Supplementary Material

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N-Acetylcysteine amide protects against oxidative stress-induced microparticle release from human retinal pigment epithelial cells

Table S1. Antibodies and Fluorophores Used for Flow Cytometry and Confocal Microscopy

Target	Fluorophore	Host / Isotype / Class	Vendor	Catalog #	Lot #	Concentration
Phosphatidylserine	Annexin V-Alexa Fluor 488	Molecular Conjugate	Life Technologies	V13241	1557794	5 µL/10^5 cells
DNA	Propodium Iodide	Fluorescent Molecule	Life Technologies	V13241	1557794	1 µg/mL
Phosphatidylserine	MFG-E8-FITC	Molecular Conjugate	Haematologic Technologies	BLAC-FITC	DD0806-1ml	160 nM
CD46	APC	Mouse / IgG1 / Monoclonal	Life Technologies	A15711	519500	5 µg/mL
CD55	PE	Mouse / IgG1 / Monoclonal	Life Technologies	A16334	E17988101	5 µg/mL
CD59	APC	Mouse / IgG2a / Monoclonal	Life Technologies	A15705	514010	5 µg/mL
IgG1	APC	Mouse	Life Technologies	MG105	1391556A	5 µg/mL
IgG2a	APC	Mouse	Life Technologies	MG2A05	1627128A	5 µg/mL
Phosphatidylethanolamine	Duramycin-FITC	Molecular Conjugate	Molecular Targeting Technologies	D-1001	MTTI-018-247	160 nM

Table S2. Primary and Secondary Antibodies Used for Fluorescent Western Blot Analysis

Target	Fluorophore	Host / Isotype / Class	Vendor	Catalog #	Lot #	Concentration
CD46	N/A	Mouse / IgG1 / Monoclonal	AbD Serotec	MCA2113	0113	2 µg/mL
CD59	N/A	Mouse / IgG2a / Monoclonal	AbD Serotec	MCA1054GA	0814	1 µg/mL
CD63	N/A	Mouse / IgG1 / Monoclonal	Life Technologies	10628D	DAQ-PF150624T1	2 µg/mL
β-Actin	N/A	Mouse / IgG2a / Monoclonal	Sigma-Aldrich	A5316	123M4876	1:3000
GAPDH	N/A	Rabbit polyclonal IgG	Santa Cruz Biotechnology	sc-25778	G2909	1 µg/mL
Mouse immunoglobulins	Alexa Fluor 488	Chicken / IgY /Polyclonal	Life Technologies	A21200	1696214	10 µg/mL
Rabbit immunoglobulins	Alexa Fluor 647	Goat / IgG/ Polyclonal	Life Technologies	A21244	1555500	10 µg/mL

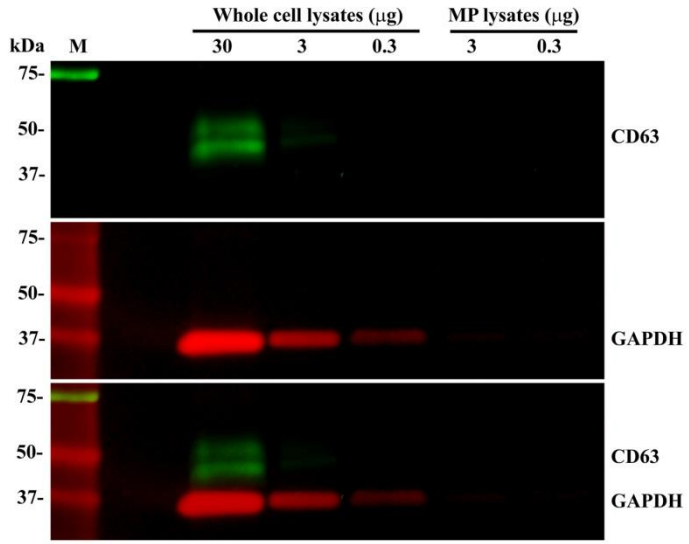

Figure S1. Fluorescent Western Blot Analysis of CD63 Protein in RPE cells and RPE-Derived Microparticles.

Human RPE cells and microparticles (MPs) were harvested and isolated. Intact cells or isolated MPs were lysed and proteins were measured. Proteins of whole cell lysates or of MP lysates were subjected to Western blot analysis of CD63 (green; upper panel), a marker of exosomes. The anti-CD63 primary antibody was detected with Alexa Flour 488-conjugated chicken anti-mouse IgG, and protein bands were pseudocolored green. The primary antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (red; middle panel) was detected with Alexa Flour 647-conjugated goat anti-rabbit IgG, and single bands were pseudocolored red. Lower panel shows an overlay of green (CD63) and red (GAPDH) images. Although GAPDH is detected in both whole cell lysates and MP lysates, CD63 is not detectable in MP lysates. M indicates protein size marker.

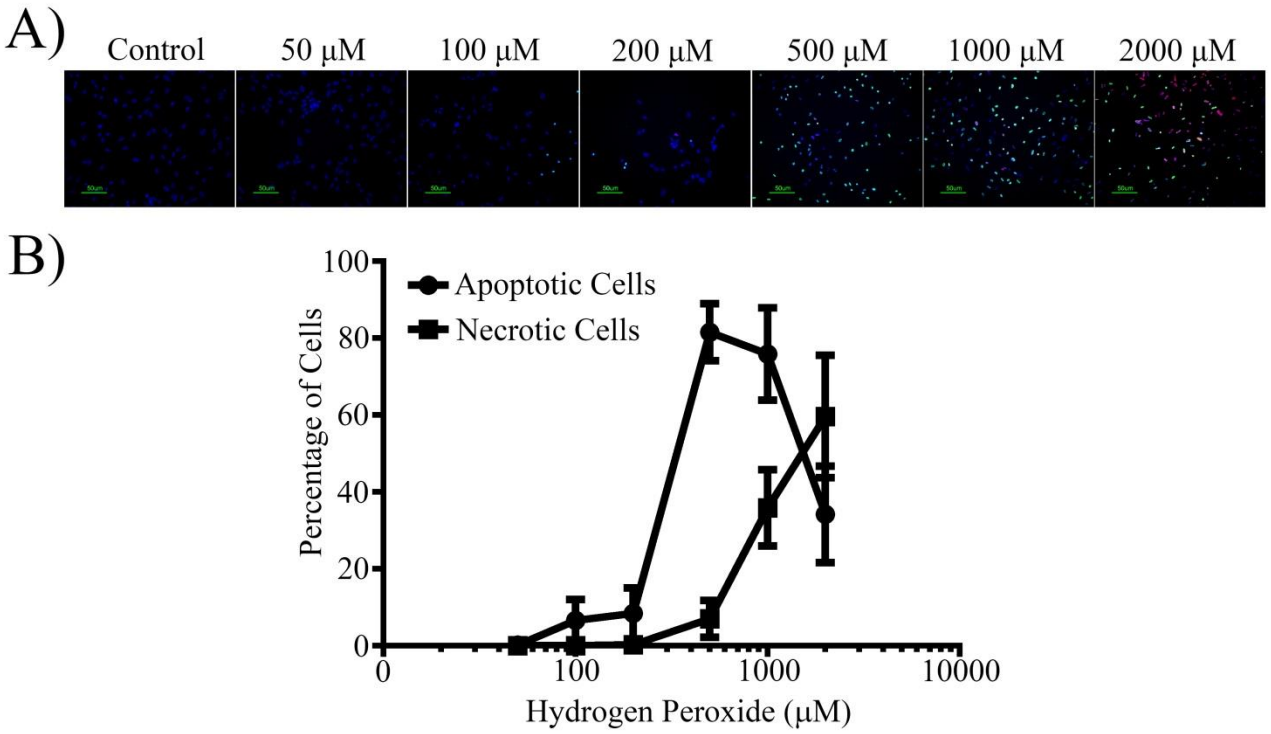

Figure S2. Dose-Response of Apoptosis and Necrosis Induced by Hydrogen Peroxide. Cultured human RPE cells were treated with 0-2000 μM H_2O_2 for 16 hours and stained with DAPI (blue), TUNEL (green), and PI (red). (**A**) Representative images. (**B**) Quantification of apoptotic (TUNEL-positive) and necrotic (PI-positive) cells with ImageJ software. Data are presented as mean ± SD (n = 3).

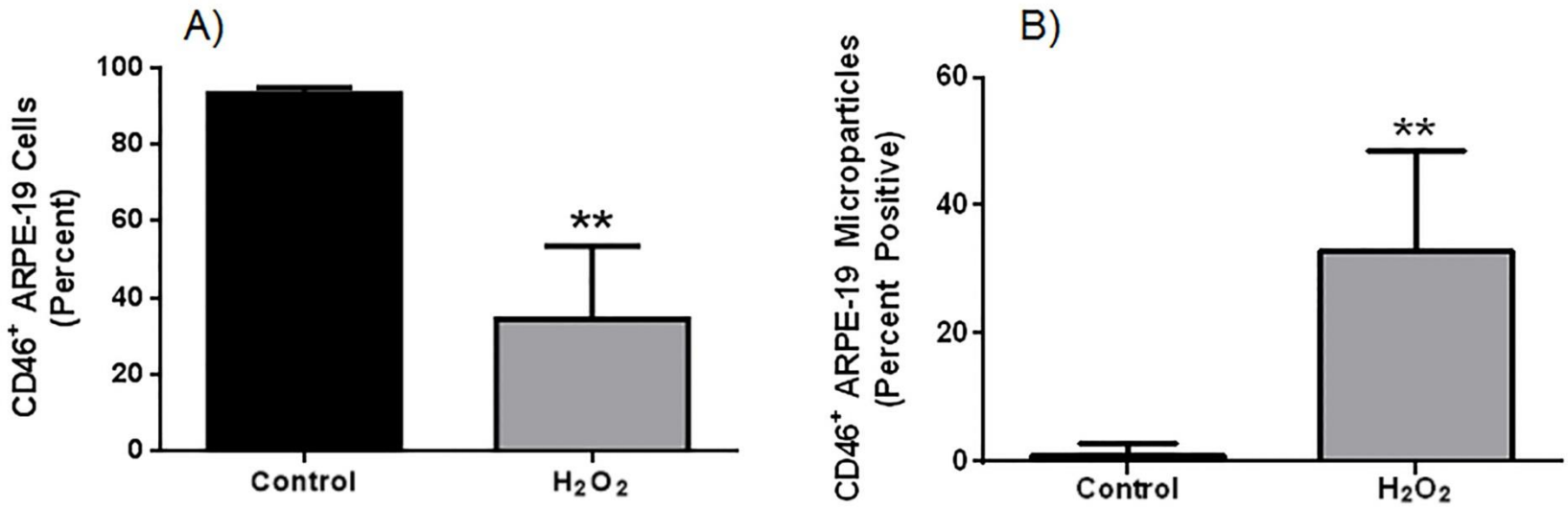

Figure S3. H_2O_2 Treatment Decreases the Abundance of CD46 and Increases CD46-Positive Microparticle Release in ARPE-19 Cells. (**A**) Control and hydrogen peroxide (H_2O_2; 500 µM for 16 hr)-treated ARPE-19 cells were stained with a CD46 antibody and analyzed by flow cytometry. (**B**) Microparticles isolated from control and H_2O_2 (500 µM for 16 hr)-treated ARPE-19 cells were stained with CD46 antibody. Data are presented as mean ± SD (n = 5-7) ***P* < 0.005.

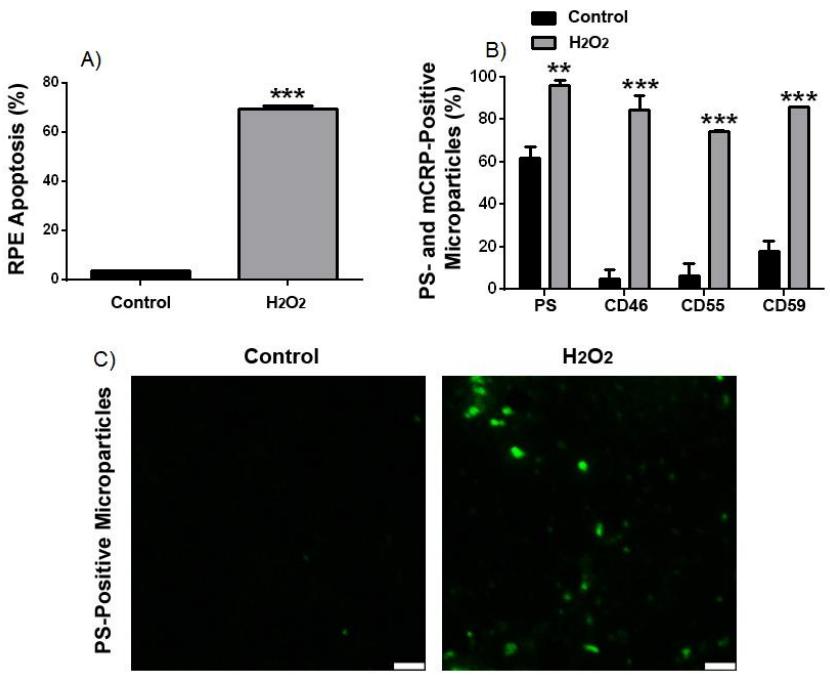

Figure S4. H_2O_2 Treatment Increases Apoptosis and Microparticle Release in Cultured Human RPE Cells. Cultured human RPE cells derived from a 61-year-old male Caucasian donor were treated with serum free media with or without 500 µM hydrogen peroxide (H_2O_2) for 16 hr. (**A**) Cells stained with annexin V-Alexa Fluor 488 and propidium Iodide were quantified for percent apoptosis. (**B**) Microparticles derived from control or H_2O_2-treated cells were stained with MFG-E8-FITC for Phosphatidylserine (PS) and membrane complement regulatory protein antibodies as indicated. (**C**) Representative images of MFG-E8-FITC stained microparticles. Scale bar represents 2.5 µm. Data are presented as mean ± SD (n = 3) ****P* < 0.0005 compared to control.

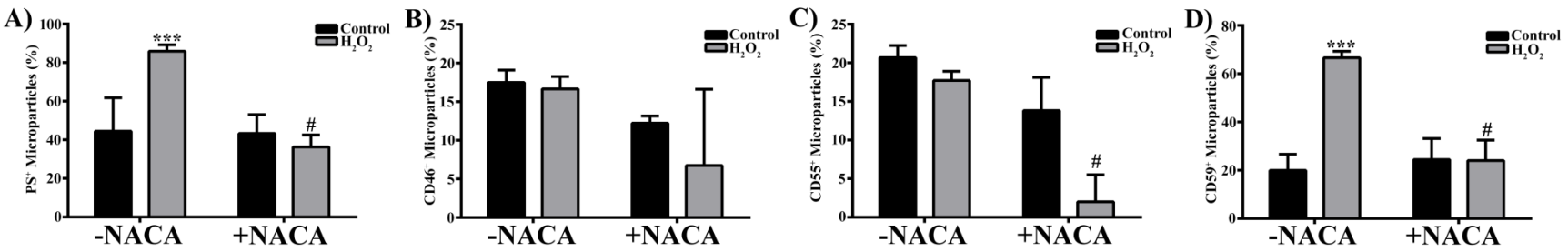

Figure S5. *N*-Acetylcysteine Amide Attenuates the Effects of 200 μM H_2O_2 on RPE-Derived Microparticles. Cultured human RPE cells derived from a 61-year-old male Caucasian donor were treated with serum free media with or without 200 μM hydrogen peroxide (H_2O_2) for 16 hr. Microparticles isolated from control and H_2O_2-treated RPE cells with *N*-acetylcysteine amide (+ NACA) or without NACA (- NACA) pretreatment were stained with MFG-E8-FITC for Phosphatidylserine (PS) (**A**) or stained with fluorescently labeled antibodies to CD46 (**B**), CD55 (**C**) or CD59 (**D**), and quantified by flow cytometry. Data are presented as mean ± SD (n = 3). ****P* < 0.005 compared to controls in the absence of NACA (- NACA); #*P* < 0.005 compared to H_2O_2-treated groups in the absence of NACA (- NACA).