PURPOSE. To investigate the protective effect of pioglitazone on the rat retina after ischemia/reperfusion (I/R) injury and to explore its possible mechanisms.

METHODS. Retinal ischemia was induced by increasing the intraocular pressure to 110 mm Hg for 60 minutes, and pioglitazone was delivered 3 hours before the I/R. Retinal damage was quantified by measuring the thickness of the retina, the functional changes of visual evoked potential (VEP) and electroretinography (ERG), and the number of retinal ganglion cells (RGCs) at 7 days after I/R injury. Real-time PCR and Western blot analysis were performed to measure the glial fibrillary acidic protein (GFAP) expression. Retinal cell apoptosis was detected by TUNEL assay at 24 hours after reperfusion. Nuclear factor-κB (NF-κB), Bax, and Bcl-2 in the retina were determined by Western blot analysis.

RESULTS. The I/R produced a degenerative effect primarily in the ganglion cell layer, inner plexiform layer, and inner nuclear layer. Pioglitazone maintained the retinal thickness, promoted the survival of RGCs, and attenuated the destruction of ERG and VEP caused by I/R. Pioglitazone pretreatment also suppressed NF-κB activation and altered GFAP overexpression. The number of TUNEL-labeled cells significantly decreased in the retinas pretreated with pioglitazone, and the Bax–Bcl-2 ratio was much lower in the retinas pretreated with pioglitazone than in the I/R group.

CONCLUSIONS. Pioglitazone could inhibit activation of the glia cells, prevent cell apoptosis, and protect the retina from subsequent cellular damage caused by the retinal I/R. The possible mechanism might involve the NF-κB pathway.

Keywords: retinal ischemia/reperfusion, pioglitazone, PPAR-γ, glia activation, NF-κB
Protective Effect of Pioglitazone on Retinal I/R Injury

Retinal ischemia/reperfusion (I/R) injury is thought to be a contributor to several ocular diseases including glaucoma, central retinal artery occlusion, ischemic optic neuropathy, and diabetic retinopathy; and to cause damage to the whole retina. Several mechanisms including inflammation, oxidative stress, excitotoxicity, and apoptosis are responsible for the retinal damage after I/R insult.\(^1\)\(^-\)\(^4\) Increased evidence suggests that retinal neuronal cells are damaged by apoptosis after retinal I/R injury.\(^5\)\(^-\)\(^6\) and retinal glial activation during the process of an acute intraocular pressure (IOP) elevation might play an important role in retinal neuronal cell death as well.\(^6\)

Pioglitazone is an exogenous ligand of the proliferator-activated receptor \(\gamma\) (PPAR-\(\gamma\)), which is a ligand-activated transcription factor belonging to the nuclear receptor superfamily.\(^7\) Originally, because PPAR-\(\gamma\) activity was once thought to be mainly involved in lipid metabolism and glucose homeostasis, pioglitazone received U.S. Food and Drug Administration approval in July 1999 with the indication to treat type 2 diabetes mellitus. However, many experimental data now support that PPAR-\(\gamma\) agonists including pioglitazone could be potential neuroprotective drugs in traumatic, ischemic, and neurodegenerative diseases in the central nervous system (CNS).\(^8\)\(^-\)\(^14\) Recent studies show that PPAR-\(\gamma\) agonists protect the neurons through the regulation of several gene expression targeted glial activation, inflammation, oxidative stress, and apoptosis.\(^8\)\(^-\)\(^10\)\(^,\)\(^14\)\(^-\)\(^15\) These findings inspired a number of studies investigating the utility of PPAR-\(\gamma\) agonists in a broad range of disease indications in the CNS. Although the research into PPAR-\(\gamma\) agonists as neuroprotectants has provided increased evidence, their potential protective effect in the retinal neurons has received little attention.

The present study aimed to investigate the protective effect of pioglitazone in the rat retina after I/R injury and to explore its possible mechanisms.

Materials and Methods

All experimental procedures using animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. One hundred twenty male Sprague-Dawley rats (Shangai Laboratory Animal Center, Shanghai, China), 8 weeks old and weighing 250 g each, were used for the experiments. The animals were anesthetized by intraperitoneal injection of 10% (w/v) chloral hydrate (4 mL/100 g; Sangon Biotech Co., Ltd., Shanghai, China). The pupils of the rats were fully dilated, and topical anesthesia was used to reduce the discomfort of the animals. After dark adaptation, the pupils of the rats were fully dilated, and topical anesthesia was used to reduce the discomfort of the animals. Because the fragments of dead RGCs could be phagocyted by the microglia and could become fluorochrome positive, a method described by Chauhan et al.\(^19\) was employed to ensure that only the apparently surviving RGCs were counted. The numbers of RGCs were obtained in 12 distinct areas of \(\times200\) fields by two investigators in a blinded manner, and the obtained scores were averaged.

Electroretinography (ERG)

Flash ERG was assessed 7 days after I/R in each group (RetiScan; Roland Consult, Brandenburg, Germany). After dark adaptation, the pupils of the rats were fully dilated, and topical anesthesia was used to reduce the discomfort of the animals. The flash ERG responses were recorded from the eyes by corneal electrodes, with the negative electrode placed in the subcutaneous space of the cheek and the ground electrode clipped to the back leg. All procedures were performed under a dim red light. The responses to a light flash (3.0 cd/m\(^2\)) from a photic stimulator were amplified, and the signals were filtered by a preamplifier set at 1 to 300 Hz.

Visual Evoked Potential (VEP) Recording

The flash VEP were recorded after the ERG recordings (RetiScan; Roland Consult). The positive electrode was inserted under the scalp overlying the visual cortex and the negative electrode clipped into the skin of the cheek. The grounding electrode was then placed into the tail. The opposite eyes were covered with black tape to block out light. The impedance between the scalp and reference electrodes was \(<2.0\) k\(\Omega\). The stimulus was a 1.3 Hz, 3.0 cd/\(\text{m}^2\) Ganzfeld strobe flash, and 100 responses per eye were averaged.
In Situ TUNEL Labeling

The terminal deoxynucleotidyl transferase-mediated dUTP (2'-deoxyuridine 5'-triphosphate) nick-end labeling (TUNEL) assay was used to evaluate apoptosis of the retinal neurons after I/R injury. The eyes were enucleated 24 hours after I/R injury, and retinal paraffin sections were prepared per the previously described method, as mentioned in the previous section (Histopathology and Morphology of Retina). Apoptosis of the retinal tissue was detected using the In Situ Cell Death Detection Kit (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. The sections were examined using light microscopy under ×400 magnification. Six microscopic fields of each eye with three adjacent areas on both sides of the optic nerve head (1 mm from the optic nerve head) were used to count the TUNEL-positive cells, and the obtained scores were averaged.

Real-Time Polymerase Chain Reaction (PCR)

Total ribonucleic acid (RNA) of the retinal tissue was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA (2 μg) was reverse transcribed into first-strand complementary deoxyribonucleic acid (cDNA) (ReverTra Ace RT PCR Kit; Toyobo, Osaka, Japan). PCR reactions were performed in the ABI 7500 Detection System using a SYBR green PCR kit (Toyobo). The primer sets were as follows: gial fibrillary acidic protein (GFAP), 5'-TGTAG GAGTTGGGAGGGC-3' (forward), 5'-CTGAGCCAAACGAGGATA GAC-3' (reverse; accession number NM_017009.2, http://www.ncbi.nlm.nih.gov/nuccore/NM_017009.2); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CATGCCACAGTC TCC-3' (reverse; accession number NM_017008.3, http://www.ncbi.nlm.nih.gov/nuccore/NM_017008.3). The parameters were set at 95°C for 1 minute and 1 cycle, then 95°C for 15 seconds, 60°C for 45 seconds, and 72°C for 45 seconds and 40 cycles. The fold change in target gene expression was analyzed using the 2−ΔΔCT method.

Western Blot Analysis

Retinas were dissected at 24 hours and 7 days after I/R. Whole retinas were dissected and homogenized on ice in Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The protein extracts were separated and transferred onto a nitrocellulose membrane. After blocking, the membrane was probed with the primary antibody against GFAP (1:2000; Epitomics, Burlingame, CA), nuclear factor-kB (NF-kB) p65 (1:1000), phosphorylated NF-kB (phospho-NF-kB) p65 (1:1000), Bax (1:1000), Bcl-2 (1:1000), p-ERK (1:1000), ERK (1:1000) (all from Cell Signaling Technology, Danvers, MA), and GAPDH (1:2000; Millipore, Billerica, MA), followed by the appropriate horseradish peroxidase (HRP)-conjugated goat antimouse or goat antirabbit secondary antibody (Santa Cruz Biotechnology, Inc.). Specific bands were visualized by a standard enhanced chemiluminescence procedure (Millipore). The signals were analyzed using Image Lab software (version 3.0; Bio-Rad Laboratories, Inc., Hercules, CA). The band densities of each sample were normalized to the GAPDH band. The band densities of each sample were normalized to the GAPDH band. The band densities of each sample were normalized to the GAPDH band. The band densities of each sample were normalized to the GAPDH band. The band densities of each sample were normalized to the GAPDH band.

Statistical Analysis

All results are expressed as the mean ± standard error of the mean. Statistical analysis was performed using one-way ANOVA followed by the Fisher least significant difference test for between-group comparison using SPSS 15.0 software (SPSS, Inc., Chicago, IL). Differences were considered statistically significant at P < 0.05.

RESULTS

Protective Effect of Pioglitazone on the Histologic and Morphologic Changes in Retina Following I/R

In the control group, the whole retinal thickness was 190.1 ± 2.455 μm, and the inner plexiform layer (IPL) was 49.93 ± 1.68 μm. Seven days post-I/R, a significant decrease in the thickness of the whole retina and IPL was evident in the eyes subjected to I/R (retinal thickness: 119.3 ± 3.593 μm; IPL: 13.45 ± 0.476 μm; both P < 0.01 versus control; n = 6; Fig. 1). Pioglitazone delivered either by periocular injection (retinal thickness: 158.2 ± 5.848 μm, P < 0.01 versus I/R; IPL: 54.84 ± 1.429 μm, P < 0.01 versus I/R; n = 6) or intraperitoneal injection (retinal thickness: 143.7 ± 7.967 μm, P < 0.01 versus I/R; IPL: 25.54 ± 2.018 μm, P < 0.01 versus I/R; n = 6) maintained the thickness of the whole retina and IPL after I/R. There was no significant difference in the whole retinal thickness between the two routes of administration, while the IPL was thicker in the group that received pioglitazone through the periocular route (P < 0.01).

Effect of Pioglitazone on the Survival of RGCs

Evaluation of the survival of RGCs was performed by retrograde labeling. The RGCs labeled with DiI were counted 7 days following the retinal I/R with or without pioglitazone treatment (Fig. 2). The mean density of RGCs in the control group was 254.92 ± 56.92/mm². Seven days post-I/R, the number of RGCs was 1141.14 ± 82.89/mm², which was recorded as a decrease of approximately 53% compared with the control group (P < 0.01; n = 6). Under pioglitazone treatment, the number of surviving RGCs was 1954.43 ± 38.16/mm² (approximately 77% of the control, P < 0.01 versus I/R group; n = 6) in the group that received periocular injection, and 1890.53 ± 68.96/mm² (approximately 74% of control, P < 0.01 versus I/R group; n = 6) in the group that received intraperitoneal injection, respectively. There was no statistical difference in the number of RGCs between the two pioglitazone-pretreated groups.

Effect of Pioglitazone on Retinal Function After I/R Insult

Electroretinography. The I/R injury caused a 50% and 71% reduction in the a- and b-wave amplitudes, respectively (P < 0.01 versus control; n = 14; Fig. 3). However, in the pioglitazone-pretreated groups, the reductions in a- and b-wave amplitudes were significantly improved (for a-wave, P < 0.05 versus I/R in the I/R+Pi oip. group and I/R+Pi periocu lux groups; for b-wave, P < 0.05 versus I/R in the I/R+Pi oip. group and P < 0.01 versus I/R in the I/R+Pi periocu lux group; n = 14). The reductions in the b-wave amplitudes were approximately 49% and 24%, respectively, in the intraperitoneal- and periocular-injected groups. No significant difference in the a- and b-wave amplitudes was observed between the two different routes of pioglitazone administration.

Visual Evoked Potential. To functionally evaluate the retinal neuron and optic nerve injury after retinal I/R insult, flash VEP were recorded and analyzed. Figure 4 shows typical VEP records for the experimental groups. A significant reduction in the flash VEP N1-P1, P1-N2, and N2-P2 components was observed in the eyes of the group subjected to I/R compared to the control group (P < 0.01; n = 14). Pioglitazone-pretreated groups significantly attenuated the...
Effect of the retinal I/R injury on N1–P1, P1–N2, and N2–P2 amplitude components as shown in Figure 4B. The amplitude of N1–P1 and P2–N2 components in the pioglitazone periocular-injected group was higher than in the intraperitoneal-injected group (\(P < 0.01\) in N1–P1 and \(P < 0.05\) in N2–P2). There was no significant difference in P1-N2 amplitude between the two routes of pioglitazone administration.

**Effect of Pioglitazone on Retinal I/R-Induced Apoptosis**

TUNEL analysis was performed to determine the effect of pioglitazone on apoptosis induced by retinal I/R (\(n = 6\)). Apoptotic cells were observed mainly in the inner retina. TUNEL-positive cells were counted in the GCL and inner nuclear layer (INL) of the retina 24 hours after I/R; a significant increase in TUNEL-labeled cells was found in the I/R group (13.85 ± 1.67 cells/field versus control, 0.33 ± 0.21 cells/field, \(P < 0.01\); Fig. 5A). In the pioglitazone-pretreated groups, TUNEL-positive cells were decreased; values were 6.2 ± 0.51 cells/field (\(P < 0.01\) versus I/R group) in the intraperitoneal injection group and 8 ± 0.58 cells/field in the periocular injection group (\(P < 0.01\) versus I/R group). No significant difference was observed in the number of TUNEL-labeled cells between the two routes of pioglitazone administration. The expression of apoptosis-associated Bax and Bcl-2 in the retina was examined by Western blot analysis (Fig. 5B; \(n = 4\)). Bax protein was upregulated 24 hours after I/R (\(P < 0.01\) versus control). No significant difference was noted between the
Pioglitazone-pretreated and I/R groups. Bcl-2 expression was upregulated in the pioglitazone-pretreated groups compared with the I/R group, and the Bax–Bcl-2 ratio was significantly lower in the retinas of groups pretreated with pioglitazone than in the I/R group (\(P < 0.01 \) versus I/R group). The Bax–Bcl-2 ratio in the periocular-injected group was lower than in the intraperitoneal-injected group (\(P < 0.01 \)).

Effect of Pioglitazone on GFAP Expression in the Retinal Tissue Following I/R Insult

To quantitatively evaluate the changes in GFAP expression levels, Western blot analysis and real-time PCR evaluation were performed in the retinal tissue for GFAP protein detection and GFAP messenger ribonucleic acid (mRNA) expression, respectively. As seen in Figure 6, Western blot analysis showed that the GFAP protein increased in retina 7 days after retinal I/R (\(P < 0.01 \) versus control; \(n = 4 \)). The increase of GFAP protein induced by I/R injury was blocked by pioglitazone pretreatment (both periocular and intraperitoneal injections, \(P < 0.01 \) versus I/R group). Real-time PCR analysis showed a trend in the GFAP mRNA level similar to that for GFAP protein expression (Fig. 6A3). Pioglitazone pretreatment significantly curtailed the post-I/R induction of GFAP transcription. No significant difference in the GFAP expression either in the mRNA level or in the protein level was noticed between the periocular injection and intraperitoneal injection groups (Fig. 6A).

Effect of Pioglitazone on NF-\(\kappa\)B Activation

As shown in Figure 6B, expression of both phospho-NF-\(\kappa\)B p65 subunit and NF-\(\kappa\)B p65 subunit was upregulated 7 days after I/R (both \(P < 0.01 \) versus control). Pioglitazone pretreatment by both periocular (\(P < 0.05 \) versus I/R group; \(n = 4 \)) and intraperitoneal injections (\(P < 0.05 \) versus I/R group; \(n = 4 \)) significantly attenuated p65 subunit expression, and the phosphorylation level of NF-\(\kappa\)B p65 was reduced (both \(P < 0.01 \) versus I/R group). No significant difference in phospho-NF-\(\kappa\)B p65 subunit and NF-\(\kappa\)B p65 subunit expression was observed between the two routes of pioglitazone administration.

DISCUSSION

In this study, we investigated the protective effect of pioglitazone on the survival of RGCs and retinal function following an I/R challenge caused by an acute IOP elevation. The study results showed that pretreatment with pioglitazone markedly reduced retinal cell injury following I/R, as demonstrated by a reduction in both the histologic damage and functional changes in comparison to the I/R groups. It was also found that pioglitazone inhibited the activation of the glial cell and prevented cell apoptosis in the retinal I/R model in rats, suggesting the mechanism of protection by pioglitazone.

The rat I/R model is a frequently used model that produces global ischemia with obstruction of both the retinal and choroidal circulation, representing pathologic features that mimic retinal vessel occlusion as well as acute angle-closure glaucoma. Retinal I/R injury can cause great loss of RGCs and destroy the integrity of the entire retinal tissue. As in the present study, quantification of RGCs was used as an important index to evaluate the neuroprotective effect of pioglitazone after retinal I/R. Retrograde labeling was used to evaluate the quantity of the remaining RGCs after I/R, considering that...
retinal I/R can induce not only the loss of RGCs but also impairments of retrograde axonal transport. Therefore, not all the RGCs that survive ischemia retain their capacity for retrograde axonal transport. Jehle et al. also proposed the labeling of RGCs after I/R insult, and they pointed out that only cells with intact retrograde transport were stained and counted as “vital” RGCs with this method. The results of the present study showed that retinal I/R caused a reduction of approximately 53% in the loss of RGCs, and a significant decrease in the whole retinal thickness was observed with the marked thinning of the IPL, as well as extensive disorganization of the retinal histoarchitecture 7 days after I/R. Pioglitazone pretreatment promoted the survival of RGCs and reduced retinal morphologic change during the induction of I/R. This study also showed that the thickness of the outer segment was decreased by I/R; this was in accordance with some previous studies. The exact mechanisms that lead to the loss of photoreceptors are still not totally clear. In the present study, the influence of increased IOP on choroidal blood flow, on which the outer retina depends, might have contributed to the degeneration of the outer segment. High IOP similar to that in the current study has been demonstrated to cause nearly complete cessation of blood flow in both the retina and choroid. Apart from the blood effect, other effects such as I/R-induced increased levels of nitric oxide radical and reactive Müller cells have also been suggested to cause injury to photoreceptor cells.

Good visual function is based on the morphologic and functional integrity of the retina as a whole. ERG is a sensitive physiological measurement for retinal health, displaying retinal activity of the cells apart from the RGCs, and VEP reflects the function of the visual pathway up to the visual cortex. In the present study, it was observed that the amplitudes of the a- and b-waves were drastically reduced 7 days after I/R, which was in line with several previous investigations. As all the electrophysiologic parameters were correlated significantly with the integrity of the retinal tissue and the state of resident cells, the histologic analysis revealed severe destruction of the central retina; and ERG of the I/R group might reflect mainly the activity of the peripheral retina, which appeared to be relatively spared after the ischemic injury. Pioglitazone pretreatment significantly ameliorated the reduction of b-wave amplitude, which was thought to be proportional to the severity of the ischemic episode and extent of retinal damage, showing an improvement of retinal function in accordance with the histologic changes brought about by pioglitazone. The amplitude of the a-wave was also improved by pioglitazone pretreatment, implying a retention of retinal photoreceptor–bipolar cell signaling function after I/R. Furthermore, significant improvement of the flash VEP in pioglitazone-pretreated groups was observed, which could provide additional evidence that pioglitazone could act as a neuroprotectant in retinal I/R injury.

Neuronal cell death in the retina during I/R may be an important factor that contributes to damage to visual function. It is a complicated process, and the underlying mechanisms are unclear. In a previous study, ganglion cells died in a way that was morphologically and biochemically consistent with apoptosis. The present study further showed significant reduction of TUNEL-positive cells in the GCL and INL of retina sections in pioglitazone-pretreated groups 24 hours after I/R, suggesting that pioglitazone could protect the retinal cell from apoptosis. The Bcl-2 family of proteins consists of antiapoptotic (Bcl-2 and Bcl-xl) and proapoptotic members (Bax and Bak).
Bcl-2 and Bcl-xL could inhibit apoptosis by preventing the release of cytochrome c from mitochondria into the cytoplasm. The dynamic balance between the antiapoptotic and proapoptotic members of the Bcl-2 family may determine the susceptibility of a cell to apoptosis. The present study showed that retinal I/R caused a significant suppression of Bcl-2 protein level, which was not seen with the group pretreated with pioglitazone. The Bax–Bcl-2 ratio was significantly lower in pioglitazone-pretreated retinas than in the I/R group, suggesting an antiapoptotic role of pioglitazone in retinal I/R through the upregulation of Bcl-2.

Glial cell activation is a typical response to CNS injury. The retina is considered a part of the CNS and therefore could react in a similar way. Increased immunoreactivity of GFAP is a hallmark of reactive glial activation and could be modulated by various retinal pathological activities. Elevated IOP may result in the upregulated expression of GFAP in reactive Müller glia and astrocytes. Activation of glia cells in the retina produces cytokines and other molecules involved in the inflammatory response, which could contribute to neuronal damage. Data from the present study provide direct evidence for the activation of retinal glia as shown by drastically upregulated GFAP at both the mRNA and protein level after I/R. The study demonstrated that pioglitazone pretreatment significantly curtailed the post-I/R induction of GFAP overexpression. A similar phenomenon was observed in the CNS by Heneka et al. and Shibata et al. In this regard, it could be considered that the beneficial effect of pioglitazone on the survival of RGCs and the functional improvement during retinal I/R might, at least partly, involve a mechanism of the regulation of glial cell function.

To further reveal the probable mechanism of the protective effect exerted by pioglitazone, the immunoreactivity of NF-κB in the retina was examined. In the present study, I/R insult caused overexpression of the NF-κB p65 subunit and upregulation of the phosphorylation level of NF-κB p65 subunit. The phosphorylation of the NF-κB p65 subunit plays a key role in its transcriptional activation after nuclear translocation, which could involve a wide range of biological functions associated with excitotoxic, oxidative, and inflammatory events. NF-κB activation is thought to be controversial; it could exert a dual role in the modulation of neuron survival. It has been proposed that transient activation of NF-κB in neurons could improve their survival after ischemic insult, while persistent activation of NF-κB could make the neurons vulnerable to ischemic insult. In cerebral ischemia, NF-κB was shown to be activated in the neurons and contribute to cell death, and pioglitazone showed a neuroprotective effect against ischemic brain injury through the inhibition of NF-κB signaling pathway. Furthermore, it has been demonstrated that GFAP is a target gene of NF-κB, implying a role of NF-κB in the glial activation. Therefore, our study findings provide evidence that pioglitazone protects the retinal neuronal cell from death and alters visual function after
ischemic damage through the inhibition of persistent activation of NF-kB and interaction with glia cells.

The mechanism underlying the neuroprotective effects of pioglitazone is complicated and interesting. Several PPAR-γ agonists have been proposed as an alternative for the neuroprotection in CNS, and their neuroprotective effects could occur either through a PPAR-γ-dependent way or through a PPAR-γ-independent way. The protective effect of pioglitazone may be mediated, at least partially, by PPAR-γ activation, as our ongoing study showed a significant increase of pioglitazone may be mediated, at least partially, by PPAR-γ. However, investigation of the molecular interaction involved in PPAR-γ transcriptional activity or direct interaction between PPAR-γ and DNA binding was beyond the scope of the present study. Thus, it is yet to be fully elucidated whether pioglitazone could act in a PPAR-γ-dependent manner or a PPAR-γ-independent way. The precise molecular mechanism by which pioglitazone exerts its effect will be explored in further research.

In conclusion, the present study findings demonstrate that pioglitazone can protect the RGCs and the histologic integrity of retina and retinal function after retinal I/R. This protective effect seems to be due, at least in part, to the inhibition of NF-kB immunoreactivity and glial activation as well as the antiapoptotic effect after I/R. Such information could be helpful in developing new potential therapy for retinal diseases associated with I/R. Further investigations into the mechanisms of pioglitazone as a neuroprotectant to retinal neurons are recommended.

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