Aspirin at Low-Intermediate Concentrations Protects Retinal Vessels in Experimental Diabetic Retinopathy Through Non–Platelet-Mediated Effects

Wei Sun, Chiara Gerhardinger, Zeina Dagher, Todd Hoehn, and Mara Lorenzi

The prevention of diabetic retinopathy requires drugs that leverage the benefits of glycemic control without adding the burden of side effects. Aspirin at dosages of 1–1.5 g/day has prevented manifestations of diabetic retinal microangiopathy in a clinical trial as well as in studies with dogs. Because lower and safer doses of aspirin could be used if its beneficial effects on retinopathy were due to antithrombotic effects, we compared the effects of a selective antiplatelet drug (clopidogrel) to those of aspirin in streptozotocin-induced diabetic rats. Clopidogrel did not prevent neuronal apoptosis, glial reactivity, capillary cell apoptosis, or acellular capillaries in the retina of diabetic rats. Aspirin, at doses yielding serum levels (<0.6 mmol/l) well below the anti-inflammatory range for humans, prevented apoptosis of capillary cells and the development of acellular capillaries but did not prevent neuroglial abnormalities. The aldose reductase inhibitor sorbinil, used as the benchmark for the effect of the other drugs, prevented all abnormalities. The diabetic rat retina showed increased expression of the transcription factor CCAAT/enhancer-binding protein-β, one of the known targets of low-intermediate concentrations of aspirin. Thus we found a spectrum of drug efficacy on the prevention of experimental diabetic retinopathy, ranging from the absent effect of a selective antiplatelet drug to the prevention of all abnormalities by an aldose reductase inhibitor. Aspirin at low-intermediate concentrations selectively prevented microangiopathy. The minimal effective dose of aspirin should now be sought.

Diabetic retinopathy remains a sight-threatening complication of diabetes despite decades of efforts to identify drugs that can leverage the prevention afforded by glycemic control. Aspirin has been studied several times in this context, with results that may appear inconsistent but actually are worthy of scrutiny in light of evolving knowledge. The Early Treatment Diabetic Retinopathy Study (ETDRS), the largest of the clinical trials testing aspirin, concluded that aspirin (650 mg/day) has no clinically important beneficial effects on the progression of retinopathy (1). The trial was performed in patients with mild to severe nonproliferative or early proliferative diabetic retinopathy. Having learned from the Diabetes Control and Complications Trial that intervening after retinopathy is clinically evident is much less successful than primary prevention (2), it is safe not to extrapolate the ETDRS results to prevention. The Dipyridamole Aspirin Microangiopathy of Diabetes Study, which enrolled patients with early diabetic retinopathy (a less-severe degree of retinopathy than that seen in the ETDRS), showed that aspirin (990 mg/day) was able to slow the progression of microaneurysms by >50% over the 3-year duration of the study (3). Microaneurysms are only a surrogate end point of diabetic retinopathy, but they have high predictive value for worsening retinopathy (4,5). When aspirin (the equivalent of 1.4 g/day in a 70-kg person) was given to diabetic dogs for 5 years from the time diabetes was induced, it prevented retinal hemorrhages and the development of acellular capillaries (6), the ultimate index of vascular failure in the diabetic retina (7).

The above doses of aspirin that prevented the characteristic vascular lesions of diabetic retinopathy are classified in pharmacology as low-intermediate doses (8,9). They are larger than the very small doses used in man for selective inhibition of platelet cyclooxygenase (COX) I and antithrombotic effects (40–325 mg/day) but are not quite in the range for achieving global inhibition of COX I and II and analgesia/antipyresis (2–4 g/day) and are well below the doses required for anti-inflammatory effects in rheumatic disorders (5–8 g/day). Aspirin thus may be beneficial in diabetic retinopathy through its antiplatelet effects. Such a possibility would be consistent with the fact that in human diabetes the retinal vessels show platelet-fibrin microthrombi (10), and in a clinical trial the selective antiplatelet agent ticlopidine reduced the progression of microaneurysms (11). However, the effects of ticlopidine were detected in only one subset of the patients studied (11) and were not confirmed in a subsequent trial (12). It is thus unclear whether antiplatelet agents can prevent diabetic retinopathy. Yet the information would have major clinical importance because the antiplatelet doses of aspirin are well tolerated on a long-term basis and are already indicated in diabetic patients for the primary prevention of cardiovascular disease.

We undertook the present study with the goal of ascertaining whether antiplatelet therapy is sufficient for the...
prevention of the ultimate and unequivocal histological vascular lesions of diabetic retinopathy. The study was performed in streptozotocin (STZ)-induced diabetic rats, which model human diabetic retinopathy conveniently and comprehensively. Diabetic rats show most features of human diabetic retinal microangiopathy (13), including microthrombosis (14,15) as well as the neuroglial abnormalities that are now known to occur early in the diabetic retina (16–18). The model thus permitted the evaluation of multiple relevant outcomes in response to the drugs tested. We compared and contrasted three drugs: clopidogrel, aspirin, and sorbinil. Clopidogrel was used as the reference antiplatelet agent given that aspirin, because of its multiple mechanisms of action (8,9), would affect other systems in addition to platelets. Clopidogrel, a thienopyridine derivative that is safer than ticlopidine, interferes with platelet aggregation by selectively and irreversibly inhibiting the binding of ADP to its receptor on platelets. It has no effects on COX I or II, works downstream of the site of aspirin action, and in clinical trials has been found to be superior to aspirin in the prevention of recurrent ischemic events (19). Aspirin, at doses comparable with those used in the canine study, was used to identify the effects of aspirin on diabetic retinopathy beyond those achieved by inhibition of platelet aggregation. Sorbinil, an aldose reductase inhibitor known from previous studies (13,17) to prevent the whole spectrum of neural, glial, and vascular abnormalities in diabetic rat retina, was used as the benchmark for the effect of the other treatments.

RESEARCH DESIGN AND METHODS

The procedures involving animals were approved by the Animal Care and Use Committee of the Schepens Eye Research Institute. Sprague-Dawley male rats were randomly assigned to one of the following groups: control, diabetic, diabetic treated with clopidogrel, or diabetic treated with aspirin. In experiments carried out for 2.5 months, a group of diabetic rats treated with sorbinil as well as control rats treated with clopidogrel, aspirin, or sorbinil were also included. Diabetes was induced with STZ (57.5 mg/kg body wt) dissolved in citrate buffer (pH 4.5) and injected via the tail vein. The development of diabetes (blood glucose >250 mg/dl) was verified 2 days after STZ injection. Body weight was recorded three times a week in the diabetic rats, and 2–4 units of NPH insulin were administered subcutaneously as needed to prevent weight loss without preventing hyperglycemia. Clopidogrel (SinoPharmSynthelabo, Toulouse, France) at 10 mg · kg⁻¹ · day⁻¹ (20), aspirin at 30 mg · kg⁻¹ · day⁻¹, or sorbinil (Pfizer, Groton, CT) at 65 mg · kg⁻¹ · day⁻¹ (17) was given to diabetic and control rats. Clopidogrel and aspirin were incorporated into pellets of the customary grain-based rodent diet (Bio-Serv, Frenchtown, NJ) with red and green food dye, respectively, to prevent incorrect delivery of treatment. Colored stools permitted precise verification of treatment assignment at each step of the experiments. Sorbinil was mixed into the powdered diet. The diabetic rats and age-matched controls were killed after the indicated duration of diabetes, and blood was obtained by cardiac puncture for the assay of glycohemoglobin (Glyc-Affin GHb assay; Dako, Carpenteria, CA) and goat anti-rat intercellular adhesion molecule 1 (ICAM-1; 1:200; R&D Systems, Minneapolis, MN). Negative controls were obtained by substituting the primary antibodies with an equivalent concentration of nonimmune IgG of the appropriate species.

Immunochemistry. Retinal cryosections (6 μm) were fixed in buffered formalin or ice-cold acetone for 10 min. Immunohistochemistry was performed as previously described (13). The primary antibodies were rabbit anti-cow glial fibrillary acidic protein (GFAP; 1:4,000; Dako, Carpenteria, CA) and goat anti-rat intercellular adhesion molecule 1 (ICAM-1; 1:200; R&D Systems, Minneapolis, MN). Negative controls were obtained by substituting the primary antibodies with an equivalent concentration of nonimmune IgG of the appropriate species.

Immunoblotting. Isolation of retinal proteins, SDS-PAGE, and immunoblotting were performed as previously described (13,24). GFAP and ICAM-1 were detected with the antibodies described above and CCAAT/enhancer-binding protein-β (CEBP-β) was detected with a rabbit anti-rat CEBP-β (Santa Cruz Chemical). The blots were homogenized by sonication in 100 μl EIA buffer to which 100 μmol lindomethacin had been added to inhibit COX. Ten microliters of the homogenate were used for the protein assay, and the remainder was extracted with acetone and further processed according to the EIA instructions. Purification of the samples was not required because different dilutions gave consistent results, differing by <20%. Recovery experiments yielded 100 ± 9% when PGE2 standards were added to control samples and 100 ± 12% when they were added to diabetic samples (n = 6 for each).

Statistical analysis. Data are given as means ± SD or as median and range for noncontinuous variables such as scores and counts. The multiple treatment groups were compared with ANOVA followed by Fisher’s protected least significant differences test or with the nonparametric Kruskal-Wallis test followed by multiple comparisons with the Mann-Whitney test when the results were noncontinuous variables.

RESULTS

The doses of clopidogrel and aspirin used have anti-platelet effects. In rats with 11 months of diabetes, ADP-induced platelet aggregation did not differ from that observed in control rats but was significantly impaired by clopidogrel in all but one of the rats tested (Fig. 1A and B).

Initial dose-response studies on the antiplatelet effect of aspirin performed in control rats (Fig. 1C) showed that aspirin incorporated into diet pellets to yield the very
small dosage of 1.5 mg·kg⁻¹·day⁻¹ did not inhibit TXB₂ formation significantly ($P = 0.08$) in contrast to the 90% inhibition observed in humans treated with such a dosage (22). The ~90% inhibition of TXB₂ formation was achieved in rats treated with aspirin at the dosage of 30 mg·kg⁻¹·day⁻¹, the dosage that was thus chosen for the experiments. The serum levels of salicylate in rats treated with 30 mg·kg⁻¹·day⁻¹ aspirin in pellets were consistently <100 mg/l (0.6 mmol/l), lower than those (1–5 mmol/l) required for anti-inflammatory effects in rheumatic disorders (8,9). TXB₂ levels were similar in control rats and rats with 2.5 months of diabetes, and TXB₂ formation was significantly decreased by aspirin treatment for 2.5 months in both groups (Fig. 1D). Among the diabetic rats treated with aspirin for 8 or 11 months, ~30% failed to show an effect of aspirin on TXB₂ formation.

None of the drugs tested altered the severity of the diabetic state as measured by body weight and glycohemoglobin (Table 1). Cataract formation was present in 75% of rats after 2.5 months of diabetes and was completely prevented by sorbinil but not by clopidogrel (present in 69%) or aspirin (present in 53%).

**Neither clopidogrel nor aspirin prevents early neuroglial abnormalities in the diabetic retina (2.5 months of diabetes).** Apoptosis of retinal ganglion cells (16) and reactivity of Müller glial cells (18) could be caused by ischemia (25,26). We thus tested to see if these processes could be prevented by antiplatelet therapy. Neither clopidogrel nor aspirin prevented the neuronal apoptosis induced by diabetes, whereas the dose of sorbinil that completely suppresses polyol pathway activity in the diabetic rat retina (17) did prevent apoptosis (Fig. 2A). Similarly, among the drugs tested, only sorbinil prevented the diabetes-induced changes in the topography and level of expression of GFAP. GFAP is a cytoskeletal protein normally expressed by astrocytes in the innermost retina, but it is induced in the Müller cells by retinal injury or disease and thus is used as an index of retinal response to damage. The retina of diabetic rats showed GFAP immunoreactivity in the Müller cell processes spanning the

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<td>Weight and glycohemoglobin of control rats and diabetic rats treated or not with drugs, by duration of diabetes</td>
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<td>n</td>
<td>Body weight (g)</td>
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<td>310 ± 51*</td>
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<td>Diabetic treated with aspirin</td>
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<td>14.9 ± 2.4*</td>
<td>13</td>
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<tr>
<td>Diabetic treated with sorbinil</td>
<td>12</td>
<td>318 ± 58*</td>
<td>14.0 ± 2.7*</td>
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Data are means ± SD. *$P < 0.001$ vs. control rats.
thickness of the retina (Fig. 2B) and increased retinal GFAP levels (Fig. 2C and D). The GFAP abnormalities were not prevented by clopidogrel or aspirin.

Control rats treated over the same 2.5-month period with clopidogrel, aspirin, or sorbinil showed no effects on body weight, glycohemoglobin, number of apoptotic retinal neurons, or GFAP levels when compared with untreated control rats.

**Aspirin, but not clopidogrel, prevents microvascular cell apoptosis and acellular capillaries (8–11 months of diabetes).** Apoptosis of retinal capillary pericytes and endothelial cells could be caused by microthrombosis and transient ischemia (10). In the retina of rats with 8 or 11 months of diabetes, capillary cells labeled with the TUNEL reaction were significantly more frequent than in control rats. Aspirin, but not clopidogrel, prevented the apoptosis of capillary cells induced by diabetes (Fig. 3A and B). Apoptosis is expected to irreversibly decrease the number of pericytes because pericytes do not replicate. When we counted the number of pericyte nuclei, the trend was the mirror image of the results for TUNEL-positive cells, which confirmed our observation that pericyte loss is prevented by aspirin but not by clopidogrel (13 [range 12–15] pericyte nuclei/mm capillary length in control rats, 10 [9–12] in diabetic rats, 14 [13–15] in diabetic rats treated with aspirin, and 10 [9–12] in diabetic rats treated with clopidogrel; n = 3 in each group). The number of acellular capillaries counted throughout the retina (Fig. 3C) had tripled in the diabetic rats when compared with age-matched control rats. Aspirin almost completely prevented the development of acellular capillaries, whereas clopidogrel had no effect (Fig. 3D).

**Seeking mechanisms for the effects of aspirin: measurements of ICAM-1 levels, COX activity, and C/EBP-β levels in diabetic retina (2.5–8 months of diabetes).** It has been proposed that increased ICAM-1 levels are responsible for the occlusion of retinal capillaries induced by diabetes (27), and it has been reported that large doses of aspirin (equivalent to 3.5 g/day in a 70-kg person) reduce ICAM-1...
levels and leukostasis in rats with uncontrolled diabetes of acute onset (28). Rats with 2.5 months of diabetes manifested increased levels of retinal ICAM-1 when compared with nondiabetic controls (Fig. 4A and B). The increase was not prevented by the dose of aspirin used in our study, which did however partially reduce ICAM-1 levels given that they were no longer statistically different from those of controls. Clopidogrel had no effect and sorbinil achieved complete prevention.

We also investigated the topography of the increased ICAM-1 in the retinas of diabetic rats, which has never been reported. In the retina of control rats, ICAM-1 immunoreactivity was faint and almost exclusively localized to the wall of large blood vessels, whereas in diabetic rats it became prominent along the inner limiting membrane and also around the wall of large vessels, where it co-localized with GFAP (Fig. 4C). The pattern indicates upregulation of ICAM-1 expression in the processes of Müller cells and astrocytes.

Another approach to determining the mechanisms by which aspirin protects retinal vessels in diabetes is to look at the effects of aspirin, other than its antiplatelet effects, that are exerted at the low-intermediate circulating concentrations achieved in our diabetic rats. Three such effects have been reported: inhibition of COX activity (29), inhibition of C/EBP-β activation (30), and protection of endothelial cells from oxidative stress (31). To test COX activity, we measured the levels of PGE₂, a major prostaglandin responsible for most features of inflammation in other tissues (32). In rats with 4.5 or 8 months of diabetes, retinal PGE₂ levels were low and similar to those of nondiabetic controls (Fig. 5). This indicated that COX activity throughout the retina was not increased in diabetes. The second effect of aspirin that can be expected at low concentrations is inhibition of the activation of C/EBP-β (30), a transcription factor that in different tissues regulates the expression of genes involved in energy metabolism, differentiation, and inflammation (33). Because C/EBP-β activity is also regulated transcriptionally (33), we measured the levels of the transcription factor. In rats with 6 months of diabetes, there was a small but significant increase in the levels of retinal C/EBP-β (Fig. 6), indicating that this factor is a potential relevant target for the action of aspirin in diabetic retinopathy.

**DISCUSSION**

Our observation that both aspirin and clopidogrel inhibit platelet aggregation, but that only pleiotropic aspirin protects retinal vessels, indicates that in the STZ-induced diabetic rat, selective antiplatelet therapy was not sufficient to prevent retinal microangiopathy and that the beneficial effect of aspirin was exerted through mechanisms other than inhibition of thrombosis. Aspirin, but not clopidogrel, prevented capillary cell apoptosis, which is known from previous work to precede (23) and predict (34) the development of acellular capillaries. The present observations were yet another example where the re-
response of capillary cell apoptosis to interventions was concordant with the response of acellular capillaries (34), thus furthering the concept that apoptosis has a causal role in capillary obliteration. Vascular cell apoptosis may thus represent the critical event prevented by aspirin.

Aspirin protected the retinal vessels without preventing neuronal apoptosis, Müller cell overexpression of GFAP, and increased levels of ICAM-1 (although it lowered the levels slightly). The increased ICAM-1 in the diabetic rat retina appears to be a glial abnormality, both on account of its topography (this study) and an earlier finding of increased ICAM-1 mRNA levels in Müller cells isolated from diabetic rats (35). It is possible that aspirin did not reach neurons and glia in concentrations comparable with those available to vascular endothelial cells. Aspirin and salicylate (into which aspirin is rapidly hydrolyzed) are

![Image](image1.png)

**FIG. 4.** ICAM-1 in the retina of rats with 2.5 months of diabetes. A: Representative immunoblot of retinal ICAM-1. B: Quantitation of the signals from immunoblots of retinal ICAM-1. C: ICAM-1 and GFAP immunostaining of retinal sections. The increased ICAM-1 staining in diabetic retinas overlaps with structures staining for GFAP. ILM, internal limiting membrane (constituted by the foot processes of Müller cells). Large vessels are indicated by arrows. The negative controls were performed on sections from the same diabetic rat for which retinal GFAP and ICAM-1 are shown. Bar = 50 μm. ASA, aspirin; C, control rats; Clop, clopidogrel; D, diabetic rats; INL, inner nuclear layer; ONL, outer nuclear layer; Sorb, sorbinil. *P < 0.03 vs. control rats; **P < 0.02 vs. diabetic rats.

![Image](image2.png)

**FIG. 5.** PGE₂ levels in the retina of rats with 4.5 or 8 months of diabetes. PGE₂ levels were measured by competitive enzyme immunoassay.

![Image](image3.png)

**FIG. 6.** C/EBP-β levels in the retina of rats with 6 months of diabetes. A: Representative immunoblot of retinal C/EBP-β showing a band with an apparent molecular weight of 45 kDa; +, positive control (nuclear extract from rat K-Ras -transformed kidney cells; Santa Cruz). C1–C4, four different control rats; D1–D4, four different diabetic rats. B: Signals from immunoblots of retinal C/EBP-β in individual rats showing upward shift of values in the diabetic rats. Arrowheads point to the mean. C, control rats; D, diabetic rats. *P = 0.05 vs. control rats.
>80% protein bound at low plasma concentrations (8), and the volume of distribution reached in our experiments may have been low and not included retinal neurons and glia. On the other hand, the cytoplasm of retinal endothelial cells is itself beyond several mechanisms of the blood-retinal barrier (36), and we have at present no way of telling if it may be more accessible than neural and glial cells. An additional possibility is that the retinal neuroglial abnormalities induced by diabetes are caused by mechanisms insensitive to the actions of aspirin. Indeed, the abnormalities were all prevented by sorbinil, indicating a causative role of the polyol pathway. Because sorbinil also prevents capillary cell apoptosis and acellular vessels (13), this may mean that there are redundant or overlapping pathways for the late capillary lesions that are susceptible to diverse interventions, whereas only one pathway (the polyol pathway) is operative in causing the neuroglial abnormalities.

Although the rescue of retinal capillaries did not require inhibition of neuroglial abnormalities, our data do not exclude a possible contribution of the latter to vascular lesions. Aspirin may have acted downstream of events initiated by the neuroglial abnormalities and/or prevented Müller cell changes (35) other than GFAP and ICAM-1 overexpression that may have been pathogenic for vessels and that we did not measure. One mechanism by which the rescue of retinal capillaries by aspirin does not occur is the prevention of ICAM-1 overexpression in retinal vessels, given that no ICAM-1 overexpression was detected in isolated preparations of diabetic retinal vessels (C.G., Z.D., M.L., unpublished observations).

The preventative effects of aspirin on capillary obliteration occurred at plasma concentrations of salicylate below those (1–5 mmol/L) required in humans for anti-inflammatory activity in rheumatic disorders (8,9) or for correction of metabolic abnormalities in type 2 diabetes (37). These larger doses or concentrations inhibit the activity of the serine kinase IkB kinase-β and thus the activation of nuclear factor-κB (NF-κB) and the proinflammatory program that the transcription factor can elicit (38). NF-κB activation does occur in retinal vascular cells in diabetes, although it is uncertain whether it occurs only in pericytes (39) or also in endothelial cells (40) and what the consequences of this are. Because NF-κB inhibition requires high doses of aspirin also in rodents (41), we anticipate that the aspirin treatment of our rats did not work through inhibition of IkB kinase-β and NF-κB. Similarly, we anticipate that the treatment did not work through inhibition of other cellular kinases, also requiring concentrations of aspirin and salicylate in the millimolar range (29).

We sought to identify relevant targets for the low-intermediate concentrations of aspirin that prevented retinal microangiopathy in diabetic rats. COX activity is a candidate for this, but we found no evidence for increased levels of prostaglandins in the retina of rats with various durations of diabetes. Our data on unchanged retinal PGE2 levels are in agreement with previous findings in diabetic rat retina (42) and in other diabetic tissues (43); we currently do not have an explanation for the discrepant findings by Du et al. (44). A second action of aspirin exerted at micromolar concentrations is the inhibition of ribosomal protein S6 kinase, which phosphorylates and activates the transcription factor C/EBP-β (30). C/EBP-β is involved, in a complex fashion, in multiple cellular activities, including inflammatory responses (33). Aspirin could inhibit C/EBP-β phosphorylation in the diabetic retina and thus work by limiting the consequences of the small increase in the level of the transcription factor induced by diabetes. Increased levels of C/EBP-β have also been reported in the kidney of diabetic rats (45). Rigorous understanding of aspirin’s action in diabetic retinopathy will eventually require ascertainment of COX and C/EBP-β activities, specifically in retinal vessels. A third action of aspirin exerted at low concentrations is protection of endothelial cells from oxidative stress (31), an effect that would also be relevant in diabetes.

The observations made in this study are a bridge to translational projects. The prevention of diabetic retinopathy is a long-term goal, and it can already be approached with good glycemic control. Hence, if drugs are to be used successfully in a prevention regimen, they must be safe. Antiplatelet therapy is well tolerated, but this study in rats indicates that it is not sufficient to prevent the hallmark histopathology of diabetic retinopathy. A limitation of our study is that we do not know if the thrombotic contributions to diabetic vascular disease and the response to antiplatelet drugs are precisely the same in rats and humans. The rat is, however, a model for the diabetes-induced proneness to microthrombosis (14,15,46,47) and is widely used in preclinical studies of antiplatelet drugs (see PubMed citations under “antiplatelet therapy in rats” or “aspirin in rats”). The poor success of clopidogrel in rat diabetic retinopathy does resonate with the inconsistent results obtained with selective antiplatelet agents in human trials for retinopathy (11,12). The intriguing implication of our study is that the documented beneficial effects of aspirin on human diabetic retinopathy (3), although probably not due to antithrombotic effects, may require less that the previously tested 990 mg/day, which did have gastrointestinal side effects (3). We should seek to precisely identify the mechanisms through which aspirin exerts its protective effect on diabetic retinal vessels so that we can learn about the lowest efficacious dose of aspirin (or salicylate) in rodents and humans.

The only drug that, to date and to our knowledge, has proven capable of preventing the whole spectrum of neural, glial, and vascular abnormalities of retinopathy in the diabetic rat is an aldose reductase inhibitor (13,17). Drugs capable of comprehensive prevention may ultimately be the most desirable, and it is thus encouraging that new aldose reductase inhibitors (48–50) are being tested for clinical safety and efficacy. As an alternative or additional strategy, aspirin could be used to provide protection to the retinal vessels if it were found to be active at low, safe concentrations.

ACKNOWLEDGMENTS

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Measurements of serum salicylate performed with improved sensitivity in additional diabetic rats treated with 30 mg · kg⁻¹ · day⁻¹ aspirin yielded values of 0.08 ± 0.04 mmol/l.

### REFERENCES


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