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Pharmacological protection of retinal pigmented epithelial cells by sulindac involves PPAR-α

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The retinal pigmented epithelial (RPE) layer is one of the major ocular tissues affected by oxidative stress and is known to play an important role in the etiology of age-related macular degeneration (AMD), the major cause of blinding in the elderly. In the present study, sulindac, a nonsteroidal antiinflammatory drug (NSAID), was tested for protection against oxidative stress-induced damage in an established RPE cell line (ARPE-19). Besides its established antiinflammatory activity, sulindac has previously been shown to protect cardiac tissue against ischemia/reperfusion damage, although the exact mechanism was not elucidated. As shown here, sulindac can also protect RPE cells from chemical oxidative damage or UV light by initiating a protective mechanism similar to what is observed in ischemic preconditioning (IPC) response. The mechanism of protection appears to be triggered by reactive oxygen species (ROS) and involves known IPC signaling components such as PKG and PKC epsilon in addition to the mitochondrial ATP-sensitive K^+ channel. Sulindac induced iNOS and Hsp70, late-phase IPC markers in the RPE cells. A unique feature of the sulindac protective response is that it involves activation of the peroxisome proliferator-activated receptor alpha (PPAR-α). We have also used low-passage human fetal RPE and polarized primary fetal RPE cells to validate the basic observation that sulindac can protect retinal cells against oxidative stress. These findings indicate a mechanism for preventing oxidative stress in RPE cells and suggest that sulindac could be used therapeutically for slowing the progression of AMD.

preconditioning | oxidative stress | sulindac | retinal pigmented epithelial cells | age-related macular degeneration

Oxidative damage, resulting from excess production of reactive oxygen species (ROS), has been implicated in the progression of key ocular disorders such as cataracts, glaucoma, and age-related macular degeneration (AMD). Death of retinal pigmented epithelial (RPE) cells has been shown to be an important contributor to AMD pathophysiology. RPE cells are known to be highly metabolically active, and there is strong evidence that the RPE cells are sensitive to oxidative stress (1). It has been reported that the pathophysiology of AMD is due to cumulative oxidative damage to RPE cells resulting from an imbalance between the generation of ROS and the ability of these cells to destroy and/or protect against ROS damage to macromolecules (2, 3). Hence, strategies for protecting RPE cells against oxidative damage may be particularly important in maintaining retinal function and preventing the development or progression of AMD.

Sulindac was one of the first nonsteroidal antiinflammatory drugs (NSAIDs) used to treat inflammation. It is a prodrug, composed of R and S epimers, whose NSAID activity is dependent on the reduction of the epimers to sulindac sulfide, the active cyclooxygenase (COX) inhibitor (4). This reduction is catalyzed by two members of the methionine sulfoxide reductase (Msr) family, MsrA and MsrB, that reduce the sulindac S and R epimers, respectively (5). Because substrates of the Msr system, such as methionine sulfoxide in proteins, could theoretically function as part of an ROS scavenger system (6), sulindac was previously tested for its protective effect in cultured normal human lung cells and shown to protect these cells against oxidative damage. However, the observed protection did not involve either the Msr system or COX inhibition (7). A more detailed study, examining the effect of sulindac on protecting the intact heart against ischemia/reperfusion oxidative damage using a Langendorff procedure, provided preliminary evidence that the sulindac protection that was observed involved an ischemic preconditioning mechanism (IPC), dependent on ROS formation (8).

Sulindac has also been shown to be an inhibitor of phosphodiesterase type 5 (PDE5) (9) and has been reported to react with both the peroxisome proliferator activator receptors (PPARs) and a truncated retinoic acid receptor (RXR) (10). The members of the PPAR nuclear receptor family are involved in certain key protective pathways in a variety of cell types and are known to complex with the RXR family (11). The three classes of PPAR, PPAR-α, PPAR-β, and PPAR-γ, are normally activated by fatty acids and eicosanoids. PPAR-α agonists have been reported to be cardioprotective and to up-regulate antioxidant genes in diabetic rats (12). A role for PPAR-α in protective

Significance

Oxidative stress-induced damage to retinal pigmented epithelial (RPE) cells is implicated in the progression of age-related macular degeneration (AMD), which is one of the primary causes of vision loss in the elderly. The present studies show that sulindac, a known nonsteroidal antiinflammatory drug, can protect an established RPE cell line, low-passage human fetal RPE, and polarized primary human fetal RPE cells against oxidative damage. The results with the RPE cell line indicate that the protective response is similar to that seen with ischemic preconditioning. Our results suggest that preventing oxidative damage in RPE cells by this drug-induced protective mechanism could be an inexpensive and relatively nontoxic therapeutic approach for AMD treatment.

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pathways in AMD models also has been highlighted in studies demonstrating a potent effect of PPAR-α agonists on inhibiting pathological neovascularization in the retina (13, 14).

As mentioned, we have previously shown that sulindac protects cardiac cells against ischemia/reperfusion damage by what appeared to be a drug-induced IPC response (8). However, the signaling pathways that were involved in this pharmacological protective response were not elucidated. In the present study we provide strong evidence that sulindac can protect RPE cells against oxidative damage by initiating a protective response, similar to that seen with IPC, that involves both mitochondrial reactions and PPAR-α.

In this study, we primarily used ARPE-19 cells but also low-passage human fetal RPE and polarized primary human fetal RPE cells to validate the protective response of sulindac against oxidative stress. Fetal RPE cells grown as a polarized monolayer have been shown to be a more relevant model to what may occur in human retinal cells in vivo (15, 16).

Results

Sulindac Protection of RPE Cells Against Oxidative Damage Involves Activation of PPAR-α. Results of previous studies on the protection of the heart against ischemia/reperfusion oxidative damage indicated that sulindac acts by initiating an IPC response (8). We wanted to extend these studies to RPE cells that are known to be sensitive to oxidative stress-induced damage. In the initial experiments, fenofibrate, a PPAR-α agonist, was also tested with RPE cells because it was reported to be an IPC agent in a cardiac system (17). As described in the *Materials and Methods*, two types of oxidative stress were used in these experiments; either exposure of the RPE cells to the chemical oxidizing agent tert-butylhydroperoxide (TBHP) or exposure to UVB light. As shown in Fig. 1*A*, both sulindac, and to a lesser extent fenofibrate, afforded significant protection against TBHP-dependent loss of cell viability at TBHP concentrations up to 325 μM. GW 6471, an antagonist of PPAR-α, significantly reversed the protection by sulindac of RPE cells against TBHP (Fig. 1*A*), indicating that PPAR-α is also involved in the sulindac protective effect. Fig. 1*B* shows that sulindac, sulindac sulfone, the oxidized metabolite of sulindac, and fenofibrate also significantly protect RPE cells against photooxidative stress induced by UVB exposure. It should be noted that sulindac sulfone is not an NSAID or a substrate for the Msr system.

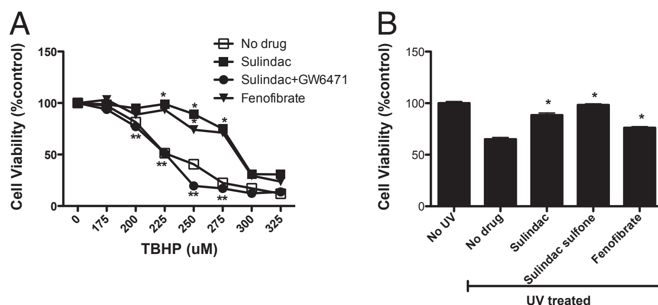

Fig. 1. Activation of PPAR-α is required for the protection of RPE cells by sulindac. (*A*) The protective effect of preincubating RPE cells with sulindac, fenofibrate, or sulindac + the PPAR-α antagonist (GW6471) before exposing them to chemical oxidative stress induced by TBHP. Concentrations used: 200 μM sulindac, 6 μM fenofibrate, and 4 μM GW6471. (*B*) The comparative protective effect of sulindac, sulindac sulfone, and fenofibrate on cultured RPE cells following UVB light exposure. Concentrations used: 500 μM sulindac, 200 μM sulindac sulfone, and 6 μM fenofibrate. *, shows significant difference from no drug. **, shows significant difference from treatment with sulindac.

It has also been reported that a class of PPAR-γ agonists known as glitazones can protect neuronal cells against oxidative damage (18). Three known PPAR-γ agonists, troglitazone, rosiglitazone, and pioglitazone, were also tested. Only troglitazone gave protection similar to sulindac (Fig. S1). In addition, the sulindac effect was not reversed by the presence of PPAR-γ antagonist, T0070907 (Fig. S2). These results suggest that the sulindac protective effect in RPE cells most likely does not involve PPAR-γ and that the effect of troglitazone in these experiments is independent of PPAR-γ. In summary, sulindac's protective effect is independent of its NSAID activity, the Msr system, and PPAR-γ, but appears to involve activation of PPAR-α.

Sulindac Protection of RPE Cells Involves Both Mitochondrial and Nuclear Events. The results in Fig. 1 indicate the involvement of PPAR-α activation in the sulindac protective effect on RPE cells exposed to oxidative stress. However, it was not known whether this protection involved a drug-initiated mechanism similar to that seen with IPC. The IPC response in tissues, normally initiated by hypoxic conditions, can be triggered by ROS and/or nitric oxide (NO), which activate PKG and mitochondrial PKCε, resulting in the activation of the mitochondrial ATP-sensitive K^+ [mK(ATP)] channel (19). This blocks the formation of the mitochondrial permeability transition pore (MPTP) and prevents the cell from initiating an apoptotic response (20). To obtain more direct evidence that an IPC-like response was responsible for the protection of RPE cells seen with sulindac, a number of components known to be involved in IPC were tested for their effect in this system.

A well-established trigger of IPC is the increased generation of ROS by pharmacological preconditioning agents (21). To test the role of ROS in the sulindac protection of RPE cells, cells were incubated with sulindac and the ROS scavenger tiron before TBHP exposure. As shown in Fig. 2, tiron causes significant reversal of sulindac's protective effect providing evidence that increased ROS levels are involved in the observed IPC effect. As mentioned above, another component reported to play an important role in the preconditioning pathways is PKG (22). Sulindac has been reported to be an inhibitor of PDE5, which raises cGMP levels and activates PKG (9). In the present study we tested the effect of inhibiting PKG using the known PKG inhibitor, Rp–Br-8–PET–cGMPS. As shown in Fig. 3, when RPE cells were coincubated with sulindac and Rp–Br-8–PET–cGMPS before exposing them to either 300 μM or 325 μM TBHP (Fig. 3*A*) or UVB-induced oxidative stress (Fig. 3*B*), the protective effect of sulindac was significantly reduced.

PKCε has been identified as the PKC isoform involved in the IPC response (20). In our previous cardiac study the IPC effect of sulindac was shown to be dependent on the activation of PKC, but not specifically PKCε (8). In the present study, we also have shown that the effects of both sulindac and fenofibrate were significantly reversed by chelerythrine, a broad spectrum PKC inhibitor (Fig. S3 *A* and *B*). To demonstrate that PKCε was involved in the sulindac protection we used V1-2, a known peptide inhibitor of PKCε (23). As shown in Fig. 4, the protective effect of sulindac was completely reversed by V1-2. Other experiments provided evidence that rottlerin, when used at a concentration of 3 μM, which has been reported to inhibit PKCδ (24), did not reverse the sulindac protection (Fig. S4). The opening of the mK (ATP) channels that prevents the formation of MPTP is also considered a key step in the protection of cells against oxidative damage by IPC agents (25). To determine whether the mK(ATP) channels are involved in sulindac's protective mechanism, RPE cells were incubated with sulindac and 5-hydroxydecanoic acid (5-HD), a chemical blocker of mK(ATP) channels. As shown in Fig. 5, the presence of 5-HD results in almost complete reversal of sulindac's protective effect, indicating the involvement of mK(ATP) channels in the sulindac protection. Finally, to obtain

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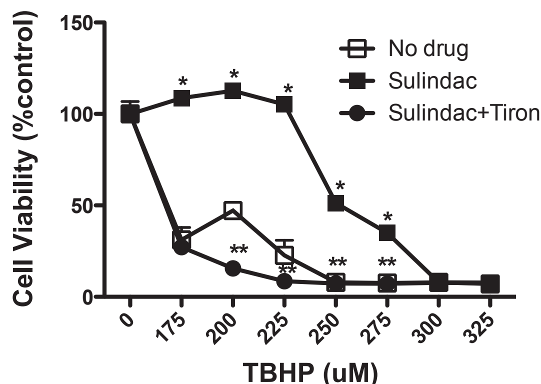

Fig. 2. Protection by sulindac involves increased intracellular ROS. Effect of the ROS scavenger, tiron (1 mM), on the sulindac (200 μM) protection against TBHP. *, shows significant difference from no drug. **, shows significant difference from treatment with sulindac.

further support that sulindac was protecting the RPE cells by initiating an IPC-like response we determined the induction of two well-established late preconditioning markers, iNOS and Hsp70. The results are shown in Fig. S5 *A* and *B*. Incubation of RPE cells with sulindac for 48 h resulted in a significant induction of iNOS and Hsp70. As expected, the induction of these late-stage markers in these experiments was prevented if the cells were treated with chelerythrine. All of the above results indicate that sulindac is inducing a protective response in the RPE cells that is similar to that seen with IPC.

Previous experiments have indicated conflicting results on cell death of RPE cells exposed to oxidative damage. Cai et al. (26) reported that RPE cells exposed to TBHP show death by apoptosis under their conditions, whereas Roduit and Schorderet (27) report that RPE cells exposed to UV show cell death by apoptosis. In contrast, Hanus et al. (28) found that RPE cells exposed to TBHP primarily die through necrosis. In addition to the MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay, which we have used to measure cell viability, we have examined cell death of RPE cells after TBHP treatment using the TUNEL system. Under our conditions about 25% of the cells appear to be killed by apoptosis (Fig. S6*A*). We have additionally used lactate dehydrogenase (LDH) release as a measure of cell death (Fig. S6*B*).

Studies Using Fetal RPE Cells. There has been some concern that the results obtained from established RPE cell lines may not reflect what occurs in vivo (29, 30). Recent studies have indicated that the behavior of human fetal cells grown as a monolayer appears to be more relevant to what occurs in vivo (31). Therefore, we validated the basic observation that sulindac can protect retinal cells against oxidative stress using human highly differentiated polarized RPE cells and nonpolarized fetal RPE cells. As shown in Fig. 6, sulindac completely protects fetal RPE cells against TBHP oxidative stress. It should be noted that the highly differentiated RPE cells (Fig. 6*B*) are more resistant to TBHP oxidative damage, consistent with what has been reported previously (32).

Discussion

The initial impetus for the studies with sulindac was based on it being a substrate for the Msr system (5) and its possible function in cells as a catalytic antioxidant. However, this does not appear to be the case for sulindac protection of cardiac tissue (8) and of RPE (ARPE-19) cells described in the current investigation. The oxidized metabolite of sulindac, sulindac sulfone, can replace sulindac in these studies, and because sulindac sulfone is not an NSAID, or a substrate for the Msr system, these data point to a protective mechanism for sulindac that is independent of NSAID or Msr activity. The major goal of the present study was to determine the key components of this NSAID-independent pathway by which sulindac elicits protection of RPE cells. The results from the present study provide strong evidence that sulindac protects ARPE-19 cells against oxidative damage by its ability to initiate an IPC response and that this response involves PPAR-α. As shown in Fig. 6, we validated the relevance of sulindac's protective response against oxidative stress in RPE cells by using human fetal polarized monolayer RPE cells that mimic the human RPE cells in vivo (16).

In the present study using ARPE-19 cells, sulindac protection against TBHP-induced damage was found to be dependent on activation of PPAR-α. Both sulindac and the PPAR-α agonist fenofibrate offered protection of RPE cells against oxidative stress, and the protection by sulindac was reversed in the presence of a PPAR-α antagonist (Fig. 1). In a previous study a therapeutic effect of IPC through PPAR-α activation was observed against myocardial infarction in rabbit myocardium (17). Interestingly, and consistent with our RPE data, this previous myocardial ischemia study using PPAR-α showed an increase in mRNA levels of iNOS resulting from activation of PPAR-α and IPC (17). The finding that sulindac's protective effect may involve activation of PPAR-α could be related to the known ability of PPARs to complex with RXRs (11). In this regard, sulindac has previously been reported to induce apoptosis in an embryonic carcinoma cell line (F9) by binding to a truncated form of the retinoid-X–receptor-α (RXRα) (10).

To determine the possible role of PPAR isoforms other than PPAR-α, we tested three different PPAR-γ agonists, troglitazone, rosiglitazone, and pioglitazone, on cultured RPE cells subjected to oxidative stress. Of these three, only troglitazone successfully protected the cells against both TBHP and UVB light-induced loss of viability. This cytoprotective effect of troglitazone was not observed with the two other PPAR-γ agonists, suggesting a selective modulation of PPAR-γ by the different PPAR-γ agonists or a mechanism completely independent of PPAR-γ. In fact, differential effects of PPAR-γ agonists have been reported in previous studies with cultured RPE cells exposed to oxidative stress (33), although no evidence was presented that a preconditioning response was involved. However, because treatment of RPE cells with a PPAR-γ antagonist did not result in significant reversal of the sulindac protection, it appears PPAR-γ is not involved in the sulindac protection of RPE cells described here. A property of PPAR-α which adds further clinical potential to our findings is that PPAR-α is also known to influence the activity of key functional components,

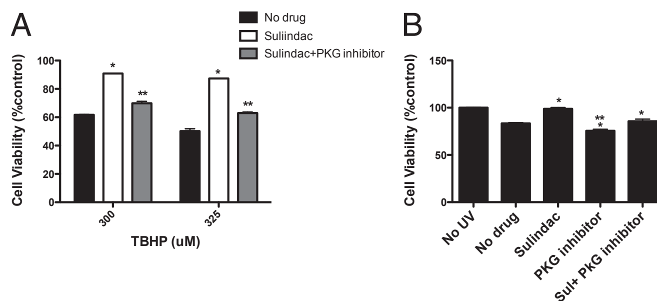

Fig. 3. The sulindac protection effect involves PKG. (*A*) Effect of the PKG inhibitor, Rp-Br-8–PET–cGMPS (250 nM), on sulindac protection of RPE cells exposed to two concentrations of TBHP. (*B*) The effect of inhibiting PKG on sulindac protection after UVB light exposure. The sulindac concentration was 200 μM. *, shows significant difference from no drug. **, shows significant difference from treatment with sulindac.

Fig. 4. PKCε is involved in protection of RPE cells by sulindac. Effect of the specific PKCε inhibitor peptide, V1-2 (10 μM), on sulindac (200 μM) protection of RPE cells against TBHP damage. *, shows significant difference from no drug. **, shows significant difference from treatment with sulindac.

such as vascular endothelial growth factor (VEGF), docosahexanoeic acid (DHA), and matrix metalloproteinases (MMP), which participate in the progression of AMD and are themselves possible targets for therapeutic intervention (13, 14). DHA has been shown to be critical for maintaining the integrity of photoreceptors (34), and its protective effect on RPE cells to oxidative stress is mediated by neuroprotectin D1 (NPD1), a derivative of DHA, that possesses antiapoptotic and anti-inflammatory properties (35, 36).

The first evidence that an IPC-like response was involved in the sulindac protective effect was that the protection was also dependent on PKCε as demonstrated by a loss of sulindac protection when cells were treated with either a nonspecific PKC blocker or a specific PKCε peptide inhibitor. More definitive evidence indicating that protection against oxidative stress in RPE cells is mediated through preconditioning-like pathways was demonstrated by showing the involvement of ROS, PKG, and the mK(ATP) channel. In addition, it was shown that PKC was required for both the sulindac protection of RPE cells and induction of the downstream markers iNOS and Hsp70 following sulindac pretreatment for 48 h. Our results suggest that the protective mechanism involving sulindac and PPAR-α in RPE cells is similar to what is known for IPC and is summarized in Fig. 7. The sulindac protective effect initially involves activation of PKCε and PKG, opening the mK(ATP) channel, resulting in blocking the formation of the MPTP. Notably, in this process there are two types of mitochondrial PKCε involved, one acting on mK(ATP) and the other on MPTP (37). This mitochondrial protective signaling mechanism also involves nuclear gene expression. Sulindac protection involves PPAR-α, which has been reported to increase mitochondrial biogenesis through elevated PGC-1-α levels (38) and regulate peroxisome proliferation (39) as well as increase iNOS transcription (17). Induced iNOS is believed to be a mediator in late-phase ischemic preconditioning (20), and its downstream effects may include modulation of the respiratory chain by inhibiting complex I, which in turn has the potential to elicit decreased mitochondrial ROS generation and enhanced cellular protection (40). In addition, induction of key protective proteins, such as heat shock proteins Hsp27 and Hsp70, was shown to be protective against ischemia/reperfusion damage in cardiac systems (8, 41).

Oxidative stress is the underlying cause of multiple ocular diseases in addition to AMD. Oxidative stress is believed to play a role in glaucoma by affecting the trabecular meshwork (TM) of the eye. In an in vivo study, treatment of TM cells with H_2O_2 resulted in decreased efficiency of drainage of aqueous humor (42), and in an in vitro study of ganglion cells from rats with elevated intraocular pressure (IOP), better survival occurred upon treatment with antioxidants (43). Uptake of antioxidants has also been shown to reduce the levels of oxidation of lens proteins and formation of cataracts (44). Sulindac has several potential advantages as a therapeutic agent for diseases involving oxidative stress including AMD. Based on the previous cardiac ischemic/reperfusion studies, sulindac and its metabolites were highly effective in protecting the heart at oral doses that were only 15% (based on milligrams of drug per kilogram of body weight) of the dose used as an antiinflammatory agent (8). The findings of the current study and of the previous cardiac study (8) suggest that the protective effect of sulindac could provide a possible therapy against AMD, in addition to other ROS-induced ocular disorders, such as glaucoma or cataracts. Sulindac appears to be a unique protective agent in that it shows excellent activity in both a cardiac system and RPE cells and is inexpensive with relatively low toxicity, similar to other NSAIDs.

In conclusion, we present in vitro evidence that pharmacological protection by sulindac could have future clinical applications in protecting RPE cells against oxidative stress and may be effective in preventing the initiation and progression of AMD.

Materials and Methods

Materials. Sulindac, fenofibrate, and TBHP were purchased from Sigma. PPAR inhibitors were obtained from Tocris Bioscience. MTS assay reagents were purchased from Promega. Cell culture medium and supplements were purchased from Life Technologies.

Cell Culture Experiments. The human RPE cell line ARPE19 (American Type Culture Collection #CRL 2302, Rockville, MD) was used in these studies. Cells were maintained in DMEM/F-12 supplemented with 300 μg/mL L-glutamine, 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% (vol/vol) CO_2. In most cases, cells from passages two to five were treated with either no drug or a range of concentrations of the experimental drugs before exposing them to oxidative stress. However, with each newly purchased ARPE19 cell line it was necessary to optimize the passage number, concentration of TBHP required to obtain killing, and the sulindac concentration that afforded protection. In all experiments with sulindac, cells were pretreated with sulindac for 24 h before exposure to oxidative stress. Cells were then washed and exposed to either the TBHP or UVB light to induce oxidative stress. RPE cells were plated in 96-well plates at a concentration of 10,000 cells per well. The concentrations of sulindac and other drugs that were used are noted in the figure legends. Cell viability was measured by using the CellTiter 96 Aqueous One Cell Proliferation Assay (Promega), herein referred to as the MTS assay, following the manufacturer's instructions. This assay uses a tertrazolium salt that is converted to a formazan dye by the activity of mitochondrial NADH oxidase. The change in color due to this conversion was detected by measuring absorbance at 490 nM using a colorimetric microtiter plate reader (Spectramax Plus 384, Molecular Devices).

Fig. 5. Protection by sulindac involves activation of the mK(ATP) channels. Effect of the mK(ATP) channel blocker, 5-HD (75 μM), on sulindac (200 μM) protection of RPE cells exposed to TBHP. *, shows significant difference from no drug. **, shows significant difference from treatment with sulindac.

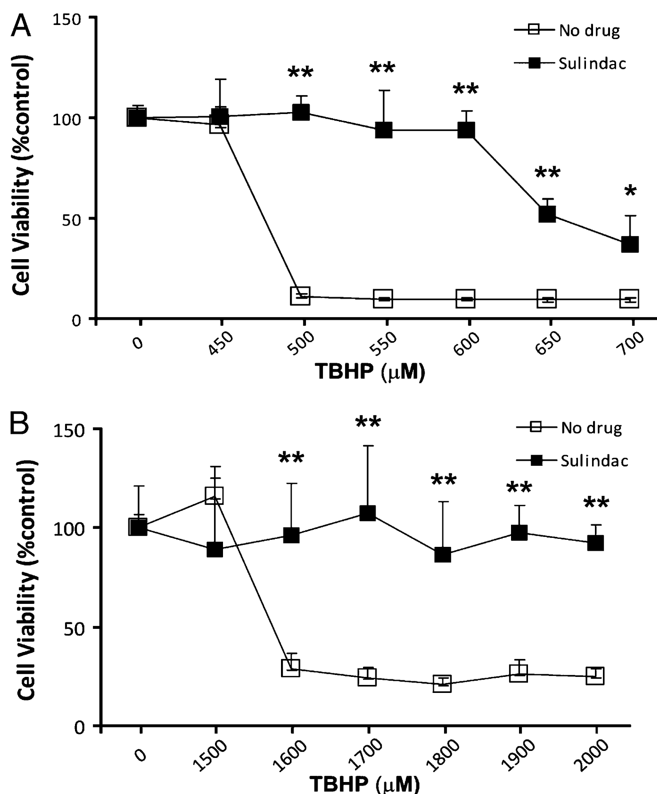

Fig. 6. Sulindac protects human fetal RPE from oxidative stress. The protective effect of sulindac was shown in both passage-three nonpolarized RPE (*A*) and polarized, highly differentiated primary RPE cells (*B*). Human fetal RPE cells were preincubated with 400 μM sulindac for 48 h before exposing them to chemical oxidative stress induced by TBHP. $P < 0.05$ (*); P,0.01 (**).

Cell death was also determined by assaying for LDH (Promega) release following manufacturer's instructions. Apoptotic cell death was determined using the DeadEnd Fluorimetric TUNEL system (Promega), in which apoptotic cells are identified by measuring DNA fragmentation. Apoptotic cells were detected following the manufacturer's protocol and visualized under a Zeiss fluorescent microscope. Three different fields in three different treatments were counted, and the experiments were repeated three times.

Polarized Fetal RPE Cell Culture. The Institutional Review Board (IRB) of the University of Southern California approved the use of human RPE cells under protocol #HS-947005. Human fetal eyes (18–20 wk of gestation) were obtained from Novogenix Laboratories, and written informed consent was obtained from all donors. RPE were isolated from these eyes as described previously (15). The cells were confirmed to be RPE cells by immunocytochemical positivity for cytokeratin (>95%) and the lack of immunoreactivity for endothelial-cell–specific von Willebrand factor (Dako) and glial fibrillary acidic protein (Chemicon). Cells were used from passages two to four. The nonpolarized primary human RPE cells were cultured in DMEM supplemented with 300 μg/mL L-glutamine and 10% FBS at 37 °C and 5% CO_2. Highly differentiated human fetal RPE were seeded in matrigel (BD Biosciences) coated plates or transwells at the density of 1×10^5/cm^2 and cultured in the defined hfRPE medium (Miller medium) supplemented with 1% FBS for 4 wk with medium changed twice weekly (16). Polarized cultures were used once they obtained a transepithelial resistance greater than 300 Ohms·cm^2. Passage-three nonpolarized cells and passage-one highly differentiated polarized cells were used for drug and oxidant treatments. Each experiment was repeated at least three times under independent conditions.

Oxidative Stress in RPE Cells. For TBHP-induced oxidative stress, RPE cells were grown for 24 h in 96-well plates in DMEM/F-12 complete media. The experimental cells were treated with no drug or preincubated with the experimental drug for 24 h, whereas the control cells received no drugs. On the next day the cells were exposed to a range of TBHP concentrations for 24 h. On the following day cell viability was measured using the MTS assay.

For UVB radiation assays the RPE cells were plated in 96-well plates. After 24 h of incubation with or without the drug of interest, the cells were exposed to a UVB light source (Ultraspec 2000, Pharmacia Biotech) that emitted wavelengths between 290 and 370 nm. UVB light at an intensity of 1,200 mJ/cm^2 was used for the experiments. The duration of exposure was determined using the formula: $H\lambda = t \times E\lambda$, where $H\lambda$ is the energy level (J/cm^2), t is the exposure duration in seconds, and $E\lambda$ is the irradiance (W/cm^2) of the UVB source. Irradiance was measured at 1.3 W/cm^2, and the exposure time for an energy level of 1,200 mJ/cm^2 was calculated to be 14 min and 24 s. Immediately after the UVB exposure the media was replaced with fresh DMEM/F-12 medium. After 24 h of incubation at 37 °C and 5% CO_2, cellular viability was measured using the MTS assay.

Studies on the Mechanism of Sulindac Protection. To investigate the involvement of the PKC pathway in the sulindac protection mechanism, the PKC inhibitor chelerythrine (Sigma) was used at a concentration of 2 μM. The inhibitor was added simultaneously with the drug 24 h before exposing the cultured RPE cells to oxidative stress. To further analyze which specific isoform of PKC is involved in the sulindac protective mechanism, specific inhibitors were used for the two PKC isoforms, PKCε and PKCδ. The peptide V1-2 (Anaspec) was used to inhibit PKCε, and rottlerin (Sigma) was used to inhibit PKCδ. The inhibitors were added at the same time as the sulindac, 24 h before exposing the cells to TBHP. See legends to Fig. 4 and Figs. S3 and S4 for further details. For studying the involvement of PKG in the sulindac protection effect, Rp–Br-8–PET–cGMPS (Sigma), a known chemical inhibitor of PKG, was used. The PKG inhibitor, at a concentration of 250 nm, was added at the same time as sulindac, 24 h before exposing the RPE cells to either TBHP- or UV-induced stress.

Western Blotting. This was performed according to an established protocol (45). Proteins were isolated from RPE cells cultured in 60-mm dishes with no drug, sulindac, or a combination of sulindac and chelerythrine. β-actin was used as a loading control for the protein isolation procedure. Hsp70 (1:1,000) and iNOS (1:200 dilution) were detected with primary antibodies from Santa Cruz Biotechnology.

Quantification of the Western Blots. The Western blotting gel images of three independent experiments were scanned and quantified by densitometric analysis. ImageJ software (Image J version 1.46r, Java 1.6.0_65 (32 bit), available at imagej.nih.gov/ij/, National Institutes of Health) was used for the quantification of the bands. Band intensities of Hsp70 and iNOS were measured using the gel tool and normalized with the β-actin bands on the same blot.

Statistical Analysis. Unless otherwise noted, results of all cell viability experiments represent the mean of three replicates of a representative experiment. Data are presented as mean ± SE. The means were compared using standard t tests, and P values <0.05 were considered to be statistically significant. Statistical analyses were conducted using GraphPad Prism 4.0.

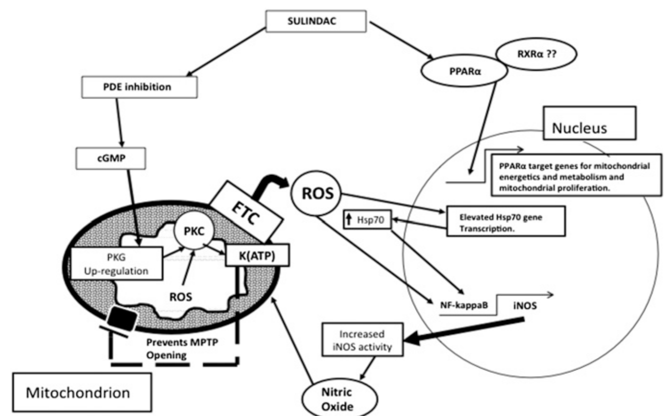

Fig. 7. Summary of the proposed mechanism involved in the protection of RPE cells by sulindac. This protective mechanism appears to be similar to what has been described for ischemic preconditioning, as discussed in the text.

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