Original Article

Inhibition of Caspase-1/Interleukin-1β Signaling Prevents Degeneration of Retinal Capillaries in Diabetes and Galactosemia

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The proinflammatory cytokine, interleukin (IL)-1β, is known to induce vascular dysfunction and cell death. We investigated the role of IL-1β and caspase-1 (the enzyme that produces it) in diabetes-induced degeneration of retinal capillaries. Caspase-1 activity is increased in retinas of diabetic and galactosemic mice and diabetic patients. First, we investigated the effect of agents known to inhibit caspase-1 (minocycline and tetracycline) on IL-1β production and retinal capillary degeneration in diabetic and galactose-fed mice. Second, we examined the effect of genetic deletion of the IL-1β receptor on diabetes-induced caspase activities and retinal capillary degeneration. Diabetic and galactose-fed mice were injected intraperitoneally with minocycline or tetracycline (5 mg/kg). At 2 months of diabetes, minocycline inhibited hyperglycemia-induced caspase-1 activity and IL-1β production in the retina. Long-term administration of minocycline prevented retinal capillary degeneration in diabetic (6 months) and galactose-fed (13 months) mice. Tetracycline inhibited hyperglycemia-induced caspase-1 activity in vitro but not in vivo. Mice deficient in the IL-1β receptor were protected from diabetes-induced caspase activation and retinal pathology at 7 months of diabetes. These results indicate that the caspase-1/IL-1β signaling pathway plays an important role in diabetes-induced retinal pathology, and its inhibition might represent a new strategy to inhibit capillary degeneration in diabetic retinopathy. *Diabetes* 56:224–230, 2007

Diabetes and experimental galactosemia (another model that develops a diabetic-like retinopathy) lead to the development of acellular (nonperfused) capillaries, pericyte ghosts, foci of vessel sudanophilia, and capillary basement membrane thickening, i.e., lesions characteristic of the early stages of diabetic retinopathy in both rats and mice (1–3). Accelerated death of retinal capillary cells likely contributes to the development of acellular nonperfused capillaries in diabetic retinopathy and, if extensive, to retinal neovascularization.

Caspases, a family of 14 known cysteine proteases, participate in one of the two distinct signaling pathways: *1*) activation of proinflammatory cytokines and *2*) promotion of apoptotic cell death (4). The members of the caspase-1 family seem to be an exception among the caspase families, as they are involved both in the production of active cytokines and in apoptosis. Caspase-1 produces interleukin (IL)-1β and IL-18 (interferon γ–inducing factor), explaining its original name of IL-1β–converting enzyme (ICE) (5). The role of caspase-1 in apoptosis is controversial. To date, no detailed study has been done to determine if active caspase-1 is sufficient for apoptosis induction or if caspase-1–induced apoptosis is a consequence of caspase-1–dependent cytokine production. Inflammation and apoptosis, including the activation of caspase-1 and its subsequent cytokines, are common features in several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (6–8) as well as in ischemic events in the brain (9,10). Inhibition of caspase-1 has been shown to be beneficial in at least slowing down the progression of these diseases or decreasing the size of damaged areas (11,12).

Our previous studies revealed that caspase-1 becomes activated in the retinas of diabetic and galactosemic mice, diabetic patients, and retinal Müller cells incubated in elevated concentrations of glucose (13,14). These data suggest that activation of this caspase subfamily might play an important role in the formation of diabetic retinopathy, since activation of caspase-1 seemed to be a common event in hyperglycemic conditions. Previously, we have also shown that minocycline was able to prevent diabetes-induced activation of caspase-1 in the early phase of diabetic retinopathy (14), thus offering one means to investigate the role of the caspase-1 family in the development of the retinopathy.

Minocycline is a member of the group of second-generation chemically modified tetracycline (15) and exerts pleiotropic actions including anti-inflammatory effects distinct from its antimicrobial action (16,17). Minocycline itself has neuroprotective qualities in models of cerebral ischemia, traumatic brain injuries, Huntington's disease, Parkinson's disease, and amyothrophic lateral sclerosis in mice (17–20). It has been speculated that its neuroprotective action is mediated by the inhibition of caspase-1 activation, subsequent inhibition of caspase-3, and iNOS transcriptional upregulation and activation (18,21). Recent studies have demonstrated that minocycline inhibits acti-

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IL, interleukin; WT, wild-type.

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vation of retinal microglia induced either by lipopolysaccharide or diabetes (22,23). Minocycline also prevented early caspase-3 activity and neuronal apoptosis in retinas of diabetic rats (22). The study, however, did not address effects of minocycline on diabetes-induced degeneration of retinal capillaries, a critical component of diabetic retinopathy. Other studies in diabetic rats have shown that minocycline inhibits metalloproteinases, depression of skin collagenase, and tooth loss (24,25).

In this study, we investigated the role of the caspase-1/IL-1β signaling pathway in the degeneration of retinal capillaries, one of the most fundamental lesions of diabetic retinopathy, using two different therapeutic approaches (minocycline and genetic deletion of the IL-1β receptor) and two different models of diabetic retinopathy (streptozotocin-induced diabetes and experimental galactosemia).

RESEARCH DESIGN AND METHODS

Minocycline and 7-amino-4-trifluoro-methylcoumarin (AFC) were purchased from Sigma (St. Louis, MO). Caspase substrates were purchased from Calbiochem (San Diego, CA). IL-1β enzyme-linked immunosorbent assays were obtained from Pierce/Endogen (Rockford, IL).

Animal models. Male mice (C57BL/6) weighing 20 g were randomly assigned to be made diabetic, galactosemic, or left as normal controls. Diabetes was induced by streptozotocin injections (60 mg/kg body wt i.p. on 5 consecutive days) as described previously (13). Insulin was given to diabetic animals as needed to achieve slow weight gain without preventing hyperglycemia and glycosuria (0.1–0.2 units of NPH insulin subcutaneously, two to three times a week). Galactosemia was induced by feeding normal mice a diet enriched with 30% galactose. Animals were caged in pairs, had free access to food and water, and were maintained under a 14 h on/10 h off light cycle. Treatment of animals conforms to the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research. Diabetic animals with fasted blood glucose levels initially >300 mg/dl were used for the studies. The severity of blood hexose elevation was estimated by measuring the level of nonenzymatically glycated hemoglobin (GHb) using affinity chromatography (Glyc-Affin; Pierce, Rockford, IL).

IL-1R1 knockout mice (Jackson Laboratories; strain name: B6.129S7-Il1r1^{tm1Jmx} in a C57BL/6J background) were bred using homozygous breeding pairs. Male mice (C57BL/6) and IL-1R1 knockout mice weighing 20 g were randomly assigned to be made diabetic as described above or to remain as controls.

Minocycline studies. Minocycline was administered intraperitoneally (5 mg/kg) three times or seven times a week for 2 months (short-term study) or three times a week for 6 months (long-term study). Inhibitor therapy was initiated 1 week after induction of diabetes to minimize the possibility that administration of the inhibitor might inhibit the severity of islet damage and thus the severity of diabetes. For comparison, tetracycline (5 mg/kg) was administered to diabetic animals using the same protocol as described for minocycline. After 2 months' duration of diabetes, animals were killed and retinas were isolated. Retinal lysates were used for caspase activity and cytokine enzyme-linked immunosorbent assays.

For long-term studies using the streptozotocin or galactosemia model, minocycline was injected intraperitoneally (5 mg/kg) three times a week. At 6 months of diabetes or 13 months of galactosemia, respectively, animals were killed to determine retinal pathology.

Tissue culture experiments. The retinal Müller cell line rMC-1, a glia-like cell type, was obtained from Dr. V.R. Sarthy (Northwestern University, Evanston, IL) (26). rMC-1 cells (4×10^6 cells) were grown in 100-mm Petri dishes in 10 ml Dulbecco's modified Eagle's medium (5 mmol/l glucose) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (growth medium) at 37°C/5% CO_2 overnight. The following day, medium was replaced by 10 ml Dulbecco's modified Eagle's medium containing 2% fetal bovine serum, 1% penicillin/streptomycin, and either 5 mmol/l glucose, 25 mmol/l glucose, 25 mmol/l glucose plus minocycline (100 μmol/l), 25 mmol/l glucose plus tetracycline (100 μmol/l) or 25 mmol/l glucose plus IL-1β neutralizing antibody (10 μg/ml) (treatment media). Cells treated with 5 mmol/l glucose served as controls. Treatment medium was changed every day. At 96 h of incubation in treatment medium, rMC-1 cells were lysed and caspase activities were measured.

Preparation of retinal and cell lysates. Retinas or rMC-1 cells were lysed in 200 μl lysate buffer [100 mmol/l HEPES, pH 7.5, containing 10% sucrose, 0.1% 3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate (CHAPS), and the general protease inhibitors EDTA (1 mmol/l), phenylmethylsulfonyl fluoride (1 mmol/l), and leupeptin (10 μg/ml)], sonicated for 10 s, and centrifuged at 9,000*g* for 5 min at 4°C, followed by protein measurement of the supernatant. Supernatants were retained for experiments.

Caspase activity assay. Caspase activities were measured as described previously (13,27). Briefly, equal amounts of retinal lysates or cell lysates (15 μg) were incubated in lysate buffer containing the fluorogenic caspase substrate (2.5 μmol/l) in a total volume of 100 μl at 32°C for 1 h. Cleavage of the substrate emits a fluorescence signal that was quantified by a Tecan Spectra FluorPlus fluorescence plate reader (excitation: 400 nm; emission: 505 nm).

Interleukin-1β enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assays were performed using kits from Endogen for mouse IL-1β according to the manufacturer's instructions. A total of 50 μl retinal lysates was added to precoated 96-well plates followed by the addition of 50 μl biotinylated antibody reagent for 2 h. Plates were washed, and 100 μl streptavidin–horseradish peroxidase solution was added for 30 min. A total of 100 μl 3,3′5,5′-tetramethylbenzidine base substrate solution was added and enzymatic reaction was allowed to develop for 30 min at room temperature in the dark. Then 100 μl of stopping solution was added. Absorbance was measured at 450 and 550 nm using a Tecan Spectra FluorPlus plate reader. Values were compared with a standard curve and normalized to milligrams of total protein.

Histological assessment of retinal vascular pathology. The retinal vasculature was isolated by the trypsin digest technique as reported previously by us after 5 days of formalin fixation (3). Briefly, isolated retinas were washed in running water overnight and then digested in 3% crude trypsin solution containing 0.2 mol/l NaFl for ~90 min. Retinas were gently brushed to dislodge neural elements. The cleaned vessel network was rinsed three times in pure clean water, dried onto a mounting slide, stained with hematoxylin and periodic acid (0.5%), dehydrated, and covered with coverslip. Acellular capillaries were quantitated in four to seven field areas in the mid-retina (200× magnification) in a masked manner.

Statistical analysis. Data were analyzed using one-way ANOVA (correlated samples, $P < 0.05$) followed by Tukey's post-analysis to determine statistical significance among groups. Ordinal data were analyzed using Kruskal-Wallis ($P < 0.05$) followed by Dunn's post-analysis to determine statistical significance among groups.

RESULTS

Minocycline inhibits caspase-1 activity and production of IL-1β in the retina of diabetic mice. We have previously shown that diabetes induced caspase-1 activation in retinas of diabetic and galactose-fed mice and retinal Müller cells, suggesting that caspase-1 might play a role in the formation of pathology in diabetic retinopathy (13,14). We tested the effect of minocycline, a potential inhibitor of caspase-1 activation, on diabetes-induced caspase-1 activity and IL-1β production in the retina of diabetic mice. One week after diabetes induction, minocycline was injected intraperitoneally either three times or seven times per week at a concentration of 5 mg/kg body wt. Administration of the drug did not alter glycemia, as indicated by GHb levels throughout the experiments (3.3 ± 0.3 for normal control animals, 12.6 ± 1.3 for diabetic, and 12.3 ± 1.3 and 12.2 ± 1.8 for diabetic animals injected with minocycline either three times or seven times per week, respectively). At 2 months, retinas of normal, diabetic, diabetic + minocycline (three times per week), and diabetic + minocycline (seven times per week) C57BL/6 mice were isolated, and caspase-1 activity of retinal lysates was measured. Figure 1 shows that minocycline inhibited caspase-1 activity in the retina of diabetic mice. Both administration regimes strongly inhibited caspase-1 activity by 74 and 69%, respectively. Minocycline also inhibited the activities of caspase-2 (45%), -6 (87%), and -8 (54%) at 2 months of diabetes (data not shown).

Caspase-1 is the enzyme responsible for the production of mature IL-1β. Diabetes increased IL-1β levels from undetectable levels in retinas of normal animals to 39.0 ±

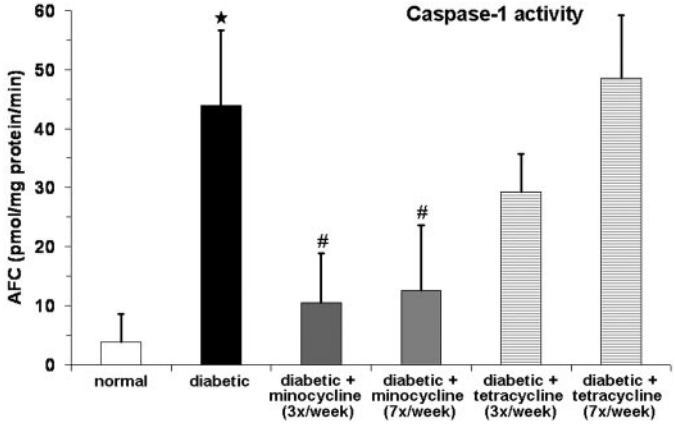

FIG. 1. Inhibition of diabetes-induced caspase-1 activity in the retina by minocycline. Retinas of diabetic (n = 12), normal (n = 10), and diabetic mice injected with minocycline (5 mg/kg body wt i.p.) either three times (n = 11 minocycline, n = 8 tetracycline) or seven times (n = 7 minocycline, n = 9 tetracycline) per week for 2 months were isolated, and caspase-1 activity was measured. Caspase-1 activity is presented as pmol 7-amino-4-trifluoro-methylcoumarin (AFC)/mg protein/min (means ± SD). ⋆P < 0.05 compared with normal; #P < 0.05 significantly different from diabetic.

1.6 pg · ml^{-1} · mg^{-1} protein in retinas of diabetic animals (Fig. 2). Treatment with minocycline significantly reduced IL-1β levels to 16.0 ± 0.9 pg · ml^{-1} · mg^{-1} protein (three times per week) and to 12.0 ± 0.4 pg · ml^{-1} · mg^{-1} protein (seven times per week).

Tetracycline, a related compound of this class of antibiotics differing only in the ability to cross the blood-brain (and presumably blood-retinal) barrier, had no comparable ability to inhibit caspase-1 activity or IL-1β production in vivo (see Figs. 1 and 2).

Minocycline inhibits the formation of acellular capillaries in the retina of diabetic and galactosemic mice. The short-term study (2 months) showed that minocycline injected intraperitoneally three times per week at a concentration of 5 mg/kg body wt effectively inhibited diabetes-induced caspase-1 activity in the retinas of diabetic mice. Based on this result, we initiated a long-term study using three experimental groups (normal, diabetic, and diabetic + minocycline [three times per week] mice). Administration of the drug again had no effect on glycemia. After 6 months of diabetes, retinal vasculatures were isolated, and the number of acellular capillaries/mm^2 retina was counted. Figure 3 shows that diabetes significantly increased the number of acellular capillaries/mm^2 by 2.2-fold compared with normal, and minocycline inhibited the diabetes-induced increase in numbers of acellular capillaries/mm^2 by 84%.

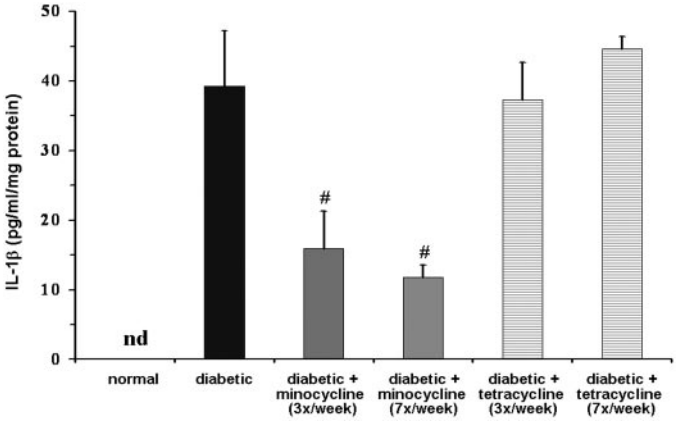

FIG. 2. Inhibition of diabetes-induced IL-1β in the retina by minocycline. IL-1β production was measured from same retinal lysates as described in Fig. 1. IL-1β levels are expressed as the mean ± SD in pg · ml^{-1} · mg^{-1} protein. #P < 0.05 compared with diabetic.

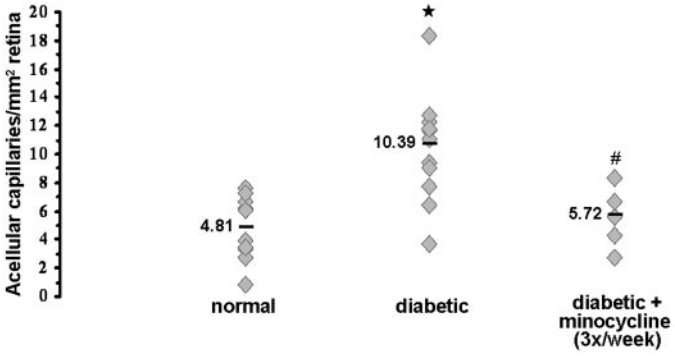

FIG. 3. Minocycline inhibits diabetes-induced degeneration of retinal capillaries. The retinal vasculature of normal (n = 12), diabetic (n = 11), and diabetic mice injected with minocycline (5 mg/kg body wt i.p.) (n = 6) three times per week was isolated (6 months duration of diabetes), and acellular capillaries were counted (mean ± SD). ⋆P < 0.02 compared with normal; #P < 0.05 compared with diabetic.

Galactosemia, another model that leads to diabetic-like retinopathy, strongly induces caspase-1 activity as previously demonstrated by us (13). To test if blocking caspase-1 activation is also beneficial in the galactose model of retinopathy, galactosemic animals were injected with minocycline three times per week at a concentration of 5 mg/kg for 13 months. Figure 4 shows that galactosemia significantly increased the number of acellular capillaries/mm^2 retina by 2.8-fold compared with normal, and minocycline significantly inhibited galactosemia-induced formation of acellular capillaries/mm^2 by 69% in this model as well.

High glucose–induced caspase-1 activation and subsequent apoptosis in retinal Müller cells is suppressed by minocycline. Müller cells, an important retinal cell type, become altered in diabetes and are postulated to play a role in the development of diabetic retinopathy. Diabetes-like concentrations of glucose have been shown to induce the activation of caspase-1 and

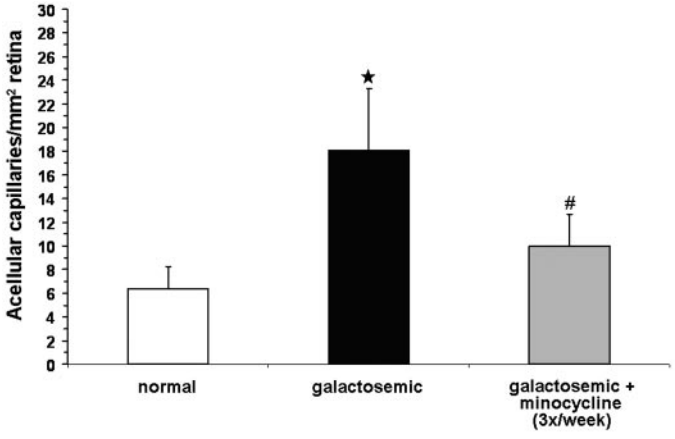

FIG. 4. Minocycline inhibits galactose-induced retinal pathology. Retinas of normal mice (n = 10), galactosemic mice (n = 12), and galactosemic mice injected with minocycline (5 mg/kg body wt i.p.) (n = 15) three times per week were isolated (13 months' duration) and acellular capillaries were counted. ⋆P < 0.02 compared with normal; #P < 0.05 compared with galactosemia.

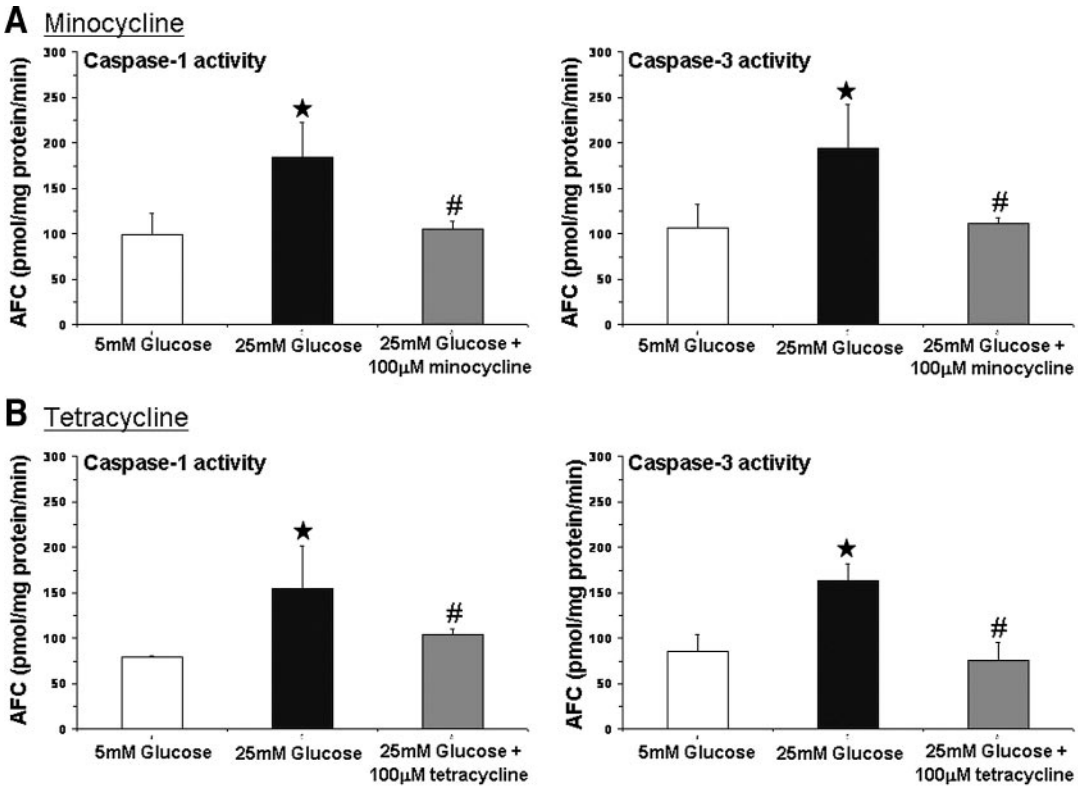

FIG. 5. Inhibition of high glucose–induced caspase activities in retinal Müller cells by minocycline: rMC-1 cells were incubated in medium containing 5 mmol/l glucose, 25 mmol/l glucose, 25 mmol/l glucose plus 100 μmol/l minocycline (*A*), or 25 mmol/l glucose plus 100 μmol/l tetracycline (*B*). After 96 h, activities of caspase-1 and caspase-3 were measured and expressed as means ± SD. ⋆*P* < 0.05 compared with normal glucose; #*P* < 0.05 compared with high glucose.

caspase-3, as well as apoptosis in retinal Müller cells (13,28–30). Therefore, we tested the effect of minocycline and tetracycline on high glucose–induced caspase-1 activation and apoptosis in retinal Müller cells in vitro. rMC-1 cells were incubated in medium containing 25 mmol/l glucose in the presence or absence of 100 μmol/l minocycline or 100 μmol/l tetracycline for 96 h and compared with rMC-1 cells incubated in medium containing 5 mmol/l glucose. Activities of caspase-1 and caspase-3, an apoptosis executioner caspase, were increased in high glucose, and minocycline completely inhibited the high glucose–induced increase in caspase-1 and caspase-3 activities by 93 and 94%, respectively (Fig. 5*A*). To further demonstrate that 100 μmol/l minocycline prevented high glucose–induced apoptosis in Müller cells, we tested for apoptosis using Annexin V staining. Minocycline significantly inhibited the number of Annexin-positive cells by 70% compared with cells treated in high glucose (data not shown).

In contrast to the poor effectiveness of tetracycline in vivo, in the in vitro studies, tetracycline did inhibit caspase-1 and caspase-3 activity. As Fig. 5*B* shows, 100 μmol/l tetracycline significantly inhibited high glucose–induced activity of caspase-1 by 67% and activity of caspase-3 by 99%.

Inhibition of IL-1β action prevents high glucose–induced caspase-3 activation in retinal Müller cells. Caspase-1 activation predominantly leads to the formation of mature IL-1β but has also been associated with the induction of apoptotic pathways by direct activation of caspase-3. To identify whether high glucose–induced apoptosis is a consequence of increased IL-1β production rather than direct activation of an apoptotic pathway by caspase-1, rMC-1 cells were treated with IL-1β neutralizing antibody. After 96 h of treatment in 5 mmol/l glucose, 25 mmol/l glucose, and 25 mmol/l glucose plus 10 μg/ml IL-1β neutralizing antibody, caspase-1 and caspase-3 activities were measured. Figure 6 shows that IL-1β neutralizing antibody significantly attenuated high glucose–induced activity of caspase-3 by 68 ± 22%, indicating that high glucose–induced apoptosis of retinal Müller cells is a consequence of increased autocrine production of IL-1β by these cells. Interestingly, although caspase-1 is responsible for the production of IL-1β and is therefore upstream, treatment with IL-1β neutralizing antibody also significantly inhibited caspase-1 activity by 83 ± 12% at 96 h of high glucose incubation of rMC-1 cells.

Diabetes does not induce caspase activation and capillary degeneration in IL-1R1 knockout mice. The action of IL-1β is mostly mediated via the binding to its specific IL-1R receptor, also known as the type I receptor. To further establish the role of IL-1β in formations of lesions in diabetic retinopathy, we examined whether inhibition of IL-1β signaling using IL-1R1 receptor knockout mice (IL-1R1$^{-/-}$ mice) prevents diabetes-induced caspase activation and retinal capillary degeneration in the retina of diabetic mice. Wild-type (WT) C57Bl6 mice and IL-1R1$^{-/-}$ mice were made diabetic using streptozotocin, and untreated WT and IL-1R1$^{-/-}$ mice served as the control. GHb values for the different experimental groups demonstrated that this genetic modification did not alter the severity of diabetes (4.4 ± 0.8 for WT and 3.7 ± 0.7 for normal IL-1R1$^{-/-}$ animals, 13.2 ± 0.8 for WT diabetic and 9.8 ± 1.1 for diabetic IL-1R1$^{-/-}$ animals). At 5 months of diabetes, diabetes significantly increased the activities of the initiator caspases-1 and -8 as well as the activities of executioner caspases-3 and -6. Caspase activities were

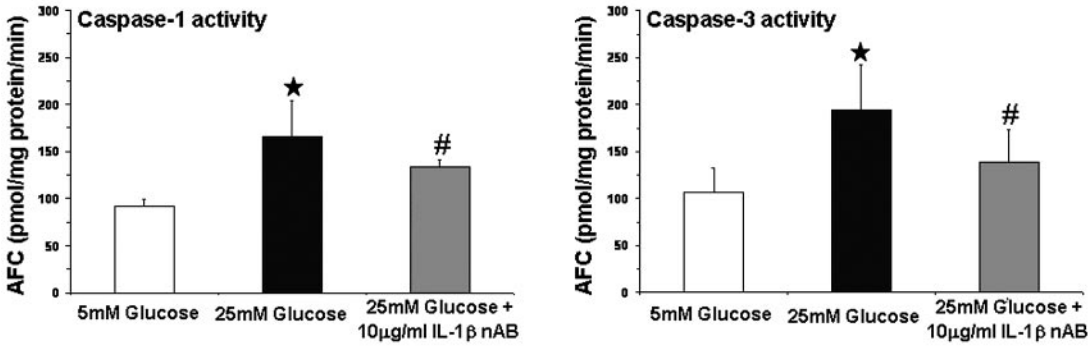

FIG. 6. Inhibition of IL-1β action prevents high glucose–induced caspase-3 activation in retinal Müller cells. rMC-1 cells were incubated in medium containing 5 mmol/l glucose, 25 mmol/l glucose, and 25 mmol/l glucose plus 10 μg/ml IL-1β neutralizing antibody (IL-1β nAB). After 96 h, caspase-1 and caspase-3 activities were measured and expressed as means ± SD. AFC, 7-amino-4-trifluoro-methylcoumarin. *$P < 0.05$ compared with normal glucose; #$P < 0.05$ compared with high glucose.

significantly inhibited in diabetic IL-1R1$^{-/-}$ mice compared with normal IL-1R1$^{-/-}$ mice or normal WT mice. Table 1 summarizes the results.

At 7 months of diabetes, diabetes significantly increased the number of acellular capillaries/mm^2 retina 2.5-fold compared with normal WT animals but had no effect in the IL-1R1$^{-/-}$ mice. Numbers of acellular capillaries/mm^2 retina in diabetic IL-1R1 mice did not significantly differ from that found in the retinas of normal IL-1R1$^{-/-}$ mice or normal WT mice (Fig. 7). GHb values for the different experimental groups again demonstrated that this genetic modification did not alter the severity of diabetes (4.5 ± 0.3 for WT and 3.2 ± 0.2 for normal IL-1R1$^{-/-}$ animals, 15.3 ± 1.8 for WT diabetic and 12.1 ± 1.4 for diabetic IL-1R1$^{-/-}$ animals). Deletion of the IL-1 receptor did not alter the normal pattern of the retinal vasculature.

DISCUSSION

Good glycemic control has been shown to inhibit the development of diabetic retinopathy, but such metabolic control is difficult to achieve and maintain in diabetic patients. Thus, finding supplemental therapies by which diabetic retinopathy can be prevented would help to improve the quality of life for these patients. Our results revealed that caspase-1 becomes activated early and remains activated in the retina of diabetic and galactose-fed mice (13). To identify the role of caspase-1 in the development of diabetes-induced retinal pathology, we used two different strategies: first, we pharmacologically inhibited caspase-1 using the proposed caspase-1 inhibitors minocycline and tetracycline. Second, we blocked downstream caspase-1 signaling mediated by IL-1β using IL-1R1 receptor knockout mice. Using these two approaches, we have demonstrated that inhibition of the caspase-1/IL-1β pathway inhibits diabetes-induced degeneration of retinal capillaries, suggesting that this signaling pathway is involved in the formation of retinal pathology characteristic of diabetic retinopathy and believed to be critical to the later neovascular response. Selective caspase-1 inhibitors (pralnacasan, VX-765) (31), other than the Ac-YVAD-CHO or Ac-YVAD-fmk, which are toxic to the necessity of being dissolved in organic solvents, are in development.

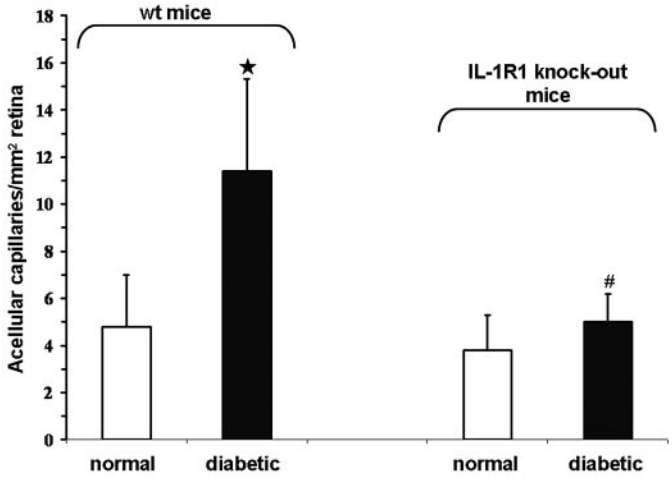

FIG. 7. Inhibition of IL-1β signaling inhibits diabetes-induced retinal pathology. Retinas of WT normal ($n = 7$) and diabetic mice ($n = 7$), and normal ($n = 8$) and diabetic IL-1R1$^{-/-}$ mice ($n = 5$), were isolated at 7 months. Diabetes-induced formation of acellular capillaries in WT mice compared with normal WT mice: ⋆$P < 0.02$. Number of acellular capillaries in diabetic IL-1R1$^{-/-}$ animals compared with diabetic WT animals: #$P < 0.05$.

Minocycline, a second-generation tetracycline derivative, has been shown to prevent caspase-1 activation in a variety of neurodegenerative diseases, including cerebral ischemia, traumatic brain injuries, Huntington's disease, and Parkinson's disease (17–20) but apparently does not inhibit caspase-1 activity directly. The exact mechanism(s) of minocycline actions remains under investigation, but minocycline-mediated protection has been associated with the inhibition of several different signaling molecules and pathways, such as inhibition of caspase-1, caspase-3, iNOS expression, and activation (18,21,32).

We conducted studies to determine whether inhibition of caspase-1 activation is specific for minocycline or is a general action of this type of antibiotic. We found that tetracycline did not prevent diabetes-induced activation of caspase-1 in the retina of diabetic mice in vivo but was able to prevent high glucose–induced caspase-1 activity in retinal Müller cells in vitro. The lack of efficacy of tetracycline in vivo was not surprising, since it is known that tetracycline does not cross the blood-brain barrier. Nevertheless, the ability of tetracycline to inhibit caspase-1 in vitro demonstrates that the ability to inhibit caspase-1 may not be restricted to minocycline but might be common to multiple drugs of this type. Screening for similar agents that are able to penetrate the blood-retinal barrier could reveal even more effective therapies.

Although clinically well tolerated, there are reports demonstrating negative results of minocycline resulting in requests for more animal studies before attempting further

TABLE 1
Diabetes-induced caspase activation is inhibited in IL-1R1$^{-/-}$ mice

	WT mice		IL-1R1$^{-/-}$ mice	
	Normal	Diabetic	Normal	Diabetic
Cas-1	35.67 ± 1.83	46.50 ± 4.33*	33.00 ± 5.67	29.83 ± 3.83
Cas-3	35.00 ± 4.33	40.00 ± 2.67*	36.00 ± 3.67	33.33 ± 1.83
Cas-6	64.50 ± 4.50	72.33 ± 3.17*	64.00 ± 8.67	65.83 ± 5.67
Cas-8	61.83 ± 4.50	73.83 ± 5.00*	61.83 ± 7.67	62.17 ± 4.67

Caspase activities are expressed as means ± SD. Retinas of WT normal ($n = 7$) and diabetic mice ($n = 7$), and normal ($n = 8$) and diabetic IL-1R1$^{-/-}$ mice ($n = 5$), were isolated at 4 months of diabetes. *$P < 0.05$, significantly different from normal WT animals compared with WT diabetic animals.

clinical trials (33,34). Concentrations of minocycline used in studies of neurodegenerative diseases (up to 50 mg/kg daily) might have played a major role in these adverse effects. A similar high dose of 50 mg/kg daily was used to investigate the effects of minocycline on degeneration of retinal ganglion cells in diabetic animals (22). Our finding of the beneficial effects of minocycline at the low dose of only 5 mg/kg body wt three times a week raises the possibility that high doses of the drug (and consequent adverse effects) are not necessary. This low dose of minocycline was sufficient to inhibit degeneration of retinal capillaries in diabetes as well as in galactosemia.

How caspase-1 becomes activated in diabetes and which retinal cell types(s) are responsible for active caspase-1 and IL-1β production has yet to be determined. Our in vitro studies with retinal cells suggest Müller cells as a source for active caspase-1. High glucose led to the activation of caspase-1, which preceded the activation of caspase-3, an executioner of apoptosis. Minocycline and tetracycline prevented hyperglycemia-induced caspase-1 activation, cytokine production, and apoptosis in these cells. Similar actions of minocycline have been observed in microglia cells (22). Although studies with these groups of antibiotics do not give a defined answer of a possible mechanism of high glucose–induced apoptosis, our results using IL-1β neutralizing antibody demonstrate that execution of high glucose–induced apoptosis is not a consequence of direct activation of downstream executioner caspases such as caspase-3 by active caspase-1 but rather caused by its predominant product IL-1β. Other studies by us have shown that high glucose–induced caspase-1 activation in retinal Müller cells is bi-phasic (S.M., unpublished observations), indicating that high glucose–induced apoptosis of retinal Müller cells might depend on the increased autocrine production of IL-1β in hyperglycemic conditions by Müller cells themselves. The idea that autocrine production of IL-1β possibly leads to the second activation of caspase-1 via IL-1β receptor activation in the later phases of hyperglycemic conditions is supported by the results that IL-1β neutralizing antibody prevented high glucose–induced caspase-1 activity at later (96-h) time points in Müller cells. IL-1β neutralizing antibody did not prevent the early phase of caspase-1 activity by high glucose (data not shown). Our in vivo data also show that in IL-1R1 receptor knockout mice, caspase-1 is not activated at 4 months of diabetes. More detailed studies need to be done to identify the exact mechanism of hyperglycemia-induced caspase-1 activation. These studies are ongoing and go beyond the scope of this article. We also speculate that production of IL-1β by Müller cells contributes to the capillary cell death observed in diabetes via a paracrine mechanism. Müller cells are the principal glia of the retina and, through their processes, span the depth of the retina and surround neuronal cell bodies, axons, and vessels. Müller cell dysfunction in diabetes has been described in the literature in diabetic patients and in animal models (28–30,35–37). Like brain astrocytes, Müller cells synthesize factors that induce the formation of tight junctions and thus confer barrier properties to the retinal vessels (38). Because Müller cells produce all these factors capable of modulating blood flow, vascular permeability, and cell survival, impaired or dying Müller cells will certainly affect these important functions.

The biological activity of IL-1β is mediated by binding to a specific cell surface receptor (IL-1R1) (39). Although it has been speculated in the literature about other possible IL-1 receptors, no such receptor has been identified to date. IL-1R1 mice develop normally (40,41). Using these IL-1β receptor knockout mice, we have shown that preventing IL-1β$^{-/-}$ signaling rescues the retinal vasculature from diabetes-induced damage. Caspase-1 is activated as early as 2 weeks in the retina of diabetic mice. Early increase in IL-1β seems to induce downstream caspase activation, since IL-1R1$^{-/-}$ mice are protected from diabetes-induced activation of initiator and executioner caspases. Interestingly, as mentioned above, IL-1β itself also seems to participate in the consistent activation of caspase-1 in the retina by diabetes.

Taken together, our results provide evidence that inhibition of caspase-1 and/or its product IL-1β play a major role in the capillary degeneration that plays a crucial role in the development of diabetic retinopathy. Inhibition of the caspase-1/IL-1β signaling pathway might represent a potential new strategy to prevent the development of this disease.

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