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Soluble Vascular Adhesion Protein-1 Mediates Spermine Oxidation as Semicarbazide-Sensitive Amine Oxidase: Possible Role in Proliferative Diabetic Retinopathy

Short running title: Acrolein Production by VAP-1/SSAO

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Abstract

Purpose/Aim of the study: To explore the possible role of vascular adhesion protein-1 (VAP-1) via its enzymatic function as a semicarbazide-sensitive amine oxidase (SSAO) in the pathogenesis of proliferative diabetic retinopathy (PDR).

Materials and Methods: The levels of soluble VAP-1/SSAO and the unsaturated aldehyde acrolein (ACR)-conjugated protein, N^ε-(3-formyl-3, 4-dehydropiperidino) lysine adduct (FDP-Lys), were measured in vitreous fluid samples of PDR and non-diabetic patients using ELISA. Recombinant human VAP-1/SSAO (rhVAP-1/SSAO) was incubated with spermine, with or without semicarbazide or RTU-1096 (a specific inhibitor for VAP-1/SSAO). Immunofluorescence assays were performed to assess the localization of VAP-1/SSAO and FDP-Lys in fibrovascular tissues from patients with PDR. The impact of ACR on cultured retinal capillary endothelial cells was assessed using a cell viability assay and total glutathione (GSH) measurements.

Results: The levels of sVAP-1/SSAO and FDP-Lys were elevated in the vitreous fluid of patients with PDR. Incubation of rhVAP-1 with spermine resulted in the generation of hydrogen peroxide and FDP-Lys and the production was inhibited by semicarbazide and RTU-1096. In fibrovascular tissues, FDP-Lys and VAP-1/SSAO were present in endothelial cells. ACR stimulation reduced GSH levels in the cultured endothelial cells in a dose-dependent manner and caused cellular toxicity.

Conclusions: Our results indicate the pathological role of sVAP-1/SSAO to generate hydrogen peroxide and toxic aldehyde ACR, both of which are associated with oxidative stress, as a consequence of spermine oxidation in eyes with PDR.

Key words: Diabetic retinopathy, Oxidative stress, Hydrogen peroxide, Acrolein,

Polyamine

Introduction

Diabetic retinopathy (DR) is a major microvascular complication of diabetes and a leading cause of blindness among people of working age in developed countries.^{1, 2} To date, preclinical and clinical studies have shown that vascular endothelial growth factor (VEGF), a potent angiogenic factor, plays a central role in the two main manifestations of DR, *i.e.* proliferative diabetic retinopathy (PDR)^{3, 4} and diabetic macular edema. (DME)^{5, 6} However, recent studies have also elucidated that, in addition to dysregulation of vascular homeostasis caused by VEGF, chronic inflammation and oxidative stress participate in the pathogenesis of DR, particularly at the early stage of this disease.^{7, 8}

Vascular adhesion protein-1 (VAP-1) is a homodimeric sialylated glycoprotein expressed in vascular endothelial cells and involved in the transmigration step of leukocyte trafficking.^{9, 10} A previous study using animal model of early DR revealed that leukocytes adhering to the endothelium damage the endothelial cells of retinal vessels.¹¹ Our prior research indicates that VAP-1 is expressed in the vascular endothelial cells of fibrovascular tissues in patients with PDR¹² and VAP-1 blockade, furthermore, has been shown to suppress the accumulation of inflammatory cells in DR animal models.¹³ Therefore, we had hypothesized that VAP-1 was involved in the pathogenesis of DR via its function as an adhesion molecule, and that it recruited inflammatory cells to the diabetic retina. However, subsequent analyses revealed that the levels of the soluble form of VAP-1 (sVAP-1) are higher in the vitreous fluid¹² and serum¹⁴ of patients with DR. In addition to its role as an adhesion molecule, VAP-1 is also known to have enzymatic function as a semicarbazide-sensitive amine oxidase (SSAO) that converts primary amines to their corresponding aldehydes,

releasing hydrogen peroxide and ammonia.¹⁵ These findings led us to hypothesize that VAP-1 acts in the pathogenesis of DR not only as an adhesion molecule, but also as an oxidative enzyme, SSAO.

Acrolein (ACR) is a highly reactive unsaturated aldehyde and reacts preferentially with Cys, Lys, and His residues on the protein with preserving the aldehyde functionality.^{16, 17} It is an exogenous pollutant that has been linked to the development of pulmonary disorders; however, recently it has been shown that ACR is also produced endogenously as a consequence of polyamine metabolism, mediated by polyamine oxidases,¹⁸ resulting in cell toxicity and oxidative stress. In the eye, the levels of polyamines in eyes with PDR patients have also been shown to be elevated.¹⁹ Previous studies demonstrated that ACR-conjugated protein, N ϵ -(3-formyl-3,4-dehydropiperidino) lysine adduct (FDP-Lys), markedly increased in glial cells of diabetic retina, and the FDP-lysine accumulation in the retina was significantly prevented by pyridoxamine, potent antioxidant, of diabetic rats.^{20, 21} In addition to the previous studies, we also reported that the FDP-Lys accumulates in cellular components of fibrovascular tissues, *i.e.*, endothelial cells, glial cells and pericytes, obtained from patients with PDR.²² Therefore, accumulating evidence indicates that sVAP-1/SSAO is associated with the production of ACR as an enzyme that catabolizes polyamine in DR. However, whether sVAP-1/SSAO, similar to polyamine oxidases, catalyzes spermine oxidation and produces ACR in eyes with DR remains unclear.

In this study, we explored the link between sVAP-1/SSAO and ACR in the pathogenesis of DR.

Materials and Methods

Specimens

Vitreous fluid samples were collected from the eyes of 12 patients with PDR (7 males and 5 females; mean age, 58.2 ± 4.1 years) that had undergone pars plana vitrectomy for prolonged vitreous hemorrhage and tractional retinal detachment resulting from macular lesions. Control vitreous fluid samples were collected from the eyes of 9 patients with non-diabetic ocular diseases (4 males and 5 females; mean age, 68.4 ± 3.1 years), *i.e.* idiopathic epiretinal membrane (ERM) and idiopathic macular hole (MH). Undiluted vitreous samples were collected into sterile tubes and were frozen rapidly at -80°C . Clinical characteristics of the patients were shown in Table 1. Fibrovascular tissues surgically removed from patients with PDR (2 males and 1 female; mean age, 52.7 ± 6.9 years) were used for immunofluorescence microscopy. All experiments were conducted in accordance with the tenets of the Declaration of Helsinki following approval from the institutional review committee of Hokkaido University Hospital (IRB #015-0124, #014-0293). Written informed consent was obtained from all patients after an explanation of the purpose and procedures of this study.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of the ACR-conjugated protein, FDP-Lys, were measured using a competitive ELISA kit (MK-150, Takara Bio, Shiga, Japan), according to the manufacturer's protocol. The protein levels of sVAP-1/SSAO in the samples were measured using an ELISA kit for human sVAP-1/SSAO (BMS259, Thermo Fisher Scientific, Waltham, MA). The vitreous samples were pre-diluted to 1:10 by assay buffer and the diluted samples (100 μL) were used for sVAP-1/SSAO measurement.

For FDP-lys measurement, vitreous samples (50 μ L) were used without dilution.

IC₅₀ Measurement of VAP-1/SSAO Inhibitor RTU-1096

To evaluate the effect of the novel VAP-1 inhibitor, RTU-1096 (Sucampo Pharma, LLC, Tokyo, Japan), for the VAP-1/SSAO activity, the half maximal inhibitory concentration (IC₅₀) of RTU-1096 was measured²³. Briefly, an enzyme suspension prepared from COS-7 cells transiently expressing human VAP-1/SSAO enzyme was pretreated with RTU-1096 at final concentrations from 100pM to 100nM in 50mM potassium phosphate buffer (pH 7.4) at room temperature for 20 min, and subsequently incubated in the presence of [¹⁴C]-benzylamine (10 μ M, 0.1 μ Ci, GE Healthcare, Buckinghamshire, UK) in a final volume of 200 μ L at 37°C for 2h. The enzyme reactions were terminated with citric acid (200 μ L, 2M). The oxidized products were extracted into 1mL of toluene/ethyl acetate (1:1, v/v) of which 750 μ L was then transferred to a counting vial containing 3mL Ultima Gold (Perkin Elmer, Wellesley, MA). Radioactivity was assessed using a liquid scintillation counter (Tri-carb 2900TR, Perkin Elmer). The VAP-1/SSAO activity was compared to that in the absence of RTU-1096, and then IC₅₀ values were calculated. For the determination of inhibitor specificity, recombinant human monoamine oxidase A or B (MAO-A or MAO-B, Sigma, St. Louis, MO) was pretreated with RTU-1096 at final concentrations from 10nM to 100 μ M for 20 min, and MAO activities were measured using a Fluoro MAO kit (Cell technology, Fremont, CA).

In vitro Incubation of VAP-1/SSAO with Spermine

Recombinant human VAP-1/SSAO (rhVAP-1/SSAO, Peprotech, Rocky Hill, NJ) was

incubated with 2.5mM spermine (S4264, Sigma) in each of the following buffers: 50mM HEPES (pH 7.4), 50mM Tris-HCl (pH 7.4), physiological HEPES (50mM HEPES, 140mM NaCl, 5mM KCl, 2mM CaCl₂, 1.4mM MgCl₂, pH 7.4), and PBS (137mM NaCl, 8.1mM Na₂HPO₄, 2.7mM KCl, 1.5mM KH₂PO₄; pH 7.4) at 37°C. For the inhibition assay, rhVAP-1/SSAO was incubated with 2.5mM spermine in PBS, with or without 100μM semicarbazide (Cell technology, Fremont, CA) or 100nM of the RTU-1096. The concentration of hydrogen peroxide was measured using a Fluoro hydrogen peroxide kit (Cell technology), according to the manufacturer's protocol. The FDP-Lys concentration was also measured using an ELISA kit (MK-150, Takara Bio), when rhVAP-1/SSAO was incubated with 2.5mM spermine and 1mg/mL human serum albumin in PBS, with or without 100μM semicarbazide or 100nM RTU-1096, at 37°C.

Immunofluorescence Microscopy

After microwave-based antigen retrieval, paraffin sections were incubated in 10% normal goat serum (Thermo Fisher Scientific) for 30 min and then incubated with a primary rabbit polyclonal antibody against VAP-1/SSAO (1μg/mL, ab42885, Abcam, Cambridge, MA) and a mouse monoclonal antibody against FDP-Lys (2μg/mL, NOF Corporation, Tokyo, Japan) at 4°C overnight prior to exposure to Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (1:400, Thermo Fisher Scientific) for 30 min at room temperature. Serial section was stained with rabbit polyclonal antibody against CD34 (1:100, ab81289, Abcam), an endothelial cell marker. Normal mouse Immunoglobulin G (IgG, 2μg/mL, Agilent, Santa Clara, CA) and normal rabbit IgG (1μg/mL, R&D systems, Minneapolis, MN) were used as negative control instead of primary antibodies. Photomicrographs were taken with a

fluorescence microscope (BIOREVO, BZ-9000, Keyence, Japan).

Cell Culture

The rat retinal capillary endothelial cell line, TR-iBRB2, was provided by Fact Inc. (Sendai, Japan)²⁴. TR-iBRB2 cells were cultured in type I collagen-coated 96-well plates with DMEM containing 5.5mM glucose (normal blood glucose concentration), L-glutamine supplemented with 10% (v/v) FBS, and 15mg/L endothelial cell growth factor (ECGF, Roche, Mannheim, Germany).

Measurement of Hydrogen Peroxide and FDP-Lys Production

Cells were washed twice with PBS and incubated with 200μM spermine in PBS, with or without 100μM semicarbazide or 100nM RTU-1096, and the concentration of hydrogen peroxide in the supernatant was measured. For FDP-Lys detection, cells were washed twice with PBS and incubated with 200μM spermine and 1mg/mL human serum albumin in PBS at 37°C, and the concentration of FDP-Lys was measured using an ELISA kit (MK-150, Takara Bio).

VAP-1/SSAO Overexpression in Cultured Microvascular Endothelial Cells

The rat *Vap-1* cDNA open reading frame (NM_031582.2) was cloned into pcDNA3.1/V5-HIS-C (Thermo Fisher Scientific) using standard cloning techniques. Rat *Vap-1*-pcDNA3.1/V5-HIS-C or Mock-pcDNA3.1/V5-HIS-C was transfected into TR-iBRB2 cells using X-treme Gene-HP (Roche).

Western Blotting

Cell lysates were sonicated 3 times for 5 sec each on ice, and centrifuged at 15000g at 4°C for 10 min. The protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific) and adjusted to 2mg/mL. The samples were boiled at 95°C for 3 min, separated by SDS-PAGE, and electroblotted to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). Membranes were incubated with 5% skim milk for blocking, then incubated with a rabbit polyclonal antibody against VAP-1/SSAO (1/1000, ab42885, Abcam) at 4°C overnight, and then finally incubated with goat anti-rabbit IgG (H+L) HRP conjugate (1:4000; Promega) at room temperature for 1 h. Signals were visualized using a Supersignal west pico chemiluminescent substrate (Thermo Fisher Scientific).

SSAO Activity Assay

Transfected cells were washed twice with serum-free DMEM and stimulated with rat recombinant TNF- α (0.1 to 1ng/mL, Peprotech) for 17 h. The cells were then incubated with 250 μ M benzylamine at 37°C for 2 h. Since benzylamine is a substrate for both SSAO and MAO-B, the cells were pretreated with 0.5mM pargylin, a MAO-B inhibitor, for 30 min. SSAO activity in the supernatant was measured using a Fluoro SSAO kit (Cell technology).

Measurement of FDP-Lys Production in Cultured Microvascular Endothelial Cells overexpressing VAP-1/SSAO

Transfected cells were washed twice with serum-free DMEM and stimulated with rat recombinant TNF- α (0.1 to 1ng/mL, Peprotech) for 17 h. The cells were then

incubated with 200 μ M spermine and 1mg/mL human serum albumin in PBS at 37°C. The level of FDP-Lys in the supernatant was determined using an ELISA kit (MK-150, Takara Bio).

Glutathione (GSH) Assay

Recombinant humanVAP-1/SSAO (Peprotech) was incubated with 2.5mM spermine in serum-free DMEM for 24 h. TR-iBRB2 cell was serum-starved and exposed to either pre-incubated mixture of rhVAP-1/SSAO with spermine or ACR ranging from 0 to 100 μ M for 3 h. Total GSH levels were measured using a total GSH assay kit (Nikken Seil Co., Ltd., Shizuoka, Japan), according to the manufacturer's protocol.

Cell Counting Assay

TR-iBRB2 cells were serum-starved for 17 h and then exposed to ACR. The number of viable cells were counted using 0.4% Trypan blue (Sigma) 24 h after stimulation with concentrations of ACR ranging from 0 to 100 μ M.

Reactive Oxygen Species (ROS) Detection Assay

Recombinant humanVAP-1/ SSAO (Peprotech) was incubated with 2.5mM spermine in serum free DMEM for 24 h. TR-iBRB2 cells were serum-starved for 17 h and exposed to the either pre-incubated mixture of rhVAP-1/SSAO with spermine or ACR ranging from 0 to 100 μ M for 24 h. Cellular oxidative stress status was measured by using DCFDA-Cellular ROS Detection Assay Kit (Abcam), according to the manufacturer's protocol.

Statistical Analysis

All results are presented as the mean $\pm$ SEM as indicated. The Mann-Whitney U Test was used for statistical comparisons between groups, and one-way ANOVA, with a Tukey-Kramer test, was used for multiple comparisons. Differences in the means were considered statistically significant if the probability values were <0.05 . Spearman's correlation coefficient was used in correlation analyses.

Results

Accumulation of the ACR-Conjugated Protein, FDP-Lys, in Eyes with DR

To determine whether the level of the unsaturated aldehyde ACR is greater in the eyes of patients with DR, the concentration of FDP-Lys was measured in the vitreous fluid samples obtained from patients with and without DR. FDP-Lys was detectable in all of the vitreous samples, and it was significantly elevated in the vitreous fluids of the PDR patients ($11.2 \pm 0.8 \text{ nmol/mL}$, $n=12$) when compared to those of the non-diabetic patients ($8.8 \pm 0.3 \text{ nmol/mL}$, $n=9$, $P < 0.05$, Fig. 1A). The protein levels of sVAP-1/SSAO were approximately 8.0-fold higher in the vitreous fluid samples of the PDR patients ($9.4 \pm 1.4 \text{ ng/mL}$, $n=12$), when compared to those of the non-diabetic patients ($1.2 \pm 0.2 \text{ ng/mL}$, $n=9$, $P < 0.01$, Fig. 1B). Furthermore, FDP-Lys levels were moderately correlated with sVAP-1/SSAO levels in the vitreous samples of the PDR patients ($p=0.67$, $n=12$, $P < 0.05$, Fig. 1C), but not with those of the non-diabetic patients ($P=0.87$, data not shown).

Specificity of the VAP-1/SSAO Inhibitor

To examine the specificity of the novel VAP-1/SSAO inhibitor RTU-1096, we quantified VAP-1/SSAO enzymatic activity in a radiochemical assay. In this assay, [^{14}C]-benzylamine is used as substrate, and the ratios of the half maximal inhibitory concentration (IC_{50}) values for VAP-1/SSAO and the functionally related MAO-A or B inhibition were obtained as an indication of the selectivity of our inhibitor. RTU-1096 showed an IC_{50} of 0.9 nM against human VAP-1/SSAO, while its IC_{50} against human MAO-A and -B were more than 100 μM (Fig. 2). The data indicates the specific

inhibitory property of RTU-1096 on human VAP-1/SSAO, but not MAO-A or B.

Production of FDP-Lys and Hydrogen Peroxide via VAP-1/SSAO Enzymatic Activity in the Presence of Spermine

To determine whether VAP-1/SSAO produces ACR and hydrogen peroxide via the oxidation of spermine, which is previously reported to increase in eyes with active PDR¹⁹, we incubated rhVAP-1/SSAO with spermine *in vitro*, and measured the concentrations of FDP-Lys and hydrogen peroxide.

Firstly, we compared four types of buffer solutions, and found that PBS is the optimal buffer for the enzymatic reaction of VAP-1/SSAO with spermine (Fig. 3A). The production of hydrogen peroxide was both dose-dependent (n=3 each, Fig. 3B) and time-dependent (n=3 each, Fig. 3C), and the reaction was suppressed by the addition of semicarbazide or the VAP-1 specific inhibitor, RTU-1096 (Fig. 3BC). Furthermore, the production of FDP-Lys increased when rhVAP-1/SSAO was incubated with spermine, and was abrogated by semicarbazide or RTU-1096 (n=3 to 5, Fig. 4AB).

Production of FDP-Lys and Hydrogen Peroxide Mediated by VAP-1/SSAO Enzymatic Activity in Retinal Microvascular Endothelial Cells

Immunofluorescence microscopy showed that VAP-1/SSAO staining was confined to the cytoplasm of vascular components including endothelial cells of the fibrovascular tissues obtained from patients with PDR (Fig. 5). Furthermore, FDP-Lys was found to be ubiquitous in the cytoplasm of these cellular components, and co-localized with

VAP-1/SSAO, indicating that FDP-Lys is generated by VAP-1/SSAO in vascular component cells including endothelial cells and potentially pericytes.

To determine whether VAP-1/SSAO activity leads to the production of ACR in endothelial cells, *in vitro* assays were performed using the rat retinal microvascular endothelial cell line, TR-iBRB2. Spermine treatment significantly increased hydrogen peroxide and FDP-Lys levels in the supernatants of the TR-iBRB2 cell line samples (n=8 each, Fig. 6AB); however, the induction was not remarkable. We then transfected rat Vap-1/SSAO into the TR-iBRB2 cell line (TR-iBRB2-Vap-1, Fig. 6C) and found that SSAO activity was unchanged in the supernatants of the Mock and TR-iBRB2-Vap-1 cells (27.7 ± 0.4 pmol/min/mL, 28.1 ± 0.3 pmol/min/mL, respectively, n=8 each, Fig. 6D). However, when pretreated with 1 ng/mL TNF- α , SSAO activity significantly increased in the supernatants of the TR-iBRB2-Vap-1 cells (37.1 ± 0.7 pmol/min/mL), when compared to those without the treatment (27.7 ± 0.4 pmol/min/mL, n=8, $P < 0.01$) and the Mock treatment (31.9 ± 0.5 pmol/min/mL, n=8, $P < 0.01$, Fig. 5D). Furthermore, TNF- α pretreatment significantly increased FDP-Lys production in the supernatant of the TR-iBRB2-Vap-1 cells (23.5 ± 3.5 nmol/mg, n=6) in comparison with those of the Mock-transfected cells (13.7 ± 1.4 nmol/mg, n=6, $P < 0.05$, Fig. 6E).

Cell Toxicity of FDP-Lys in Retinal Microvascular Endothelial Cells

To investigate the impact of ACR on endothelial cells, the intracellular GSH levels and cell numbers were measured after ACR stimulation. The GSH levels reduced (n=4 each, $P < 0.05$, Fig. 7A) and ROS activity increased in the TR-iBRB2 cells with stimulation of ACR in a dose-dependent manner (n=5 each, $P < 0.01$, Fig. 7B).

Furthermore, the number of viable cells was decreased when stimulated with 100 μ M ACR (n=4 each, P<0.01, Fig. 7C).

In accord with the *in vitro* incubation experiment, the TR-iBRB2 cells stimulated with pre-incubated mixture of rhVAP-1/SSAO with spermine also showed the reduced GSH level (n=3 each, P<0.01, Fig. 7D) and increased ROS activity (n=5 each, P<0.01, Fig. 7E).

Discussion

In the present study, we show that the levels of the ACR-conjugated protein, FDP-Lys, are elevated in the vitreous fluid of patients with DR. VAP-1/SSAO protein levels are also elevated, commensurate with FDP-Lys levels, and VAP-1/SSAO generates ACR and hydrogen peroxide as metabolic byproducts of spermine metabolism. Furthermore, we show that TNF- α stimulates the production of ACR via VAP-1/SSAO-mediated spermine metabolism in retinal endothelial cells, and that ACR reduces the levels of the critical endogenous antioxidant GSH and inversely increases ROS, eventually causes the cell death. The current data indicate that sVAP-1/SSAO originated from the membrane-anchored VAP-1/SSAO contribute as an enzyme to deteriorate an oxidative stress environment in the pathogenesis of DR.

In previous studies, we have shown that the levels of sVAP-1/SSAO are higher in the vitreous fluid samples of patients with PDR when compared with those from non-diabetic patients.¹² Consistent with the results, we found the protein levels of sVAP-1/SSAO were higher in patients with PDR than in individuals without diabetes in this study. Furthermore, we found that vitreous fluid ACR-conjugated protein levels were elevated, in correlation with the increased sVAP-1/SSAO levels, in PDR patients, indicating that sVAP-1 acts as an SSAO and generates ACR in eyes with DR.

Polyamines are low molecular weight polycations that have two or more primary amino groups, and are known to play an important role in cell proliferation and differentiation.²⁵ In mammals, there are three naturally occurring polyamines: putrescine, spermidine, and spermine.²⁶ Since putrescine, the precursor used for biosynthesis of the other two polyamines, and urea cycle enzymes that catalyze

polyamines are ubiquitously present in all tissues, polyamines are systemically distributed.²⁷ In patients with PDR, the levels of spermine have been shown to be up to 15-times higher in vitreous fluid when compared to those of non-DR patients.¹⁹ As mentioned, sVAP-1/SSAO is known to convert primary amines to the corresponding aldehydes with the release of hydrogen peroxide and ammonia.¹⁵ In patients with severe renal failure, the toxic compound ACR is produced in high concentrations from spermidine and spermine by amine oxidase, an enzyme that is inhibited by semicarbazide.²⁸ Prior evidence has suggested that the amine oxidase activity of VAP-1/SSAO is involved in the generation of ACR via the oxidation of spermine in the eyes of patients with PDR. In the current study, we demonstrate that VAP-1/SSAO indeed catalyzes spermine oxidation, indicating that VAP-1/SSAO contributes to the pathogenesis of DR through spermine oxidation, followed by generation of hydrogen peroxide and ACR. Moreover, it was also shown that specific VAP-1 inhibitor RTU-1096 could suppress the function of VAP-1/SSAO at lower concentration than semicarbazide, indicating the potent inhibitory effect of the compound RTU-1096.

Soluble VAP-1/SSAO is present in vitreous fluid, while membrane-anchored VAP-1/SSAO is constitutively expressed in vascular components of normal human retina²⁹ and fibrovascular tissues of the eyes of patients with PDR.¹² In this study, we confirmed the presence of VAP-1/SSAO and FDP-Lys in CD34-positive endothelial cells and surrounding cells, potentially pericytes,²² of fibrovascular tissues. Therefore, it is possible that ACR and hydrogen peroxide are produced via an enzymatic reaction mediated by VAP-1/SSAO in the cellular components. In addition, we previously reported that matrix metalloproteinase (MMP)-2 and MMP-9 elicited VAP-1 shedding from cultured endothelial cells.¹² Therefore, it is possible

that sVAP-1/SSAO released from the cellular components also participate in production of ACR and hydrogen peroxide.

Treatment with spermine alone resulted in only slight increases in the levels of hydrogen peroxide and ACR in cultured retinal microvascular endothelial cells, and overexpression of VAP-1/SSAO by transient transfection was not sufficient to increase ACR production. However, TNF- α pretreatment dramatically increased FDP-Lys generation in the supernatants of the VAP-1/SSAO transfectants when treated with spermine. These data indicate that TNF- α plays a key role in the oxidation of spermine by VAP-1/SSAO. Patients with inflammatory bowel diseases have been shown to display markedly increased expression levels of VAP-1,³⁰ and previously we reported that sVAP-1/SSAO secretion was enhanced by the stimulation of inflammatory cytokines, including TNF- α in retinal endothelial cells.¹² Therefore, it is possible that TNF- α induced the release of sVAP-1/SSAO into the culture media and in the vitreous of PDR eyes, leading to an increase in the reaction frequency of sVAP-1/SSAO and its substrate spermine. However, it is also possible that increased vitreous level of sVAP-1 is due to the hyperpermeability of retinal vessels caused by VEGF in eyes with PDR, since break down of blood-retina-barrier by inflammatory cytokines including VEGF causes serum component influx into the vitreous and elevation of protein concentration in PDR eyes.^{31, 32} In addition to the influx of serum ACR and its adduct FDP-Lys, it is likely that sVAP-1/SSAO contributes to production of ACR through spermine oxidation, leading to FDP-Lys accumulation in the vitreous of PDR patients.

The role of oxidative stress in the pathogenesis of DR has been extensively examined in experimental and clinical studies. The levels of various ROS, including superoxide³³ and hydrogen peroxide,³⁴ have been shown to be elevated in the

retinas of diabetic animals, and the antioxidant defense systems in the eyes of patients with DR have been shown to be functionally damaged.⁷ Furthermore, ACR and its adduct FDP-Lys have been shown to covalently bind to thiols, such as GSH.^{16, 17} Our results agree with those of the previous studies, and show that ACR stimulation reduces the intracellular reserves of GSH, and causes cell death in retinal microvascular endothelial cells. Furthermore, our data indicate that generation of ACR and/or FDP-Lys via spermine oxidation by VAP-1/SSAO deteriorate oxidative stress environment through limiting the defense system against oxidative stress as well as ROS generation. We recently reported that the thickening of the outer nuclear layer caused by laser photocoagulation was abrogated by oral administration of RTU-1096,³⁵ also used in the current study. In the previous study, it was demonstrated that laser photocoagulation caused upregulation of the leukocyte adhesion molecules, intercellular adhesion molecule-1 (ICAM-1), and infiltration of inflammatory cells at the vitreoretinal surface, both of which were suppressed by RTU-1096 administration. Since oxidative hydrogen peroxide is known to augment ICAM-1 expression in endothelial cells,^{36, 37} it is possible that hydrogen peroxide generation mediated by VAP-1/SSAO plays a role in the response caused by laser photocoagulation.

The limitations of the current study are as follows. First, the concentrations of sVAP-1 protein used for *in vitro* study were higher than concentrations measured in vitreous fluid of human samples, while the current data demonstrated that spermine was also an exogenous substrate for SSAO. Second, the current study did not include experimental animal data to support the findings of *in vitro* experiments. Additional research is needed to obtain the detailed information about spermine oxidation by sVAP-1/SSAO.

In conclusion, our results show that the amine oxidase activities of sVAP-1/SSAO and membrane-anchored VAP-1/SSAO contribute to the pathogenesis of PDR. Previous studies have shown that VAP-1 inhibition can reduce various ocular inflammatory responses, including the induction of leukocyte adhesion molecules and inflammatory cell accumulation in animal models. Our results demonstrate that VAP-1 contributes to the pathogenesis of DR through both function as endothelial adhesion molecule and enzyme SSAO, suggesting that VAP-1/SSAO inhibition may be a novel, potent therapeutic strategy for the treatment of DR.

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Declaration of Interest

The following authors reported potential conflicts of interest: K.N. was a consultant of Sucampo Pharma, LLC until October, 2016; A. Kawasaki is an employee of Sucampo Pharma, LLC. No other potential conflicts of interest relevant to this article were reported.

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Murata *et al.*

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Figure Legends

Figure 1

Vitreous Fluid Levels of FDP-Lys and sVAP-1/SSAO in Patients with or without Diabetic Retinopathy. Levels of **(A)** FDP-Lys and **(B)** sVAP-1/SSAO in the vitreous fluid samples of ERM/MH (n=9) and PDR patients (n=12). *, $P<0.05$ **, $P<0.01$. **(C)** Correlation of sVAP-1 and FDP-Lys levels in the vitreous fluid samples of patients with PDR ($\rho=0.67$, $P<0.05$, $n=12$).

Figure 2

Specificity of the VAP-1/SSAO inhibitor RTU-1096. **(A)** Inhibition curve of the RTU-1096 to human SSAO. **(B)** Inhibition curve of the RTU-1096 to human MAO-A and MAO-B.

Figure 3

Hydrogen Peroxide Production Resulting from Spermine Metabolism by sVAP-1/SSAO. **(A)** Hydrogen peroxide concentration when rhVAP-1/SSAO was incubated with spermine (2.5mM) for 3 h at 37°C in four different buffers: 50mM HEPES, 50mM Tris-HCl, physiological HEPES and PBS. **(B)** Hydrogen peroxide concentration when rhVAP-1/SSAO was incubated with spermine (2.5mM) in PBS, with or without semicarbazide (100μM) or the VAP-1/SSAO specific inhibitor RTU-1096 (100nM) at 37°C for 3 h (n=3). **(C)** Hydrogen peroxide concentration when rhVAP-1/SSAO (2μg/mL) was incubated with spermine (2.5mM), with or without semicarbazide (100μM) or the RTU-1096 (100nM) at 37°C for the indicated time (n=3). Values represent mean±SEM, *, $P<0.05$ **, $P<0.01$.

Figure 4

ACR Production by sVAP-1/SSAO via Spermine Metabolism. (A) FDP-Lys concentration when rhVAP-1/SSAO (5µg/mL) was incubated with 2.5mM spermine and 1µg/mL human serum albumin at 37°C for the indicated time (n=5). (B) FDP-Lys concentration when rhVAP-1/SSAO was incubated with 2.5mM spermine and 1µg/mL human serum albumin, with or without 100µM semicarbazide or 100nM RTU-1096, at 37°C for 24 h (n=3 each). Values represent mean±SEM, *, P<0.05 **, P<0.01.

Figure 5

Localization of FDP-Lys and VAP-1/SSAO in Fibrovascular Tissues. Representative fluorescent micrographs of immunofluorescence in fibrovascular tissues. (A) Green, FDP-Lys (Alexa Fluor® 488). (B) Red, VAP-1/SSAO (Alexa Fluor® 546). (C) Blue, counterstaining for the nuclei with DAPI. (D) Merged image. Arrows indicate the co-localization of FDP-Lys with VAP-1/SSAO in endothelial cells. (E) Red, CD34 (Alexa Fluor® 546) and (F) Negative control (mouse and rabbit normal IgG) in sequential sections. Scale bar, 10µm.

Figure 6

Hydrogen Peroxide and ACR Production via Spermine Oxidation Catalyzed by VAP-1/SSAO in Retinal Microvascular Endothelial Cells. (A) Hydrogen peroxide concentration when TR-iBRB2 cells were incubated with spermine (200µM) in PBS at 37°C for 6 h (n=8). (B) FDP-Lys concentration when TR-iBRB2 cells were incubated with 1mg/mL human serum albumin and spermine (200µM) in PBS at

37°C for 24 h (n=8). Values are mean±SEM, **, P<0.01. **(C)** Transient transfection of rat VAP-1-pcDNA3.1/V5-HIS-C or Mock-pcDNA3.1/V5-HIS-C in TR-iBRB2 cells. **(D)** SSAO activity in the supernatant collected from cells stimulated with TNF- α for 17 h (n=8 each). **(E)** FDP-Lys concentration in the supernatant collected from cells stimulated with TNF- α for 17 h (n=6 each). Values are mean±SEM, *, P<0.05 **, P<0.01.

Figure 7

Impact of ACR Stimulation on Antioxidant Defense Systems in Retinal Microvascular Endothelial Cells. **(A)** Total GSH levels when TR-iBRB2 cells were exposed to ACR for 3 h (n=4 each). **(B)** ROS activity when TR-iBRB2 cells were exposed to ACR for 24 h (n=5 each). **(C)** The number of viable cells when TR-iBRB2 cells 24 h after ACR stimulation (n=4 each). **(D)** Total GSH levels when TR-iBRB2 cells were exposed to the mixture of rhVAP-1/SSAO and 2.5mM spermine for 3 h (n=3 each). **(E)** ROS activity when TR-iBRB2 cells exposed to the mixture of rhVAP-1/SSAO and 2.5mM spermine for 24 h (n=5 each). Values are mean±SEM, *, P<0.05 **, P<0.01.

A

B

C

Figure 1

Figure 2

A

B

C

Figure 3

A

B

Figure 4

Figure 5

A **B** **C** **D** **E** **Figure 6**

A

B

C

D

E

Figure 7

Characteristics of the Patients

Case	Age(y/o)	Gender	Diagnosis	Case	Age(y/o)	Gender	Diagnosis	HbA1c (%)	HbA1c (mmol/mol)	VH	TRD
1	61	Male	ERM	1	66	Male	PDR	8.2	66	+	-
2	67	Female	ERM	2	67	Female	PDR	6.6	49	-	-
3	75	Male	ERM	3	35	Male	PDR	6.8	51	+	+
4	77	Female	ERM	4	37	Male	PDR	6.4	46	+	+
5	59	Male	ERM	5	76	Male	PDR	7.5	58	+	-
6	58	Female	MH	6	67	Male	PDR	12.9	117	+	-
7	62	Female	ERM	7	47	Male	PDR	7.5	58	+	-
8	85	Male	ERM	8	63	Female	PDR	6.8	51	-	+
9	72	Female	MH	9	68	Female	PDR	7.1	54	-	-
				10	40	Male	PDR	5.8	40	+	-
				11	67	Female	PDR	6.7	50	+	-
				12	65	Female	PDR	7	53	+	-

VH, vitreous hemorrhage; TRD, tractional retinal detachment

Table 1