediated on vascular cells, neuroglial cells, or both.

The evidence presented in this study shows that several salicylates can inhibit the diabetes-mediated degeneration of retinal capillaries in diabetic rats and raises a possibility that these therapies may inhibit capillary and neuron degeneration in patients if given in high-enough concentrations. Prior clinical studies using low doses of aspirin (9.3 mg · [kg body wt · day]$^{-1}$ in the Early Treatment Diabetic Retinopathy Study [7] or 14.1 mg · [kg body wt · day]$^{-1}$ in the DAMAD Study [6]) sufficient only to inhibit platelet and thromboxane production failed (7) or achieved only modest inhibition (6) of diabetic retinopathy. These clinical studies differed from our present and previous animal study (5) in several ways: *1*) per kilogram body weight, our animals received higher doses of salicylates than given to the patients; *2*) our animals were treated from the onset of diabetes, whereas patients already had mild (6) to advanced nonproliferative retinopathy (7) at the start of the clinical studies; *3*) the duration of these clinical trails was undesirably short, considering what is now known about needed study durations for retinopathy; and *4*) clinically available methods to assess the retinal vasculature (fundus photos or even fluorescein angiography) are not able to monitor the early stages of capillary degeneration with the sensitivity achieved by us using microscopic assessment of the isolated retinal vasculature. Thus, the clinical reports of failure or only modest benefit of salicylates to inhibit retinopathy to date have not definitively answered the potential efficacy of salicylates as a therapy against diabetic retinopathy.

We interpret the available evidence as indicating that the salicylate-mediated inhibition of capillary and neuron degeneration in retinas of diabetic animals is mediated at least in part via inhibition of NF-κB and the subsequent inflammatory response. Further testing of this hypothesis will require more selective inhibition or elimination of proinflammatory molecules and selective inhibition of activation of transcription factors. Salicylates nevertheless seem able to safely inhibit the degeneration of retinal capillaries and neurons in diabetes and, thus, can be expected to provide further insight into the pathogenesis of diabetic retinopathy and offer a basis for development of even better therapies.

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