

# Oxidative stress regulates expression of claudin-1 in human RPE cells

Communication

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**Abstract:** Age-related macular degeneration (AMD) is a neurodegenerative disease associated with irreversible loss of central vision in the elderly. Disruption of the homeostatic function of the retinal pigment epithelium (RPE) is thought to be fundamental to AMD pathogenesis, and oxidative stress is implicated in the associated RPE damage. We examined the effects of oxidative stress on the expression of junctional proteins in cultured human retinal pigment epithelial (ARPE-19) cells. Reverse transcription-PCR and immunoblot analyses revealed that expression of the tight-junction protein claudin-1 was increased at both the mRNA and protein levels 8 to 12 h after exposure of ARPE-19 cells to H<sub>2</sub>O<sub>2</sub>, whereas that of the tight-junction protein ZO-1 or the adherens-junction protein N-cadherin was unaffected. Expression of both claudin-1 and N-cadherin was down-regulated by exposure of the cells to H<sub>2</sub>O<sub>2</sub> for longer periods (24 to 48 h). Oxidative stress also induced the phosphorylation of p38 mitogen-activated protein kinase (MAPK) with a time course similar to that apparent for the up-regulation of claudin-1 expression. Furthermore, the increase in the abundance of claudin-1 induced by H<sub>2</sub>O<sub>2</sub> was blocked by the p38 inhibitor SB203580. Phosphorylation of the MAPKs ERK and JNK was not affected by H<sub>2</sub>O<sub>2</sub>. Our results suggest that modulation of claudin-1 expression in the RPE by oxidative stress may contribute to the pathogenesis of AMD.

**Keywords:** Age-related macular degeneration • Retinal pigment epithelium • Oxidative stress • Claudin-1 • Tight junction • p38 Mitogen-activated protein kinase

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## Nonstandard abbreviations:

AMD - age-related macular degeneration;  
BRB - blood-retinal barrier;  
BSA - bovine serum albumin;  
ERK - extracellular signal-regulated kinase;  
G3PDH - glyceraldehyde-3-phosphate dehydrogenase;  
JNK - c-Jun NH<sub>2</sub>-terminal kinase;  
MAPK - mitogen-activated protein kinase;  
PBS(-) - Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline;  
RPE - retinal pigment epithelium;  
RT - reverse transcription;  
TER - transepithelial electrical resistance;  
TJ - tight junction;  
ZO-1 - zonula occludens-1.

## 1. Introduction

Age-related macular degeneration (AMD) is a major cause of visual impairment in the elderly and a leading cause of blindness in the Western world [1]. AMD is characterized by a loss of photoreceptors in the central retina that is associated with dysfunction of the retinal pigment epithelium (RPE), Bruch's membrane, and the choroid [2]. The RPE lies between the photoreceptors of the neurosensory retina and the choroidal capillary bed, and it comprises a specialized monolayer of quiescent cells that perform various functions essential for the maintenance of photoreceptor activity and survival. The RPE is also required for the retinoid cycle and for formation of the blood-retinal barrier (BRB),

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a highly selective and regulatable barrier between the retina and choroid that is responsible for the transport of nutrients and ions between photoreceptors and the choriocapillaris and is essential for normal vision [6]. These various features of the RPE, which are essential to its function, require specialized intercellular junctions [3,10].

Given that RPE cells constitute a key component of the BRB, RPE damage or loss invariably results in retinal degeneration [3-5]. Indeed, degeneration of the RPE during the development of AMD leads to disruption of the BRB and to photoreceptor apoptosis. Oxidative stress has been implicated as a contributor to the development of AMD [2,7-9], although the mechanism by which oxidative stress might lead to RPE cell death has remained unclear.

The response of the RPE to oxidative stress has been examined with the use of RPE cell lines. Treatment with hydrogen peroxide ( $H_2O_2$ ) has thus been shown to affect the expression of heat shock protein 70 [11,12], fibroblast growth factor (FGF) 2 [13], and FGF receptors [14] in such cell lines. Such treatment also down-regulated the expression of the RPE marker RPE-65 [14]. Formation of the BRB by the RPE is dependent on the function of tight junctions (TJs) to restrict the diffusion of nontransported solutes. Adherens junctions also mediate intercellular adhesion, a process that plays a key role in maintaining tissue integrity and the normal morphology of RPE cells. The adherens-junction protein N-cadherin was identified as the major cadherin responsible for development of an epithelial phenotype in cultured RPE cells [15]. However, the effects of oxidative stress on the expression of junctional proteins in RPE cells have remained largely unexplored.

In this study, we have examined the effects of oxidative stress on the expression of junctional proteins and barrier function in cultured human RPE (ARPE-19) cells. Specifically, we investigated the effects of short- or long-term exposure of the cells to  $H_2O_2$  on the expression of tight- and adherens-junction proteins, on the basis of the assumption that such exposure might mimic the progression of AMD.

## 2. Experimental Procedures

### 2.1 Antibodies and reagents

Rabbit polyclonal antibodies to zonula occludens-1 (ZO-1) or to claudin-1 were obtained from Zymed (Carlsbad, CA), and those to N-cadherin were from Transduction Laboratories (Lexington, KY). Mouse monoclonal antibodies to  $\alpha$ -tubulin were from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated

secondary antibodies were obtained from Promega (Madison, WI), and AlexaFluor 488-conjugated secondary antibodies were from Molecular Probes (Carlsbad, CA). Mouse monoclonal antibodies to extracellular signal-regulated kinase (ERK) or to phosphorylated ERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to phosphorylated or total forms of c-Jun NH<sub>2</sub>-terminal kinase (JNK) or p38 were from Cell Signaling Technology (Danvers, MA). SB203580 was obtained from Calbiochem (San Diego, CA).

### 2.2 Cell culture

The human RPE cell line ARPE-19 was obtained from American Type Culture Collection (Manassas, VA). The cells were seeded in 30-mm culture dishes ( $1 \times 10^6$  cells) or in 24-well Transwell culture plates ( $5 \times 10^4$  cells per well; Costar, Cambridge, MA) and were cultured in Dulbecco's modified Eagle's medium-F-12 (Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. They were maintained in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. For examination of the effects of oxidative stress on junctional protein expression and protein phosphorylation, the semiconfluent cells were exposed to  $H_2O_2$  (500  $\mu$ mol L<sup>-1</sup>) in complete culture medium. Cell number at various times after exposure to  $H_2O_2$  was determined with the use of a Coulter Counter Z1 (Beckman, CA).

### 2.3 RT-PCR analysis

Total RNA was isolated from ARPE-19 cells with the use of an RNeasy kit (Qiagen, Valencia, CA), and portions (0.5  $\mu$ g) of the RNA were subjected to reverse transcription (RT) and PCR analysis with a One-Step RT-PCR kit based on the Platinum Taq system (Life Technologies, Carlsbad, CA). The PCR protocol was designed to maintain amplification in the exponential phase. The sequences of the PCR primers (sense and antisense, respectively) were 5'-TGCCATTACACGGTCTCTG-3' and 5'-GGTTCTGCCTCATCTTCCTC-3' for ZO-1, 5'-TTCTCGCCTTCCTGGGATG-3' and 5'-CTTGAACGATTCTATTGCCATACC-3' for claudin-1, 5'-CACTGCTCAGGACCCAGAT-3' and 5'-TAAGCCGAGTGATGGTCC-3' for N-cadherin, and 5'-ACCACAGTCCACGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (G3PDH, internal control). The RT and PCR incubations were performed with a GeneAmp PCR System 2400-R (Perkin-Elmer, Foster City, CA). RT was performed at 50°C for 30 min, and PCR was performed for 25 cycles, with each cycle comprising incubations at 94°C for 2 min, 58°C for 30 s, and 72°C for 1 min. The reaction mixture was

finally cooled to 4°C, and the amplification products were fractionated by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide. The intensity of the bands was measured with the use of an image analyzer and Multi Gauge V3.0 software (Fuji Film, Tokyo, Japan), and the values for the abundance of ZO-1, claudin-1, and N-cadherin mRNAs were normalized by the corresponding value for G3PDH mRNA. For RT and real-time PCR analysis, total RNA was subjected to RT with the use of a kit (Life Technologies) and the resulting cDNA was subjected to real-time PCR with a TaqMan Fast Advanced Master Mix kit (Life Technologies) and a 7900HT Real-Time PCR system (Applied Biosystems, Foster City, CA).

## 2.4 Immunoblot analysis

ARPE-19 cells were washed twice with phosphate-buffered saline and lysed in 300 µl of a solution containing 150 mmol L<sup>-1</sup> NaCl, 2% SDS, 5 mmol L<sup>-1</sup> EDTA, and 20 mmol L<sup>-1</sup> Tris-HCl (pH 7.5). The lysates were centrifuged at 15,000 × *g* for 15 minutes at 4°C, and the resulting supernatants were subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to blocking buffer for 1 hour at room temperature before incubation with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Immune complexes were incubated with enhanced chemiluminescence reagents (GE Healthcare UK, Little Chalfont, UK) for 5 minutes. Band intensities in the linear range were quantitated by densitometric scanning of film with the use of an image analyzer and Multi Gauge V3.0 software.

## 2.5 Immunofluorescence analysis

Cells were fixed with 100% methanol for 10 min at -20°C, washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline (PBS(-)), and incubated for 1 h at room temperature with 1% bovine serum albumin (BSA) in PBS(-). They were then incubated for 1 h with antibodies to ZO-1 or to claudin-1 at a dilution of 1:200 in PBS(-) containing 1% BSA, washed with PBS(-), and incubated for 1 h with AlexaFluor 488-conjugated secondary antibodies at a 1:1000 dilution in PBS(-) containing 1% BSA. The cells were finally examined with a laser confocal microscope (LSM; Carl Zeiss, Jena, Germany).

## 2.6 Measurement of transepithelial electrical resistance (TER)

ARPE-19 cells seeded in the apical chambers of a Transwell apparatus containing filters with a pore size of

0.22 µm were cultured for 2 days to allow establishment of barrier function before exposure to H<sub>2</sub>O<sub>2</sub> (500 µmol L<sup>-1</sup>) for the indicated times. The electrical resistance of the cell monolayer was then measured with the use of STX-2 electrodes and an EVOM Volt Ohm Meter (World Precision Instruments, Sarasota, FL). TER was calculated as the measured resistance (corrected for the background value obtained from a blank Transwell filter) normalized by the area of the monolayer.

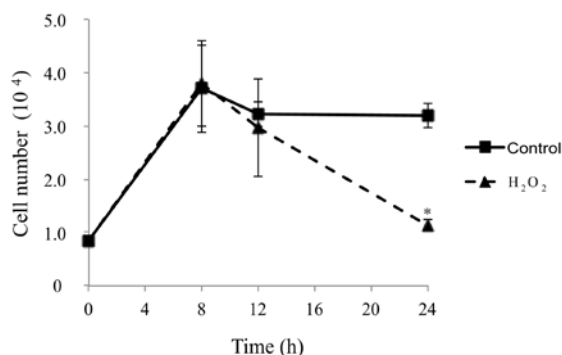
## 2.7 Statistical analysis

Quantitative data are presented as means ± SE from three independent experiments and were analyzed with Student's *t* test. A *P* value of <0.05 was considered statistically significant.

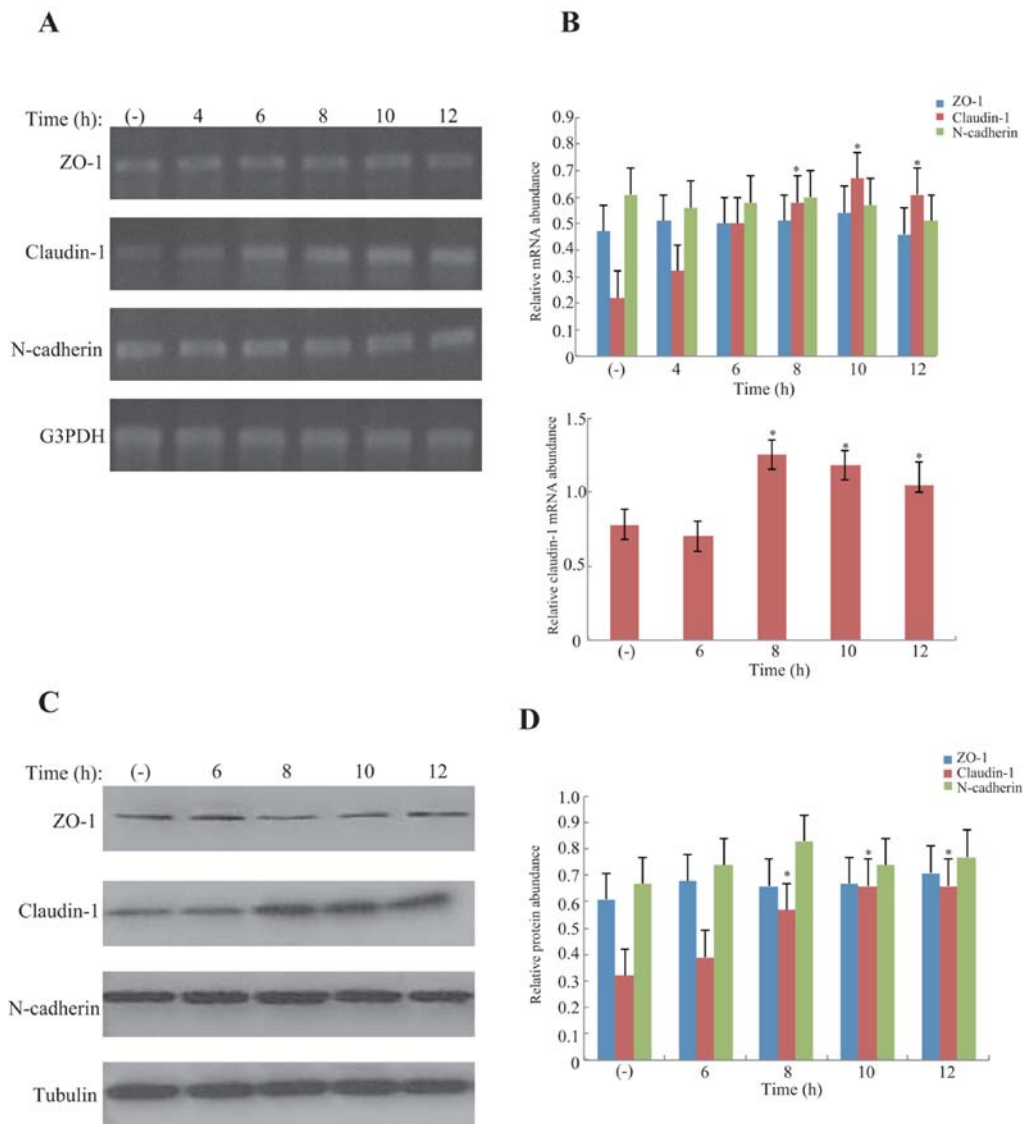
# 3. Results

## 3.1 Effects of oxidative stress on junctional protein expression in ARPE-19 cells

We first examined the possible effect of H<sub>2</sub>O<sub>2</sub> on ARPE-19 cell viability. Exposure of the cells to H<sub>2</sub>O<sub>2</sub> (500 µmol L<sup>-1</sup>) for up to 12 h had no apparent effect on cell proliferation or survival (Figure 1). The cell number was significantly reduced, however, after exposure to H<sub>2</sub>O<sub>2</sub> for 24 h. We next investigated the effects of oxidative stress on the expression of the TJ proteins ZO-1 and claudin-1 as well as the adherens-junction protein N-cadherin in ARPE-19 cells. RT-PCR analysis (Figure 2A, B) and immunoblot analysis (Figure 2C, D) revealed that the amounts of claudin-1 mRNA and protein, respectively, were significantly increased in ARPE-19 cells exposed to H<sub>2</sub>O<sub>2</sub> (500 µmol L<sup>-1</sup>) for 8 to 12 h. In contrast, such exposure to H<sub>2</sub>O<sub>2</sub> had no effect on the expression of ZO-1 or N-cadherin at the mRNA or protein level.



**Figure 1.** Effect of H<sub>2</sub>O<sub>2</sub> on ARPE-19 cell viability. Cells incubated in the absence (control) or presence of H<sub>2</sub>O<sub>2</sub> (500 µmol L<sup>-1</sup>) for the indicated times were isolated by exposure to trypsin and counted with a Coulter counter. Data are means ± SE from three separate experiments. \**P* < 0.05 versus the corresponding control value (Student's *t* test).

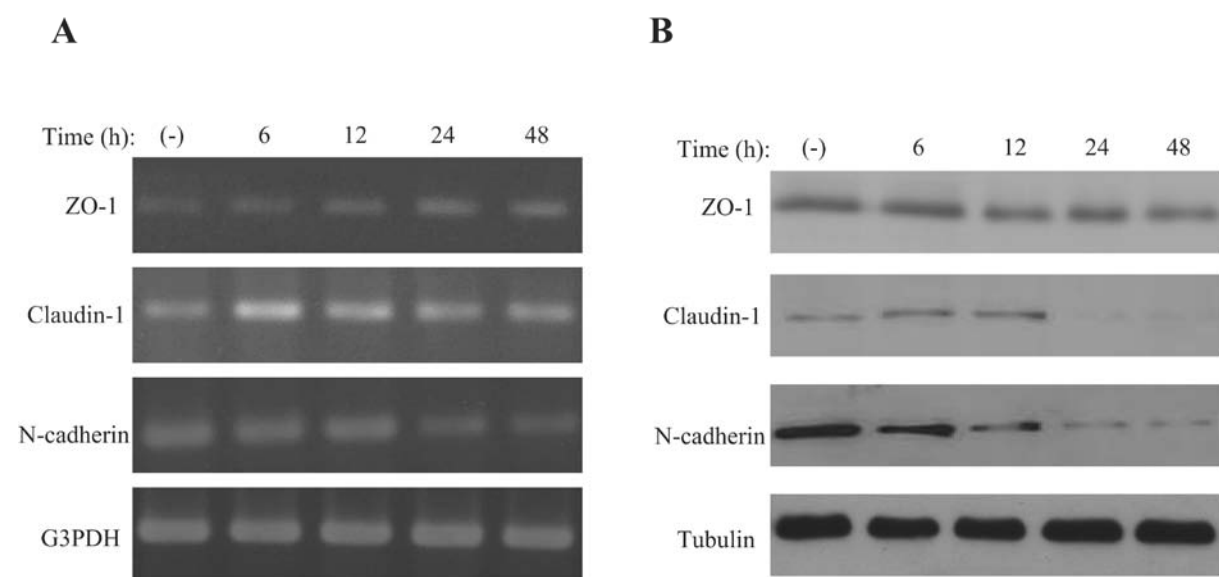


**Figure 2.** Effects of short-term exposure to  $\text{H}_2\text{O}_2$  on junctional protein expression in ARPE-19 cells. (A) Cells were incubated in the absence (-) or presence of  $\text{H}_2\text{O}_2$  ( $500 \mu\text{mol L}^{-1}$ ) for the indicated times, after which total RNA was isolated from the cells and subjected to RT-PCR analysis of ZO-1, claudin-1, N-cadherin, and G3PDH mRNAs. (B) The abundance of junctional protein mRNAs in experiments similar to that shown in (A) was quantified by image analysis and normalized by the corresponding amount of G3PDH mRNA (upper panel). The abundance of claudin-1 mRNA normalized by the amount of G3PDH mRNA was also determined by RT and real-time PCR analysis (bottom panel). (C) Cells were incubated in the absence (-) or presence of  $\text{H}_2\text{O}_2$  ( $500 \mu\text{mol L}^{-1}$ ) for the indicated times, lysed, and subjected to immunoblot analysis with antibodies to ZO-1, to claudin-1, to N-cadherin, and to  $\alpha$ -tubulin (loading control). (D) The abundance of junctional proteins in experiments similar to that shown in (C) was quantified by image analysis and normalized by the corresponding amount of  $\alpha$ -tubulin. Data in (B) and (D) are means  $\pm$  SE from three separate experiments. \* $P < 0.05$  versus the corresponding value for cells incubated without  $\text{H}_2\text{O}_2$  (Student's  $t$  test).

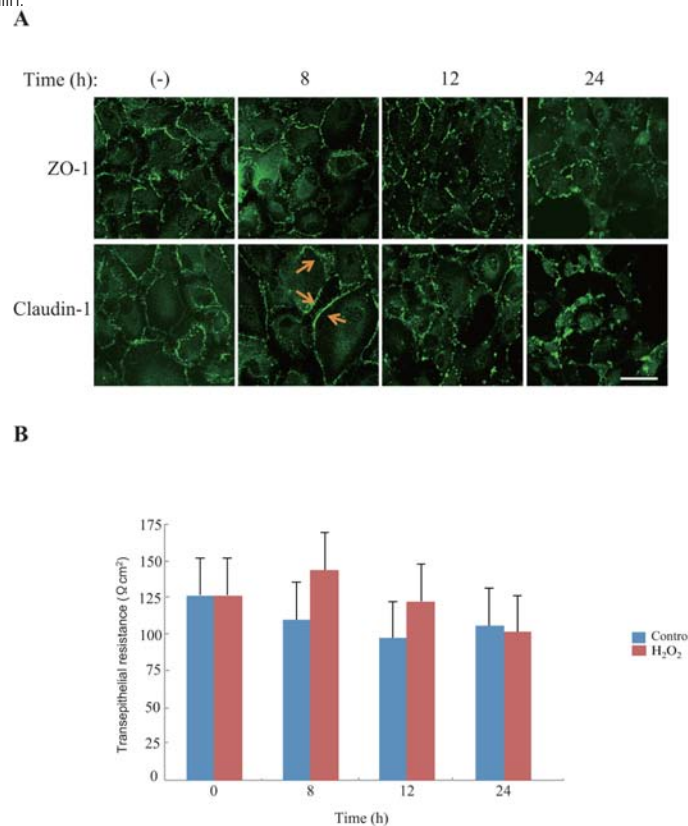
Exposure of the cells to  $\text{H}_2\text{O}_2$  for longer periods (24 or 48 h) resulted in down-regulation of the abundance of claudin-1 mRNA (Figure 3A) and protein (Figure 3B). The amounts of N-cadherin mRNA and protein were also decreased after such long-term exposure to  $\text{H}_2\text{O}_2$ , whereas those of ZO-1 mRNA and protein remained largely unchanged.

Immunofluorescence analysis also showed that the expression of claudin-1 at the interfaces of neighboring

ARPE-19 cells was markedly increased after exposure to  $\text{H}_2\text{O}_2$  for 8 h (Figure 4A). The linear pattern of claudin-1 immunoreactivity appeared disrupted and the expression level reduced, however, in cells exposed to  $\text{H}_2\text{O}_2$  for 24 h. The pattern and intensity of ZO-1 immunofluorescence appeared largely unaffected by exposure of the cells to  $\text{H}_2\text{O}_2$ . We next investigated whether these changes in the expression of claudin-1 induced by oxidative stress might affect the barrier



**Figure 3.** Effects of long-term exposure to  $\text{H}_2\text{O}_2$  on junctional protein expression in ARPE-19 cells. (A) Cells were incubated in the absence (-) or presence of  $\text{H}_2\text{O}_2$  ( $500 \mu\text{mol L}^{-1}$ ) for the indicated times, after which total RNA was isolated from the cells and subjected to RT-PCR analysis of ZO-1, claudin-1, N-cadherin, and G3PDH mRNAs. (B) Cells were incubated in the absence (-) or presence of  $\text{H}_2\text{O}_2$  ( $500 \mu\text{mol L}^{-1}$ ) for the indicated times, lysed, and subjected to immunoblot analysis with antibodies to ZO-1, to claudin-1, to N-cadherin, and to  $\alpha$ -tubulin.



**Figure 4.** Effects of exposure to  $\text{H}_2\text{O}_2$  on TJ protein expression and barrier function in ARPE-19 cells. (A) Cells were incubated in the absence (-) or presence of  $\text{H}_2\text{O}_2$  ( $500 \mu\text{mol L}^{-1}$ ) for the indicated times and then subjected to immunofluorescence analysis with antibodies to ZO-1 or to claudin-1. Scale bar,  $50 \mu\text{m}$ . Arrows indicate up-regulation of claudin-1 expression at the interfaces of cells exposed to  $\text{H}_2\text{O}_2$  for 8 h. (B) Cell monolayers were cultured in the absence or presence of  $\text{H}_2\text{O}_2$  ( $500 \mu\text{mol L}^{-1}$ ) for the indicated times and then measured for TER. Data are means  $\pm$  SE from three separate experiments.

function of an ARPE-19 cell monolayer. Incubation of the cells with  $H_2O_2$  for 8 h resulted in an increase in TER compared with that of control cells incubated in the absence of  $H_2O_2$  (Figure 4B), but this difference was not statistically significant.

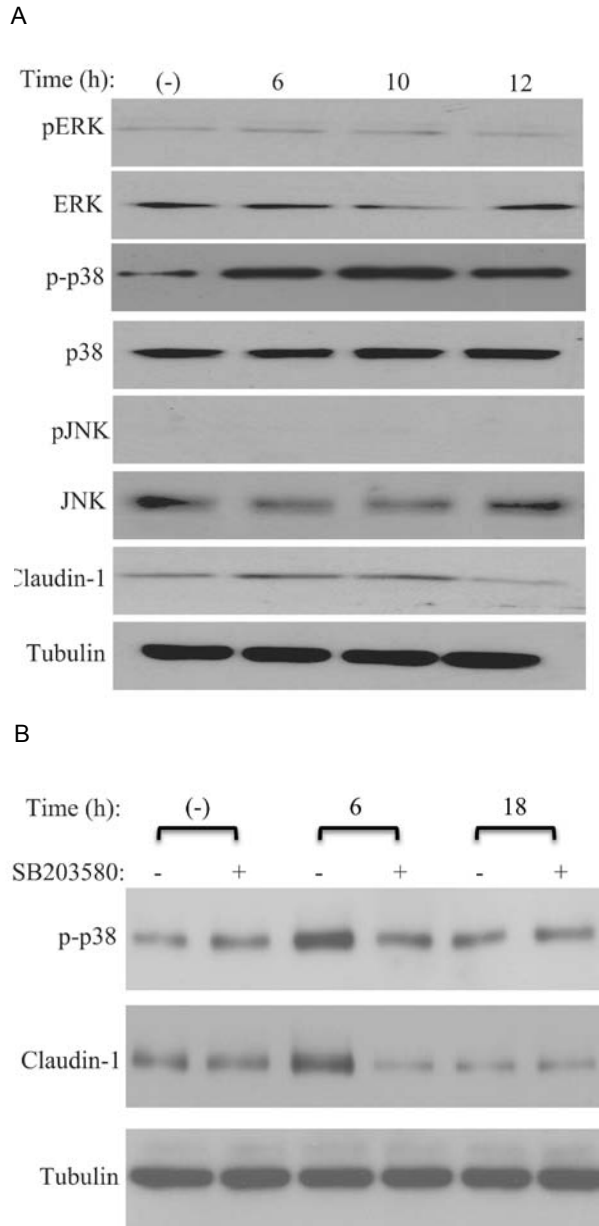
### 3.2 Role of MAPK signaling in oxidative stress-induced up-regulation of claudin-1

Given that mitogen-activated protein kinases (MAPKs) have been implicated in regulation of junctional protein expression and in mediation of the effects of oxidative stress [16-18], we finally examined whether MAPK signaling pathways might also contribute to the oxidative stress-induced up-regulation of claudin-1 in ARPE-19 cells. Exposure of the cells to  $H_2O_2$  for 6 to 12 h resulted in a marked increase in the extent of p38 MAPK phosphorylation, whereas the phosphorylation levels of ERK and JNK remained unaffected (Figure 5A). Furthermore, both the phosphorylation of p38 and the up-regulation of claudin-1 expression induced by  $H_2O_2$  were blocked by the p38 inhibitor SB203580 (Figure 5B). These results thus implicated p38 MAPK, but not ERK or JNK, in the regulation of claudin-1 expression by oxidative stress in ARPE-19 cells.

## 4. Discussion

The aim of this study was to investigate the effects of  $H_2O_2$  on junctional protein expression in ARPE-19 cells in order to shed light on the role of oxidative stress in human RPE cell death associated with AMD. We found that exposure of ARPE-19 cells to  $H_2O_2$  resulted in up-regulation of the expression of claudin-1 at the mRNA and protein levels, with this effect being significant at 8 to 12 h. Such increased expression of claudin-1 was accompanied by an increase in the TER of an ARPE-19 cell monolayer, although this effect was not statistically significant. Such short-term exposure to  $H_2O_2$  had no effect on the expression of ZO-1 or N-cadherin. Exposure to  $H_2O_2$  for longer periods (24 or 48 h) resulted in down-regulation of claudin-1 expression as well as that of N-cadherin, although cytotoxicity of  $H_2O_2$  was also evident after such long exposure times. The changes in the total abundance of claudin-1 detected by immunoblot analysis were accompanied by corresponding changes in the amount of claudin-1 immunoreactivity at the cell surface, suggesting that they reflect regulation of the TJ-associated protein. Furthermore, oxidative stress induced phosphorylation of p38 MAPK, but not that of ERK or JNK, with this effect showing a time course similar to that of the increase in claudin-1 expression. Inhibition of this phosphorylation of p38 induced by  $H_2O_2$  prevented the up-regulation of claudin-1 expression.

AMD is a leading cause of blindness in the elderly [9,19]. The loss of central vision that can occur in the later stages of neovascular or exudative AMD is thought to be related to aging-associated accumulation



**Figure 5.** Role of MAPK signaling in oxidative stress-induced up-regulation of claudin-1 expression in ARPE-19 cells. (A) Cells were incubated in the absence (-) or presence of  $H_2O_2$  ( $500 \mu\text{mol L}^{-1}$ ) for the indicated times, lysed, and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of ERK, p38, or JNK as well as with those to claudin-1 and to  $\alpha$ -tubulin. (B) Cells were incubated in the absence (-) or presence of  $H_2O_2$  ( $500 \mu\text{mol L}^{-1}$ ) or SB203580 ( $10 \mu\text{mol L}^{-1}$ ) for the indicated times, lysed, and subjected to immunoblot analysis with antibodies to phosphorylated p38, to claudin-1, and to  $\alpha$ -tubulin.

of oxidative stress in the RPE and choroidal capillaries. The prevention of oxidative stress at early stages of the disease is therefore considered a potential approach to limiting the severity of AMD. Choroidal neovascularization is responsible for the loss of vision in neovascular AMD [20]. In this process, choroidal capillaries penetrate Bruch's membrane and the RPE. The resulting disruption of junctional structures between RPE cells leads to loss of the barrier function of the BRB. Junctional proteins, especially those of TJs, including claudin, ZO-1, and occludin [21], therefore likely play an important role in the pathogenesis of AMD and other retinal diseases. The effects of oxidative and other types of stress on the expression of these proteins in the RPE have remained unclear. Several factors have been found to attenuate oxidative stress-induced disruption of junctions between RPE cells [22–24], with the cytoskeleton associated with junctional proteins having been implicated as a target of oxidative stress in these cells [22,24]. The expression of claudin isoforms has also been found to be differentially affected by oxidative stress in RPE cells [25]. However, the effects of oxidative stress on claudin expression at early time points and the intracellular signaling responsible for such effects have not previously been examined. We have now shown that oxidative stress had a bidirectional effect on claudin-1 expression in ARPE-19 cells, increasing it at shorter exposure times and reducing it after longer times.

It is possible that the initial up-regulation of claudin-1 represents a compensatory response to disruption of intercellular junctions by oxidative stress.

Oxidative stress is known to activate MAPKs including ERK, JNK, and p38 and thereby to regulate cell survival and function [26]. We have now shown that exposure of ARPE-19 cells to H<sub>2</sub>O<sub>2</sub> induced the selective phosphorylation of p38 with a time course similar to that of the initial increase in claudin-1 expression. Furthermore, the p38 inhibitor SB203580 blocked the up-regulation of claudin-1 induced by H<sub>2</sub>O<sub>2</sub>, suggesting that p38 MAPK mediates this effect of oxidative stress.

In conclusion, oxidative stress induced an initial up-regulation of the expression of claudin-1 mediated by p38 MAPK in RPE cells. This effect was followed by down-regulation of claudin-1 after longer-term exposure to such stress. These changes in the expression of this important TJ protein may contribute to the pathogenesis of AMD.

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