Supplementary

MATERIAL AND METHODS

Materials

Dulbecco's Modified Eagle Medium, Nutrient Mixture F-12 medium, Biotin-14-ATP, Alexa Fluor 488 goat anti-rabbit immunoglobulin G and Alexa Fluor 488-conjugated streptavidin were obtained from Life Technologies Corporation (Carlsbad, CA).

Paraformaldehyde, PGE2, aspirin, etodolac, diclofenac, meloxicam and pranoprofen were obtained from Wako Co (Osaka, Japan). Celecoxib was from LKT Laboratories Inc (St Paul, MN). Peptides for the assay of caspase-3-like activity were from Peptide Institute Inc (Osaka, Japan). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bromfenac, ibuprofen, glucose, sorbitol and indomethacin were obtained from Sigma Aldrich (St. Louis, MO). Terminal nucleotidyl transferase (TdTase) and the 5-bromo-2′-deoxyuridine (BrdU) enzyme immunoassay (EIA) kit were obtained from
Roche (Mannheim, Germany). VECTASHIELD was from Vector Laboratories (Burlingame, CA). 4',6-diamidino-2-phenylindole (DAPI) was from Dojindo Laboratories (Kumamoto, Japan). The RNeasy kit was obtained from Qiagen Inc (Valencia, CA). The first-strand cDNA synthesis kit was from Takara Bio (Ohtsu, Japan) and the SsoFast™ EvaGreen® Supermix was from Bio-Rad (Hercules, CA).

Antibodies against actin and lamin B were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An antibody against NFAT5 was from Thermo Fischer Scientific (Waltham, MA, USA). Diclofenac eye drops and control eye drops were a gift from Wakamoto Co (Tokyo, Japan). The PGE$_2$ EIA kit and SC-560 were purchased from Cayman Chemical (Ann Arbor, MI).

**Assay for Caspase Activity**

Caspase-3-like activity was determined as described previously. Briefly, cells were collected by centrifugation and suspended in extraction buffer (50 mM PIPES/KOH (pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM MgCl$_2$ and 1 mM dithiothreitol). Samples were sonicated and centrifuged, after which the supernatants were incubated with fluorogenic
peptide substrates (acetyl-DEVD-methylcoumarin amide) in reaction buffer (100 mM HEPES/KOH (pH 7.5), 10% sucrose, 0.1% CHAPS and 1 mg/ml bovine serum albumin) for 2 h at 37°C. The release of aminomethylcoumarin (AMC) was determined using a fluorescence spectrophotometer. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol of AMC/min.

**Real-time RT-PCR Analysis**

Total RNA was extracted using the RNeasy kit according to the manufacturer’s protocol. Samples (1 µg of RNA) were reverse-transcribed using the first-strand cDNA synthesis kit according to the manufacturer’s instructions. Synthesized cDNA was used in real-time RT-PCR experiments (CFX96™ Real time system, Bio-Rad) using SsoFast™ EvaGreen Supermix, and analyzed with Opticon Monitor software according to the manufacturer’s instructions. To normalize the amount of total RNA present in each reaction, actin was used as an internal standard. Primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used were (name, forward primer and reverse primer): bgt1,
5′-ccgaggagggagagaagtt-3′ and 5′-tccatcttggtggtcattgg-3′; actin,
5′-tgccctttctctggcact-3′ and 5′-tgtgacctctgctgtctgtg-3′; tnf-α,
5′-aggccaagccctggtatgagc-3′ and 5′-cacagggcaatgatcccaaagtag-3′; mmp-9,
5′-tgacagcgacaagaagtg-3′ and 5′-cagtaagcggtacataggg-3′.

BrdU Incorporation Assay

Cell proliferation was estimated based on the incorporation of BrdU. Cells were incubated with BrdU (10 μM) for 2 h and fixed. BrdU incorporation was determined with the BrdU EIA kit according to the manufacturer’s instructions.

Immunoblotting Analysis

Whole cell and nuclear extracts were prepared as described previously. The protein concentration of each sample was determined by the Bradford method. Samples were applied to 8% (NFAT5 and lamin B) or 12% (actin) SDS polyacrylamide gel electrophoresis, after which the proteins were immunoblotted with each antibody.
TdT-mediated Biotinylated UTP Nick End Labelling (TUNEL) and Immunohistochemical Analyses

Eyes were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4 µm-thick sections.

For TUNEL analysis, sections were incubated first with proteinase K for 15 min at 37°C, then with TdT and biotin 14-ATP for 1 h at 37°C, and finally for 2 h with Alexa Fluor 488 conjugated with streptavidin. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX73).

For immunohistochemical analysis to detect NFAT5, sections were incubated with proteinase K for 20 min for antigen activation before blocking with 3% bovine serum albumin for 30 min, incubated for 12 h with antibody to NFAT5 (1:100 dilution) in the presence of 3% bovine serum albumin. Sections were then incubated with Alexa Fluor 488 goat anti-rabbit immunoglobulin G. Samples were mounted with VECTASHIELD and inspected with the aid of a fluorescence microscope (Olympus IX73). Fluorescence intensity was determined using LuminaVision software.

