Analysis of Porcine Optineurin and Myocilin Expression in Trabecular Meshwork Cells and Astrocytes from Optic Nerve Head

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PURPOSE. To determine the cDNA sequences and analyze the expression of porcine optineurin and myocilin in trabecular meshwork cells (TMCs) and astrocytes from the optic nerve head under normal and experimental conditions.

METHODS. Both porcine optineurin and myocilin were cloned to determine the cDNA sequences. Porcine TMCs and astrocytes were isolated and treated with dexamethasone (500 nM) for 2 weeks, incubated under hypoxic conditions (7% O2) for 72 hours, or exposed to 35 mm Hg hydrostatic pressure for 72 hours. A 10% mechanical stretch for 24 hours was also performed on TMCs. The expression level of the optineurin and myocilin transcripts was analyzed by real-time quantitative PCR.

RESULTS. The sequences of porcine optineurin and myocilin cDNA were determined, and the expression of both genes was confirmed in both TMCs and astrocytes. Amino acid sequences of porcine optineurin and myocilin were homologous to those of humans by 84% and 82%, respectively, and shared protein motifs and modification sites. The expression of myocilin mRNA by TMCs and astrocytes was increased by 8.0- and 5.5-fold, respectively, after exposure to dexamethasone. In contrast, the expression of optineurin was suppressed to 68% in TMCs and 48% in astrocytes after exposure to dexamethasone. A significant reduction of myocilin expression was observed after 72 hours of incubation under hypoxic conditions in both types of cells, whereas optineurin was not affected. Hydrostatic pressure for 72 hours and mechanical stretching for 24 hours had minimal effects on gene expression of both optineurin and myocilin.

CONCLUSIONS. The high homology of porcine optineurin and myocilin to the comparable human genes indicates that pigs can be used to study changes in gene expression in hypertensive eyes. The alterations in expression of myocilin but not of optineurin under stress suggest that different mechanisms in the phenotype of glaucoma associated with the two genes are involved in development of glaucoma. (Invest Ophthalmol Vis Sci. 2004;45:2652–2659) DOI:10.1167/iovs.03-0572

Characteristic degeneration and excavation of the optic nerve head are found in glaucomatous eyes. These changes are considered to be due to ocular hypertension with the intraocular pressure (IOP) continuously more than 21 mm Hg. In contrast, there are patients with normal ocular tension who show glaucomatous changes in the optic nerve head. These patients, in whom there is no evidence of an elevation of IOP at any time, are said to have normal-tension glaucoma (NTG).

Currently three genes—myocilin (MYOC),1,2 cytochrome P4501B1 (CYP1B1),3,4 and optineurin (OPTN)5—are associated with glaucoma. Optineurin is the most recent gene to be identified and is responsible for 16.7% of families with hereditary NTG.5 It has been identified and studied by different groups under various names: NRP, NF-eB essential modulator (NEMO)-related protein6; FIP-2, adenovirus E3-14-kDa interacting protein 27; Huntington interacting protein L (HYPL)8; and transcription factor IIIA interacting protein (TFIIA-INTP).9 Optineurin is homologous to NEMO, a structural and regulatory subunit of the high molecular weight kinase complex (IKK) that is responsible for the phosphorylation of NF-eB inhibitors.6

Some of the functions of optineurin are known. They include inhibition of the tumor necrosis factor (TNF)-α pathway,7 interaction with transcription factor IIIA,9 and mediation of the interaction of Huntington and Rab8 for regulation of membrane trafficking and cellular morphogenesis.8 Optineurin is induced by TNF-α and binds to an inhibitor of TNF-α and the E3-14.7-kDa protein.7

The optineurin protein contains two leucine zippers (LZs); an N-terminal LZ responsible for the association with Rab8, and a C-terminal LZ required for Huntingtin. The gene is mapped to 10p14 and contains 16 exons encoding a 66-kDa protein. It contains two putative bZIP transcription factor motifs, a C2H2 type zinc finger, and two LZ domains.

Recently, Vittitow and Borra’s10 reported that elevated IOP, and exposure to TNF-α and dexamethasone (DEX) led to an upregulation of optineurin expression in an organ culture system. However, it is still unclear how mutations of the optineurin gene lead to glaucoma.

Another gene associated with glaucoma is myocilin, which is found in 36% of juvenile-onset POAG and 4% of adult-onset POAG.11–15 Myocilin is a 57-kDa protein that contains motifs homologous to the olfactomedin domain where nearly all mutations in patients with POAG have been identified.11–15

Pigs and miniature pigs are readily available and have been used for a wide variety of medical studies, including tissue transplantation.16,17 Because their eyes are similar in size and...
anatomy to human eyes,18 pigs have often been used to study the aqueous outflow system and the regulation of IOP.

The purpose of this study was to clone both the porcine optineurin and myocilin genes to determine their cDNA sequences, and then to use the sequences to determine the transcriptional response of isolated porcine trabecular meshwork cells (TMCs) and astrocytes from the optic nerve head after exposure to dexamethasone (DEX), increased hydrostatic pressure, hypoxia, and mechanical stretching.

Materials and Methods

Cell Cultures

Pig eyes were obtained within 3 hours of death from a local abattoir. The eyes were disinfected in 0.2% povidone iodine for 10 minutes followed by soaking in 70% alcohol for 30 seconds. The eyes were washed several times in phosphate-buffered saline (PBS) and cut into halves along the equator.

After the lens and iris were removed from the anterior half, the trabecular tissue was trimmed from the cornea at the Schwalbe’s line and then from the sclera, as described.19-20 The optic nerve head was separated from the sclera and surrounding tissues. The prelaminar region was dissected from the optic nerve head and cut into three or four pieces.21,22 The trabecular and prelaminar tissues were placed separately in 35-mm plastic Petri dishes in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen-Gibco, Grand Island, NY) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (Invitrogen-Gibco).

The tissues were incubated for 1 to 2 weeks at 37°C in humidified 5% CO₂ and 95% air until cells migrated from the tissue onto the surface of the culture dish. Cells were isolated, and fourth-passage cells were obtained for experimental use. The cells that migrated from the optic nerve head were confirmed to be astrocytes by immunostaining with antiglial fibrillary acidic protein (GFAP), a protein marker for astrocytes (Sigma-Aldrich).

Cloning of Porcine Optineurin cDNA

mRNA was isolated from cultured TMCs using mRNA isolation kits (MicroPoly(A)Pur; Ambion, St. Austin, TX). Primers (sense primer, 5′-AGGCTCCTCAAAGGGGTGAC-3′; antisense primer 5′-GTTGCTCCTGCTGGTAATAA-3′) were designed to include the conserved sequences for human, mouse (Discovery System; Celera, Gaithersburg, MD), and rat to amplify the open reading frame of porcine optineurin mRNA using a commercial system (Superscript One-Step RT-PCR System; Invitrogen-Gibco), according to the manufacturer’s protocol.

The PCR products were cloned into a TA cloning vector (pDrive; Qiagen, Valencia, CA) using a PCR Cloning Kit (Qiagen), and the inserts were sequenced using a fluorescent deoxyxynucleotide automated sequencer (CEQ2000XL DNA Analysis System; Beckman-Coulter, Fullerton, CA). The missing 3′ and 5′ ends of the cDNAs were amplified using the 3′ and 5′ rapid amplification of cDNA ends (RACE) method (Marathon cDNA Amplification Kit; BD Biosciences-Clontech, Palo Alto, CA). The full-length cDNA sequence of porcine optineurin can be obtained from GenBank under accession number AF513722 (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Cloning of Porcine Myocilin cDNA

The same mRNA used for optineurin cDNA cloning was used for myocilin cDNA amplification. The sense primer, 5′-ATGCGAGCATG/G/CTGGACGCTGCT-3′, and antisense primer, 5′-GACCATGTTGAGTTGTCCCA-3′, were designed to include the conserved sequence of human, mouse, rat, and bovine myocilin and to amplify the open reading frame of porcine myocilin mRNA, using the RT-PCR system (Superscript One-Step RT-PCR System; Invitrogen-Gibco). The PCR products were cloned into a TA cloning vector (TA Cloning Kit; Invitrogen, San Diego, CA), and the inserts were sequenced. The missing 3′ and 5′ ends of the cDNAs were amplified using the RACE method (Marathon cDNA Amplification Kit; BD Biosciences-Clontech). The full-length cDNA sequence of porcine myocilin can be obtained from GenBank under accession number AF550447.

Sequence Analysis of Porcine Optineurin and Myocilin

Amino acid sequences of both optineurin and myocilin were analyzed for domain structure and potential protein modification sites. The PROSITE scanning tool23 (http://www.ncbi.nlm.nih.gov/prosite/) provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland) was used to scan the optineurin protein sequence for the occurrence of patterns and profiles stored in the PROSITE database. Potential glycosylation and phosphorylation sites were predicted by the program developed by Hansen et al.,24 and Blom et al.,25 respectively. Sequence homology was determined by a sequence-analysis program (Omiga 2.0; Accelerlys, San Diego, CA).

Stress Experiments for Optineurin and Myocilin

All stress experiments were performed using fourth-passage TMCs and astrocytes from three different porcine eyes. For the DEX treatment. DEX stock solution (50 mM DEX/dimethyl sulfoxide) was added to cultured TMCs and astrocytes at a final concentration of 500 nM. The culture medium was replaced every 3 days and maintained for 2 weeks. For control, cultured cells were treated with dimethyl sulfoxide alone.

To examine the effect of hypoxia, both types of cultured cells were incubated in 7.0% O₂ and 5% CO₂, in a multiple gas incubator (model 9200; Wakenyaku, Kyoto, Japan) for 12, 24, 48, or 72 hours. Control cells were incubated for the same times in 5% CO₂ and 95% air in a standard CO₂ incubator.

To examine the effects of hydrostatic pressure, we exposed both types of cultured cells to a hydrostatic pressure of 33 mm Hg above atmospheric pressure for 12, 24, 48, or 72 hours in a CO₂ incubator, using the system illustrated in Figure 1. The culture flasks were filled with the medium and capped with a silicon stopper to prevent leakage. The height of the reservoir containing the medium was adjusted to control the pressure in the flask. For gas exchange, the medium was circulated with a peristaltic pump (Eyela, Tokyo, Japan), and the pressure was monitored with a pressure gauge (model PG-208; Copal Electronics, Tokyo, Japan). Control cells were exposed to hydrostatic pressure of 5 mm Hg above atmospheric pressure for 12, 24, 48, and 72 hours.

To examine the effects of mechanical stretching, cultured porcine TMCs were transferred onto a 10-cm² collagen-coated silicon chamber (S.Tec, Osaka, Japan). The silicon chamber had a 100-µm-thick transparent bottom, and the side walls were 1.5-mm thick to prevent narrowing at the bottom center. The silicon chamber was then attached to a stretching apparatus for a 10% linear stretch for 24 hours in a standard CO₂ incubator. Control cells were plated onto a collagen-coated silicon chamber without the stretching for the same amount of time.

Optineurin and Myocilin Transcript Analysis

Total RNA was isolated from cultured cells exposed to stimuli or stresses (RNAzol B; Tel-Test, Friendswood, TX). The total RNA was reverse transcribed (Superscript First Strand Synthesis System for RT-PCR; Invitrogen-Gibco) according to the manufacturer’s protocol. Real-time quantitative PCR was performed to determine the optineurin, myocilin, and glyceroldehyde-3-phosphate dehydrogenase (GAPDH) transcript with a sequence-detection system (GeneAmp 5700; Applied Biosystems, Inc. [ABI], Foster City, CA). PCR reactions were performed in 50 µL of reaction mixture containing 25 µL master PCR mix (SYBR Green PCR Master Mix; ABI), 5 µM primer pairs, and 1 µL cDNA samples. To measure myocilin transcript, 4 µL cDNA samples was used because of lower expression. The 18S ribosomal RNA gene was used as
an internal control. All the primers used in these reactions were designed on computer (Primer Express software; ABI). For myocilin cDNA amplification, sense primer 5’-GCTCAATGCCAGTGAAA-3’ and antisense primer 5’-ACGCCGTACTGGCCGTATT-3’ were used.

For amplification of optineurin cDNA, sense primer 5’-GACCA-CAACAGGCTTCTTCA-3’ and antisense primer 5’-GTCTGCAATTG-CAGCTTTCC-3’ were used. For GAPDH cDNA amplification, sense primer 5’-TCACTGAAATCGCTCTGTAC-3’ and antisense primer 5’-GTGGCAGCTGACTGCTTG-3’ were used. For 18S rRNA, sense primer 5’-GATCGAAGACGATCAGATACC-3’ and antisense primer 5’-CCAGACAAATACCTCCAC-3’ were used.

To confirm the specificity of PCR reaction, each PCR product was analyzed by agarose gel and subjected to a dissociation curve analysis, according to the manufacturer’s instructions.

RESULTS

Cloning of Porcine Optineurin cDNA

The nucleotides and deduced amino acid sequences of porcine optineurin are shown in Figure 2A. A comparison of the predicted amino acid sequences of pig, human, mouse, and rat optineurin is shown in Figure 2B. The porcine optineurin is composed of 574 amino acids, and the homology of porcine optineurin to mouse, rat, and human was 71%, 72%, and 84%, respectively. Two LZ motifs, reported in the human optineurin, were also present in porcine optineurin at residues 103-152 (Fig. 3A, dashed underscore). Two LZ motifs, reported in the human optineurin, were also present in porcine optineurin at residues 103-152 (Fig. 3A, dashed underscore). Two LZ motifs, reported in the human optineurin, were also present in porcine optineurin at residues 103-152 (Fig. 3A, dashed underscore).

Two tyrosine residue were predicted (Fig. 3A, bold italic).

The response of optineurin and myocilin to DEX was significantly decreased (to 67% and 48%, respectively), compared with that of untreated TMCs and astrocytes (Figs. 4A–C). The expression of porcine myocilin exposed to DEX increased by 8.02 ± 1.26-fold (mean ± SD) and 5.57 ± 1.05-fold in cultured TMCs and astrocytes, respectively. The expression of GAPDH was not altered in both types of cells by exposure to DEX.

Incubation under Hydrostatic Pressure and with Mechanical Stretching

After 72 hours under hydrostatic pressure or 24 hours of mechanical stretching, optineurin and myocilin expression was unchanged in both TMCs and astrocytes.

DISCUSSION

Our results demonstrated that both optineurin and myocilin were expressed in porcine TMCs and astrocytes, and their amino acid sequences were homologous to human sequences by 84% and 82%, respectively. The protein motifs and protein modification sites were also shared with humans.

The response of optineurin and myocilin to DEX was different. Optineurin expression was decreased, whereas that of myocilin was increased. The increased expression of myocilin by TMCs confirmed earlier observations, but we also detected an increase in astrocytes. Astrocytes are the major glial cells populating the optic nerve head and are probably responsible for the remodeling of the optic nerve head in glaucomatous eyes. Astrocytes are known to function as cellular support.
Porcine Optineurin

FIGURE 2. Nucleotide sequence and deduced amino acid sequence of porcine optineurin and comparison of porcine optineurin amino acid sequences with those of other species. The coding region is defined by the positions of the initiation codon (ATG) and stop codon (TAA). (A) The porcine optineurin protein is composed of 574 amino acids. Dashed underscore: LZ motifs; solid underscore: glutamic acid-rich region; circles: O-glycosylation sites. (B) Only the amino acids that differ from porcine or human optineurin sequences are shown for mouse and rat. Hyphens: the same amino acid residues as human optineurin; spaces: the absence of amino acids corresponding to the same location in human optineurin; asterisks: positions of amino acids associated with glaucoma.
Porcine Myocilin

**FIGURE 3.** Nucleotide sequence and deduced amino acid sequence of porcine myocilin and comparison of porcine myocilin amino acid sequences with those of other species. (A) Porcine myocilin is composed of 489 amino acids. Circles: O-glycosylation sites; squares: predicted N-glycosylation sites; dashed underscore: LZ motif. (B) Only the amino acids that differ from pig or human myocilin sequence are shown for mouse, rat, bovine, and monkey. Hyphens: the same amino acid residues as human myocilin. Spaces: absence of amino acids corresponding to the same location in human myocilin. Asterisks: positions of amino acids associated with glaucoma.

FIGURE 4. Quantitative PCR analysis of optineurin and myocilin under various conditions. The relative gene expression of optineurin, myocilin, and GAPDH is shown for each stimulus or stress condition. Expression level for control cells are shown as 1. (A–C) DEX treatment (500 nM for 2 weeks); (D–F) hypoxia (7.0% O₂); (G–I) hydrostatic pressure (35 mm Hg above atmospheric pressure), and mechanical stretching (a 10% linear stretch for 24 hours).
comatous process, remodeling of the extracellular matrix and reactive astrocytes induced after mechanical injury by increased IOP may play major roles in damaging the optic nerve axons. Significant changes of myocilin expression in astrocytes may alter the normal function of the astrocytes to support the optic nerve head. The tissues in the optic nerve head are central to the pathologic course in glaucomatous eyes; thus, the effect of the fivefold elevation of myocilin transcript after DEX treatment in astrocytes should be further investigated.

Optineurin, in contrast, was significantly decreased in both astrocytes and TMCs after exposure to DEX. Optineurin has been shown to interact with the E3-14.7 kDa protein, one of the three protein encoded by human adenovirus C early region 3 (E3) that use TNF-α or Fas ligand pathways to mediate apoptosis and inflammation. TNF-α plays a critical role in protecting cells from virus infection, which concurrently had been the target for virus. A downregulation of optineurin under DEX may result in the loss of the protective functions, especially in the optic nerve head.

Under hypoxic conditions that mimic the ocular hemodynamic condition in eyes with NTG the expression of myocilin was significantly reduced in both TMCs and astrocytes, whereas the expression of control GAPDH was increased more than twofold after 12 hours in both types of cells. Myocilin transcription was practically shut down in astrocytes after 72 hours, whereas the transcription of optineurin was not affected by the hypoxia. The significant changes of myocilin transcription were not due to cell death, as shown by trypan blue staining (Fig. 5).

A significant increase of GAPDH by hypoxia suggests that the transcriptional machinery is still active in TMCs under hypoxic conditions. In addition, the reduction of both OPTN and MYOC did not occur after 12 hours, which is enough time for the gas exchange in the culture medium of TMCs, suggesting that the transcriptional shut down was not triggered directly by hypoxia but by a factor(s) activated by hypoxia indirectly affecting the transcriptional regulation of both OPTN and MYOC.

Hydrostatic pressure had no affect on gene expression in both TMCs and astrocytes. Recently, Kamphuis and Schneemann34 also reported no change of optineurin gene expression by pressure elevation in an anterior chamber perfusion model. Pressure elevation in a perfusion system is likely to stress the cells by compression and mechanical stretching. Data collected under our experimental conditions fully agree with the perfusion experiments by Kamphuis and Schneemann. Vitti-tow and Borras10 have reported an increase of optineurin expression by 60% after 7 days of elevated pressure in a perfusion system. Quantification of gene expression by PCR followed by a gel scanner is usually difficult, with an accuracy within 50%, as described.10 Their results are inconsistent with those of Kamphuis and Schneemann34 and our results. Our results showed that the expression of myocilin is not affected by hydrostatic pressure or mechanical stretch, although Tamm et al.57 had previously shown induction of myocilin by mechanical stretching in human TMCs. These results demonstrated that hydrostatic pressure of +3.5 mm Hg or a mechanical stretch of 10% is not sufficient to increase the myocilin gene expression in TMCs under elevated IOP.

In this study, optineurin and myocilin behaved differently in TMCs from astrocytes during changes of cellular environment by DEX treatment, hypoxia, hydrostatic pressure, or stretching. These results suggest that different mechanisms may be involved in the development of glaucoma by defects in these two genes.

References

Optineurin and Myocilin in Trabecular Meshwork


