論文の内容の要旨

論文題目　Mechanisms of Senescence in Retinal Pigment Epithelial Cells

（網膜色素上皮細胞における加齢のメカニズム）

ムリロ　フェリックス　ロジャ

Murilo Felix Roggia

Purpose **–** Age-related macular degeneration (AMD) is a prevalent disease representing the leading cause of visual loss in individuals aged older than 60 years. Aging-related modifications in RPE cells directly contribute to the establishment of early stage AMD and, consequently, to late-stage neovascular AMD. The objective of this study was to explore the mechanism responsible for the induction of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) by photoreceptor outer segment (POS) phagocytosis in the regulation of senescence and metabolism of RPE cells, and to evaluate the influence of GPx4 expression in the RPE/choroid on the establishment of choroidal neovascularization (CNV).

Materials and Methods **–** ARPE-19 cells were treated with POS isolated from porcine eyes. Expression of PGC-1α was determined in undifferentiated and differentiated ARPE-19 cells *in vitro*, and in *ex-vivo* flat mount of the RPE. Knockdown (KD) of specific molecules enrolled in POS phagocytosis was performed to determine which stage of phagocytosis was associated with the upregulation of PGC-1α. Antibodies to specifically block the function of these molecules were used to confirm these findings. The mRNA levels of antioxidant enzymes were measured using quantitative real-time RT-PCR. ROS generation was also measured, and mitochondrial biogenesis was evaluated based on the relative mitochondrial DNA expression and in the protein levels of prohibitin using Western blot analysis. Mitochondrial function was determined by the measurement of complex I activity. Senescence of RPE cells was evaluated through senescence-associate β-galactosidase (SA-β-gal) activity. Transcription factor EB (TFEB) activity was evaluated by nuclear staining of TFEB using a specific antibody. mRNA levels of TFEB target genes were determined using quantitative real-time RT-PCR. To evaluate intracellular accumulation of lipids Oil Red O staining of RPE cells was used. Accumulation of peroxidized lipids was determined using boron dipyrromethene (BODIPY) C11 dye. Transmission electron microscopy (TEM) was performed to evaluate the RPE/choroid phenotype of PGC-1α knockout (KO) mice. Staining with biotinylated Ulex europaeus agglutinin-I (UEA-I) lectin was used to evaluate the choriocapillaris of PGC-1α KO mice, and autofluorescence of RPE cells was also evaluated to determine lipofuscin accumulation in PGC-1α KO mice. Mice expressing different levels of GPx4 were used to evaluate the effect of GPx4 in the establishment of CNV. Vascular endothelial growth factor A (VEGF-A) mRNA and protein expression in RPE/choroid were evaluated before and after laser-induced CNV using quantitative real-time RT-PCR and ELISA, respectively. Lipid peroxidation in the RPE/choroid was determined by immunohistochemistry using a specific antibody against 4-hydroxil-2-neonenal (4-HNE). To explore the protective effect of GPx4, the size of laser-induced CNV was measured 7 days after laser treatment using fluorescein isothiocyanate dextran (FITC) staining, and compared among the mice.

Results **–** POS treatment increased mRNA and protein levels of PGC-1α in undifferentiated ARPE-19 cells. Upregulation of PGC-1α shows a dose-dependent response according to the number of POS used in the treatment. POS treatment demonstrated a similar effect in differentiated ARPE-19 cells and in *ex-vivo* RPE cells in flat mount culture. To confirm the effect was specifically related to phagocytosis of POS, latex beads were used to assess the phagocytic activity of ARPE-19 cells. Treatment with latex beads did not increase the mRNA expression of PGC-1α. Furthermore, the use of Arg-Gly-Asp (RGD) peptide, which specifically inhibits the binding of POS to the ARPE-19 cells, significantly suppressed

the upregulation of PGC-1α, which excludes the possibility of other impurities present in the POS as responsible for this upregulation. When the time course of PGC-1α upregulation was analyzed, the increase began as early as 30 minutes after POS treatment. To determine which step of POS phagocytosis was related to PGC-1α upregulation, silencing of the molecules enrolled in POS phagocytosis was performed. In ARPE-19 cells where β5 integrin was silenced (abrogation of binding), upregulation of PGC-1α was significantly suppressed. In contrast, silencing of CD36 and MerTK (abrogation of internalization) led to enhanced upregulation of PGC-1α rather than suppression. Aiming to confirm these findings, antibodies to specifically block CD36 and MerTK receptors in the ARPE-19 cells were used, which revealed that upregulation of PGC-1α was not suppressed but enhanced. Focal adhesion kinase (FAK) is recognized as a major intracellular mediator of β5 integrin activation in ARPE-19 cells. When a specific inhibitor was used to abolish FAK activity, the increased expression of PGC-1α by POS treatment was significantly reduced. This suggests that FAK is associated with PGC-1α upregulation by POS binding. POS treatment increased the mRNA levels of antioxidant enzymes including glutathione peroxidase 1 (GPx1), glutathione peroxidase 4 (GPx4), superoxide dismutase 1 (SOD1), and catalase. ARPE-19 cells treated with POS showed decreased ROS level, while silencing of PGC-1α using a specific siRNA led to increased ROS level after POS treatment. POS treatment elevated the relative levels of mitochondrial DNA compared to nuclear DNA in the RPE cells, and also the expression of mitochondrial protein marker, prohibitin. Furthermore, mitochondrial complex I activity, which represents mitochondrial function, was increased after POS treatment in RPE cells. Interestingly, the elevation of mitochondrial complex I activity was abrogated by silencing PGC-1α. Next, the effect of POS and PGC-1α on the senescence of the RPE cells was evaluated through the SA-β-gal activity. Senescence of ARPE-19 cells was induced by H_2O_2, and evaluated by SA-β-gal staining after 24-h incubation. Pretreatment with POS for 3 h significantly rescued the H_2O_2-induced senescence in ARPE-19 cells. In contrast, silencing of PGC-1α aggravated H_2O_2-induced senescence and significantly suppressed the counteracting effect of POS treatment. Senescence of the RPE cells is associated with impaired lysosomal activity, and TFEB is considered a master regulator of autophagy/lysosomal pathway. Therefore, immunostaining of nuclear TFEB was performed to evaluate the activity of TFEB. When PGC-1α was silenced, nuclear staining of ARPE-19 cells with TFEB antibody was significantly weaker when compared with the cells transfected with control siRNA. Additionally, mRNA levels of TFEB target genes including galactosidase α (GLA), hexosaminidase A (HEXA), tripeptidyl peptidase I (TPPI), and cathepsin F (CTSF) were downregulated in the RPE cells transfected with PGC-1α siRNA. The same downregulation was observed in the RPE cells treated with FAK inhibitor. Silencing of PGC-1α significantly decreased cathepsin D activity, which is considered a major cathepsin in the degradation of POS. Abnormal lipid accumulation is another feature of RPE senescence. Therefore, the role of PGC-1α in POS degradation was evaluated using Oil Red O staining. Silencing of PGC-1α was associated with stronger Oil Red O staining in RPE cells when compared with control cells, both before and after POS treatment. Consistent with this finding, evaluation using BODIPY C11 showed that silencing of PGC-1α increased peroxidized lipid accumulation in ARPE-19 cells treated with POS. When the PGC-1α KO mouse phenotype was evaluated, accumulation of lipofuscin and melanolipofuscin in the RPE, thickening of the Bruch's membrane and choriocapillaris atrophy were observed. Additionally, UEA-I lectin staining confirmed choriocapillaris atrophy, and intense autofluorescence was observed in the RPE of PGC-1α KO mice. Increased GPx4 expression was associated with decreased lipid oxidation in RPE/choroid. Staining with 4-HNE antibody showed that mice overexpressing GPx4 presented decreased oxidized lipids in the RPE/choroid, while $GPx4^{+/-}$ mice demonstrated the most abundant lipid oxidation. GPx4 also influenced the development of laser-induced CNV. Increased expression of GPx4 in RPE/choroid was associated with smaller CNV size. Next, the expression of VEGF-A was measured before and after CNV induction in mice expressing different levels of GPx4. GPx4 suppressed the increase in the VEGF-A protein level after CNV induction by laser. Of note was that regardless of the difference in the expression of GPx4, VEGF-A protein level in RPE/choroid was upregulated after CNV induction while VEGF-A mRNA level was downregulated.

Discussion – The mechanisms responsible for POS degradation by the RPE cells is crucial for the comprehension of AMD pathogenesis. PGC-1α, a preeminent regulator of metabolism and oxidative stress is upregulated by POS phagocytosis in RPE cells *in vitro*. The current study demonstrated that binding of POS to the RPE cells upregulated the expression of PGC-1α through αvβ5 integrin and FAK activation at the apical portion of ARPE-19 cells. POS-induced PGC-1α upregulation conferred anti-senescent effects in ARPE-19 cells, increasing the expression of antioxidant enzymes and mitochondrial function, and decreasing generation of ROS. Furthermore, POS-induced PGC-1α protected the ARPE-19 cells against H_2O_2-induced senescence. Senescence of RPE cells was

associated with impaired lysosomal activity, causing accumulation of lipofuscine and peroxidized lipids. TFEB and PGC-1α have been described as regulators of the autophagy/lysosomal pathway in a mouse model of neurodegenerative disease. This study demonstrated that activation of PGC-1α by POS binding not only induced the nuclear translocation of TFEB in ARPE-19 cells but also upregulated the expression of TFEB target genes and the activity of cathepsin D. Therefore, the activation of αvβ5 integrin/FAK/ PGC-1α plays an important role in the regulation of lysosomal activity in ARPE-19 cells. Thus if this pathway is not activated, POS digestion by the RPE cells might be impaired, leading to abnormal lipid accumulation. Accordingly, the present study demonstrated that POS-induced PGC-1α protected the ARPE-19 cells against lipid peroxidation and intracellular lipid accumulation. In accordance with these findings, PGC-1α KO mice showed senescence-related abnormalities of the RPE/choroid tissue. Many of the AMD commemoratives were present, including accumulation of lipofuscin and melanolipofuscin, thickening of the Bruch's membrane and atrophy of the choriocapillaris. Accumulation of lipids in the RPE cells is a consequence of aging and is also present in the pathogenesis of AMD. POS phagocytosis by ARPE-19 cells is essential for the maintenance of the retinal function. Loss of the choriocapillaris as a consequence of aging is also present in the early stages of AMD development in humans. Choriocapillaris atrophy along with thickening of the Bruch membrane contributes to RPE cell loss in AMD. The implication of VEGF-A in the pathogenesis of neovascular AMD and laser-induced CNV has been well established. Oxidative stress has also been associated in the development of neovascular AMD, wherein increased antioxidant activity protects against neovascular formation. The role of antioxidant enzymes in laser-induced CNV has not been sufficiently understood. Spontaneous CNV development in the RPE/choroid has been demonstrated in superoxide-dismutase 1 (SOD1) deficient mice. Although a protective effect of GPx4 against oxidative stress has been described in the retinal tissue, the role of GPx4 in CNV formation has not been explored in previous studies. Thus, the present study demonstrated that GPx4 influences VEGF-A expression in the RPE/choroid, suppressing the increase in VEGF-A protein levels after laser-induced CNV. Furthermore, GPx4 demonstrated a protective effect against CNV development *in vivo*.

Conclusion – The current study demonstrated a novel regulatory mechanism for senescence and lysosomal function in ARPE-19 cells triggered by POS binding through αvβ5 integrin/FAK/PGC-1α activation. This mechanism represents a potential target for a therapeutic approach to suppress the progression from the early stage of AMD to the late stages of the disease. Furthermore, the current study postulates that increased expression of the anti-oxidant enzyme GPx4 is associated with the regulation of VEGF-A in the RPE/choroid after laser-induced CNV protecting against CNV development. Thus, GPx4 may represent a possible target for CNV treatment. Altogether, these findings demonstrate novel mechanisms associated with the pathological alterations responsible for the establishment and progression of AMD.