Protective Effects of a Coumarin Derivative in Diabetic Rats

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PURPOSE. Retinal microvascular cells play a crucial role in the pathogenesis of diabetic retinopathy. The endothelial effects of cloricromene, a novel coumarin derivative, on diabetic retinopathy induced by streptozotocin (STZ) in the rat were investigated.

METHODS. Cloricromene (10 mg/kg intraperitoneally) was administered daily in diabetic rats, and 60 days later eyes were enucleated for localization of nitrotyrosine, ICAM-1, VEGF, ZO-1, occludin, claudin-5, and VE-cadherin by immunohistochemical analysis. The effect of treatment was also evaluated by TNFa, ICAM-1, VEGF, and eNOS protein levels measurement in the retina with the respective ELISA kits. Blood-retinal barrier (BRB) integrity was also evaluated by Evans blue.

RESULTS. Increased amounts of cytokines, adhesion molecule, and nitric oxide synthase were observed in retina. Cloricromene treatment significantly lowered retinal TNFa, ICAM-1, VEGF, and eNOS. Furthermore, immunohistochemical analysis for VEGF, ICAM-1, nitrotyrosine (a marker of peroxynitrite), and tight junctions revealed positive staining in the retina from STZ-treated rats. The degree of staining for VEGF, ICAM-1, nitrotyrosine, and tight junctions was markedly reduced in tissue sections obtained from diabetic rats treated with cloricromene. Treatment with cloricromene suppressed diabetes-related BRB breakdown by 45%.

CONCLUSIONS. This study provides the first evidence that the new coumarin derivative cloricromene attenuates the degree of inflammation preserving the BRB in diabetic rats. (Invest Ophthalmol Vis Sci. 2009;50:3846–3852) DOI:10.1167/iovs.08-3328

Diabetic retinopathy is a leading cause of adult vision loss in developed countries, and is frequently complicated by macular edema as direct consequence of blood–retinal barrier (BRB) breakdown. Diabetic retinopathy in rats recapitulates much of the pathology of human diabetic retinopathy. In experimental diabetes, leukocyte binding to the retinal vascular endothelium is important in the pathogenesis of diabetic retinopathy, as it results in early BRB breakdown, capillary nonperfusion, and endothelial cell injury and death. Various mediators contribute to the upregulation of endothelial cell and leukocyte adhesion molecules in diabetic retinopathy. One such mediator is TNFa, a proinflammatory cytokine found in the vitreous as well as the extracellular matrix, endothelium, and vessel walls of fibrovascular tissue of eyes with proliferative diabetic retinopathy.1–4 The induction of endothelial adhesion molecules (i.e., ICAM-1) by proinflammatory cytokines is often mediated via activation of the NF-xB, a transcription factor that plays an important role in transcriptional regulation of inflammatory proteins.5–7 Increased NF-xB DNA-binding affinity has been detected both in the retinas of diabetic animals and in retinal cells cultured in elevated levels of glucose.8,9 VEGF is another important cytokine involved in the pathogenesis of diabetic retinopathy. Intraocular VEGF levels are increased in diabetic rats and humans and correlate with BRB breakdown and neovascularization, and VEGF inhibition prevents retinal neovascularization and BRB breakdown. Recently, it has been reported10 that the inhibition of endogenous retinal VEGF downregulates an intracellular adhesion molecule (ICAM-1) in diabetic vasculature and suppresses retinal leukocyte adhesion.

The BRB plays a critical role in partitioning the neural elements of the retina from the circulation and protecting them from circulating inflammatory cells and their cytotoxic products. Endothelial cells of the retinal microvasculature contribute to the function of the BRB through the formation of specialized intercellular junctions, including adherens and tight junctions. In the tight junctional complex, two proteins, occludin and claudins, span the plasma membrane, whereas the other proteins reside in the peripheral cytoplasm. The claudins are a multigene family of more than 20 members, and it has been demonstrated11 that claudin-5 is indispensable for the retinal barrier function. Occludin and claudins limit fluid flow between endothelial cells; other proteins including zonula occcludens-1 (ZO-1) act through multiple protein-interaction domains. The vascular endothelial (VE)-cadherin mediates calcium-dependent homophilic adhesion between endothelial cells, and the maintenance and regulation of the retinal barrier is likely to involve interactions between VE-cadherin and occludin and claudin-5. Breakdown of the BRB contributes to vasogenic macular edema that occurs in more than 25% of people with diabetes and is highly correlated with visual impairment in patients with diabetic retinopathy. In recent studies, inflammation has been implicated as playing a key role in the pathogenesis of diabetic retinopathy.12,13 Biological support for the role of inflammation in early diabetes is that the adhesion of leukocytes to the retinal vasculature (leukostasis) has been observed in both humans and rats.14,15 Increased adhesion of leukocytes to the retinal vasculature promotes vascular leakage. Blockage of the bioactivity of adhesion molecules such as ICAM-1 leads to decreased retinal leukocyte adhesion and reduced vascular leakage.15

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Due to the inflammatory nature of early diabetic retinopathy, and because its early lesions can be inhibited by salicylates and anti-TNF agents, we hypothesized that the nonsteroidal anti-inflammatory drug cloricromene would constitute an attractive therapeutic tool for diabetes. Cloricromene (ethyl 2-[8-chloro-3-(2-diethylaminoethoxy)-4-methyl-2-oxochromen-7-yl]oxycacetate) is a semi-synthetic coumarin derivative used as an antiplatelet drug with vasodilating and endothelium-protective activity. It has been demonstrated that cloricromene attenuates chronic inflammation and tissue damage associated with collagen-induced arthritis in rats. In this study we investigated the effects of cloricromene on diabetic retinopathy elicited by injection of streptozotocin (STZ) in the rat. In addition, we evaluated the effects of cloricromene on TNFα production, formation of ENOS and nitrotyrosine (index of nitrosative stress and a marker of peroxynitrite), increases in ICAM-1, and VEGF formation, as well as the role played by tight junctions (TJs).

**Materials and Methods**

**Animals and Reagents**

Male Sprague-Dawley rats weighing approximately 200 g were obtained from Charles River (Calco, Italy). All the animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were fed on standard laboratory chow and were allowed free access to water in an air-conditioned room with a 12 hour light/12 hour dark cycle. Final group sizes for all measurements were n = 6–9 except as noted. STZ and phenylmethyl sulfonyl fluoride were purchased from Sigma-Aldrich (St. Louis, MO). Avidin, biotin, and various antibodies were purchased from DBA (Milan, Italy). All other reagents were procured from standard commercial suppliers unless otherwise noted.

**Induction of Diabetes**

After 12 hours of fasting, the animals received a single 60 mg/kg IV injection of STZ in 10 mM sodium citrate buffer, pH 4.5 (1 ml/kg dose volume). Control (sham, nondiabetic) animals were fasted and received citrate buffer alone. After 24 hours, animals with blood glucose levels greater than 250 mg/dl were considered diabetic and randomly divided into groups of ten animals each. We performed all experiments 60 days after induction of diabetes. We confirmed the diabetic state by evaluating glycosmia daily using a blood glucose meter (Accu-Check Active; Roche Diagnostic, Milan, Italy). A group of rats was treated with cloricromene (10 mg/kg intraperitonically [IP]) daily starting from 30 minutes after STZ administration. We selected the IP route instead of the oral route to assure active levels of drug in the retina and to have a reproducible intake of drug during 2 months of treatment.

**Measurements of TNFα, VEGF, ICAM-1, and eNOS**

Rat eyes were collected 60 days after STZ administration, and each retina was homogenized in 100 μL of solution consisting of 20 mM imidazole hydrochloride, 100 mM KCl, 1 mM MgCl₂, 1 mM ethylene-glycol-bis[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid (EGTA), 1% Triton, 10 mM NaF, 1 mM sodium molybdinate, and 1 mM EDTA. The solution was supplemented with a cocktail of protease inhibitors (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland) before use. Samples were cleared via centrifugation for 10 minutes at 10,000g and assessed for protein concentration with the bicinchoninic acid (BCA) assay (Mini BCA Kit; Pierce Scientific, CA). The eNOS, TNF-α, and VEGF protein levels were estimated with the respective ELISA kits (EMD Biosciences, San Diego, CA; R&D Systems, Minneapolis, MN), according to the manufacturers’ instructions. We performed all measurements in duplicate. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

**Localization of Nitrotyrosine, ICAM-1, ZO-1, Occludin, VE-cadherin, Claudin-5, and VEGF by Immunohistochemistry**

Sixty days after the administration of STZ, the tissues were fixed in 10% PBS-buffered formaldehyde and 7 μM sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H₂O₂ in H₂O for 1 hour. Non-specific adsorption was minimized by incubating the section in 2% universal serum in PBS for 30 minutes and endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin. Sections were incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody (1:1000 in PBS), anti-ICAM-1 polyclonal antibody (CD54) (1:250 in PBS, v/v), anti-VEGF monoclonal antibody (1:100 in PBS, v/v), anti-ZO-1 monoclonal antibody (1:100 in PBS, v/v), anti-claudin 5 monoclonal antibody (1:100 in PBS, v/v), anti-occludin monoclonal antibody (1:100 in PBS, v/v), or anti-VE-cadherin monoclonal antibody (1:100 in PBS, v/v). Specific labeling was detected with a biotin-conjugated goat anti-rabbit, donkey anti-goat, or goat anti-mouse IgG and avidin-biotin peroxidase complex. To verify binding specificity, some sections were also incubated with primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody). In these situations, no positive staining was found, indicating that the immunoreactions were positive in all the experiments carried out. To confirm that the immunoreactions for the nitrotyrosine were specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. Positive staining was stained in brown.

**Measurement of BRB Breakdown**

Animals were anesthetized and BRB breakdown was measured using Evans blue dye (Sigma-Aldrich) injected through the tail vein over 10 seconds at a dosage of 45 mg/kg. After 120 minutes, the chest cavity was opened and rats were perfused for 2 minutes at a physiological pressure via the left ventricle with citrate-buffered paraformaldehyde (0.05 M, pH 3.5, 1% w/v; supplied by Sigma-Aldrich). A pH of 3.5 was used to optimize binding of Evans blue to albumin, and the perfusion solution was warmed to 37°C to prevent vasoconstriction. Immediately after perfusion, both eyes were enucleated and bisected at the equator. The retinas were carefully dissected and thoroughly dried in a concentration/drying system (SpeedVac; Thermo Fisher Scientific, Milan, Italy) for 5 hours. The dry weight was used to normalize the quantitation of Evans blue leakage. Evans blue was extracted by incubating each retina in 120 μL formamide (Sigma-Aldrich) for 18 hours at 70°C. The supernatant was filtered through centrifugal filter tubes (Ultrafree-MC tubes 30,000 MWL, UFC31TK00; Millipore, Bedford, MA) at 2500 g for 2 hours, and 60 μL of the filtrate was used for triplicate spectrophotometric measurements. Each measurement occurred over a 5-second interval, and all sets of measurements were preceded by evaluation of known standards. The background-subtracted absorbance was determined by measuring each sample at 620 nm (the absorbance maximum for Evans blue in formamide) and 740 nm (the absorbance minimum). The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide. BRB breakdown was calculated using the following equation, with results being expressed in μL plasma × g retina dry weight⁻¹ × h⁻¹.

\[
\text{BRB breakdown} = \frac{\text{Evans blue (μg/Retina dry weight (g))}}{\text{time-averaged Evans blue concentration (μg/plasma μL)/circulation time (h)}}
\]

We expressed results as percentage of non-diabetic controls (sham).

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Western Blot Analysis

Retinas were carefully dissected from normal or STZ rat eyes, and lysates were prepared with 0.1% Triton X-100 extraction buffer containing phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT). Protein concentration in tissue lysates was determined by using a protein assay (Bradford Protein Assay; Bio-Rad, Hercules, CA), and the protein concentrations were adjusted to allow equal total protein loading on the gels. Samples were mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer and analyzed for TJ protein expression by SDS-polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically to ECL membranes (Bio-Rad) and TJ proteins (ZO-1, claudin-5, occludin), along with adherens junction protein (VE-cadherin) identified with rabbit polyclonal antibodies from Zymed Laboratories at 1:3000 in 6% (wt/vol) BSA-TBS. Chemiluminescent membranes were washed and incubated with horseradish peroxidase (HRP)–tagged goat anti-rabbit Ig or HRP-tagged goat anti-mouse Ig and visualized (ECL; Amersham Biosciences, Piscataway, NJ). For quantification the densitometry was performed by the NIH Image program (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Statistical Analysis

All values are expressed as mean ± SD. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A value of $P \leq 0.05$ was pre-determined as the criterion of significance. The figures from histologic and immunohistochemical experiments are representative of at least three experiments (five slides for each eye from different animals) performed on different experimental days.

RESULTS

Glycemia and Body Weight

Sixty days after onset of diabetes, blood glucose values in diabetic rats treated with cloricromene were significantly ($P < 0.0001$) higher than corresponding values in nondiabetic rats (396 ± 31 and 98 ± 16 mg/dL, respectively; Table 1). Cloricromene does not interfere with glycemia values in nondiabetic rats (101 ± 19 mg/dL). Body weights of diabetic rats treated with cloricromene were significantly less than those of nondiabetic rats but were not different compared with diabetic group (Table 1).

Cytokines, Adhesion Molecule, and eNOS Levels

TNFα and VEGF along with ICAM-1 and eNOS represent the major contributors to the BRB breakdown in the diabetic retina. Increased TNFα, VEGF, ICAM-1, and eNOS were observed in retina 60 days after STZ injection (Fig. 1). Treatment with cloricromene significantly reduced the retinal TNFα (from 7.4 ± 1.0 pg/mg to 3.0 ± 0.5 pg/mg; $P < 0.001$), VEGF (from 6.3 ± 0.9 pg/mg to 2.3 ± 0.5 pg/mg; $P < 0.001$), ICAM-1 (from 14.0 ± 2.0 pg/mg to 5.8 ± 1.0 pg/mg; $P < 0.001$), and eNOS (16.9 ± 3.0 pg/mg to 8.0 ± 2.0 pg/mg; $P < 0.001$) in diabetic rats.

Immunohistochemistry

Immunohistochemical analysis of retinas obtained from rats injected with STZ revealed positive staining for VEGF mainly localized in the endothelium (Fig. 2). In contrast, no VEGF staining was found in retinas of cloricromene-treated or sham-

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<th>Table 1. Effects of STZ-Induced Diabetes on Body Weight and Blood Glucose Levels in Different Groups after Sixty Days</th>
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Data are expressed as the mean ± SD.

FIGURE 1. Retinal levels of TNFα (A), ICAM-1 (B), VEGF (C), and eNOS (D) 60 days after STZ injection with (●) or without (□) cloricromene treatment. *$P < 0.001$ versus sham; **$P < 0.001$ versus STZ.
treated rats. Likewise, retinas from STZ-treated rats demonstrated positive staining for ICAM-1 which was mainly localized in endothelial cells, particularly in the nuclei and cytoplasmic membrane (Fig. 2). In contrast, no positive ICAM-1 staining was found in the retina samples obtained from cloricromene-treated or sham-treated rats. Rats injected with STZ revealed positive staining for nitrotyrosine mainly localized in the endothelium (Fig. 2). In contrast, no positive nitrotyrosine staining was found in the retina tissues of cloricromene-treated or sham-treated rats.

In the retinas collected from sham-treated rats the immunosignal for ZO-1 clearly showed the normal reticular distribution of the protein (Fig. 3; ZO-1 panel a). On the contrary, significant disruption of immunosignal for ZO-1 was observed in the retina collected 60 days after STZ administration (Fig. 3; ZO-1 panel b and inset b1). In retina from cloricromene-treated rats, a substantially less irregular distribution of ZO-1 was observed (Fig. 3; ZO-1 panel c, insets c1 and c2). Similarly, in retinas collected from sham-treated rats the immunosignal for occludin or for claudin-5 clearly showed the normal reticular distribution of these proteins (Fig. 3; claudin-5 panel a), whereas an absence of the occludin or claudin-5 immunosignal was observed in retinas collected 60 days after STZ administration (Fig. 3; claudin-5 panel b). As with ZO-1, cloricromene appeared to substantially protect STZ-treated rats from this disruption of both occludin and claudin-5 distribution in the retina (Fig 3; claudin-5 panel c, inset c1). Finally, in the retinas collected from sham-treated rats, the immunosignal for VEGF, ICAM-1, or nitrotyrosine.
cadherin clearly showed the normal reticular distribution of the protein (Fig. 3; VE-cadherin panel a), whereas STZ substantially disrupted VE-cadherin (Fig. 3; VE-cadherin, panel b). and cloricromene appeared to protect STZ-treated rats from this disruption (Fig. 3; VE-cadherin panel c, inset c1).

Quantification of immunostaining images was assessed by densitometry analysis and, the data are showed in Table 2.

**BRB Breakdown**

Increased BRB permeability is an early event in diabetes, and in fact we observed a dramatic leakage in STZ-rats (Fig. 4A). Cloricromene treatment suppressed diabetes-related BRB breakdown by 45% compared with diabetic group (P < 0.05).

**Retinal Junction Proteins**

Expression of TJ proteins was evaluated by Western blot analysis. The results showed a significant (P < 0.01) reduction in ZO-1, occludin, claudin-5, and adherens junction protein VE-cadherin in retinas from STZ-rats, demonstrating a downregulation during experimental diabetes; however, the cloricromene treatment significantly (P < 0.01) attenuated this downregulation (Fig. 4B).

**DISCUSSION**

Chronic hyperglycemia in diabetes is associated with the development and progression of pathologic changes in the retinal vasculature involving breakdown of the BRB. This functional barrier has long been recognized to reside at the level of the tight junctions between adjacent endothelial cells. The appropriate expression of junctional molecules in endothelial cells is crucial for the normal functioning of tissues, and disturbances of the expression of these molecules, both congenital and acquired, contribute to various pathologic conditions such as diabetic retinopathy. Hypoxic stress has been previously shown to suppress claudin-5 localization in the retinal vasculature, and a previous study using STZ-treated rats demonstrated decreased occludin at the tight junctions of endothelial cells in retinal arterioles and capillaries, although no change in claudin-5 was observed. Further, STZ-treated rats have also been shown to have increased albumin permeability in the retina after 3 months. In the same study, treatment of bovine retinal endothelial cell cultures with VEGF caused a similar decrease in occludin. Occludin and ZO-1 have also been shown to be decreased in diabetic mouse retinas, with inducible nitric oxide synthase implicated as a key mediator of leukostasis and BRB breakdown in diabetic retinopathy with an upregulation of ICAM-1. Recently, it has been reported that the administration of neutralizing antibodies against ICAM-1 or its counter-receptor CD18 in early experimental diabetes dramatically reduces leukocyte adhesion, BRB breakdown, and endothelial injury. Various studies have clearly demonstrated that TNFα is increased in the diabetic retina as early as 1 week after the induction of diabetes. TNFα is an inflammatory cytokine produced mainly by monocyte-macrophages, activated endothelial cells, fibroblasts, and joint cartilage chondrocytes. In the diabetic retina, astrocytes and Müller cells are a major potential source of TNFα production. The induction of TNFα could be attributed to various mediators that operate during the course of diabetes, such as hyperglycemia and oxidative stress, which signal via NF-κB. Conversely, TNFα itself is a potent activator of NF-κB. In blood vessels, NF-κB expression was described initially in endothelial cells, where it regulates the transcription of several cytokines and adhesion molecules, including ICAM-1. NF-κB plays an important role in 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 other than TNFα include NOS, ICAM-1, COX-2, and IL-1β. It has been demonstrated that after 60 days of diabetes there is an upregulation of NF-κB in retinas of STZ-diabetic rats compared with nondiabetic rats. In the present study we showed an increase in TNFα and ICAM-1 expression using rats with STZ-induced diabetes. TNFα has been shown to induce ICAM-1 in endothelial cells. The observed correlation between the reduction in TNFα levels and inhibition of ICAM-1 expression combined with the marked reduction of oxidative stress (reduced
nitrotyrosine, a marker of peroxynitrite) as well as leukocyte infiltration after cloricromene treatment indicate that this cytokine plays a central role in the increased adhesiveness of leukocytes in diabetes.

In the present study, we demonstrate that treatment with the coumarin derivative cloricromene reduces TNFα, ICAM-1, VEGF, and eNOS in the diabetic rat retina. The findings of the present study are in agreement with other studies, which have clearly demonstrated that ICAM-1 levels are reduced with anti-inflammatory treatment, and previous work has also shown that VEGF-induced vascular permeability can be prevented via ICAM-1 inhibition. Furthermore, it has been shown that TNFα upregulates VEGF receptor-2 on endothelial cells. These data suggest that cloricromene may directly inhibit VEGF, or may affect VEGF expression through TNFα via its effects on VEGF receptor-2; additional studies would be required to further clarify these mechanisms. Another mechanism of cloricromene could be linked to NF-κB, in fact it is well known that increased eNOS-derived NO activates the redox-sensitive transcription factor NF-κB, subsequently upregulating adhesion molecules such as ICAM-1, therefore the inhibition of NF-κB by cloricromene could block this cascade. On the basis of these evidences and our results, we suggest that cloricromene may have a multipronged effect on tight junctions, preserving the BRB in rats with diabetic retinopathy. This latter statement is also supported by the fact that the BRB breakdown was suppressed significantly by the administration of cloricromene. Our data also demonstrated that cloricromene did not downregulate ZO-1, occludin, claudin-5, and adherens junction protein VE-cadherin in retinas from diabetes rats. Cloricromene is a drug with proven efficacy in several models of experimental shock as well as in experimental arthritis. Cloricromene protects rats from lipopolysaccharide (LPS)-induced endotoxemia by blocking NF-κB activation, leading to inhibition of NO and TNFα overproduction, reversing the LPS-induced vascular hyporeactivity. Since cloricromene influences TNFα production, the drug has recently been evaluated in an animal model of inflammatory bowel disease, where TNFα has a key role; cloricromene significantly reduced tissue concentrations of TNFα and myeloperoxidase activity, whereas no effect was seen on blood coagulation parameters. Corsini and colleagues showed that cloricromene inhibits LPS-induced transcription of TNFα and activation of NF-κB by interfering with LPS-induced cellular oxidative activity. These results demonstrated that cloricromene interferes with the early signal transduction pathway triggered by LPS. The mechanism by which cloricromene inhibits activation of NF-κB and subsequent neosynthesis of TNFα could be related to the scavenger effect against ROS. We recently proposed cloricromene for ocular applications, showing that the drug attenuates the degree of inflammation and tissue damage associated with endotoxin-induced uveitis in the rabbit eye and protects against experimental rat uveitis, reducing the expression of adhesion molecules such as P-selectin and ICAM-1. Furthermore, we demonstrated that cloricromene strongly inhibited TNFα production, cell infiltration, protein exudation, and nitrite/nitrate formation. Altogether, these data suggest that cloricromene may be useful in the treatment of diabetic retinopathy, and that clinical studies to evaluate this possibility may be warranted.

**References**


