Proteolytic Cleavage of Vascular Adhesion Protein-1 Induced by Vascular Endothelial Growth Factor in Retinal Capillary Endothelial Cells

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Running Title: VEGF induces the cleavage of VAP-1

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Abstract

Purpose: To investigate the mechanism of soluble vascular adhesion protein-1 (sVAP-1) accumulation induced by vascular endothelial growth factor (VEGF) in the vitreous of patients with diabetic retinopathy (DR).

Study Design: Experimental

Methods: Protein levels of sVAP-1 and N epsilon-(hexanoyl)lysine (HEL), an oxidative stress marker, in the vitreous samples from patients with proliferative diabetic retinopathy (PDR) with or without intravitreal bevacizumab (IVB) injection were determined by ELISA. The effect of VEGF on both mRNA expression of Vap-1 and secretion of sVAP-1 in rat retinal capillary endothelial cells (TR-iBRB2) was analyzed by real-time PCR and western blotting, respectively. In addition, the impact of VEGF on production and activation ratios of matrix metalloproteinase (MMP)-2 and MMP-9 was examined by gelatin zymography. Hydrogen peroxide production and reactive oxygen species (ROS) levels were assessed in the supernatants of TR-iBRB2 cells treated with VEGF.

Results: IVB injection decreased vitreous levels of sVAP-1 and HEL in patients with PDR. VEGF stimulation released sVAP-1 protein from TR-iBRB2 cells as a consequence of membrane-anchored VAP-1 shedding by MMP-2 and MMP-9. In addition, VEGF increased hydrogen peroxide generation and ROS augmentation through spermine oxidation by sVAP-1 as semicarbazide-sensitive amine oxidase (SSAO) in the supernatant of cultured endothelial cells.

Conclusions: The current data demonstrate that proangiogenic factor VEGF induces sVAP-1 release from retinal capillary endothelial cells and facilitates hydrogen peroxide generation via enzymatic property of sVAP-1, followed by the increase of oxidative stress, one of the crucial factors in the pathogenesis of DR.

Key words

vascular endothelial growth factor (VEGF), vascular adhesion protein-1(VAP-1),
semicarbazide-sensitive amine oxidase (SSAO) and matrix metalloproteinases (MMPs)

Introduction

Diabetic retinopathy (DR) is a leading cause of vision loss [1]. Under hyperglycemic conditions, cellular constituents of retinal vessels are damaged and the changes cause obliteration of retinal microvasculature, i.e., retinal ischemia, and eventual pathological neovascularization at the vitreoretinal surface, which is a hallmark of proliferative diabetic retinopathy (PDR) and leads to severe complications such as vitreous hemorrhage and tractional retinal detachment [2]. In addition, retinal ischemia compromises the blood-retinal barrier and results in fluid accumulation in the center of the diabetic retina, causing diabetic macular edema (DME) [2]. Extensive basic and clinical studies elucidate that vascular endothelial growth factor (VEGF) plays a crucial role in the pathogenesis of PDR [3] and DME [4, 5]. As a corollary, anti-VEGF agents have emerged as a part of first-line treatment for DR. However, the pathogenesis of DR is multifactorial, warranting further studies investigating the underlying pathophysiology of this vision-threatening disease.

Vascular adhesion protein (VAP)-1 is a moonlighting protein that possesses multiple molecular functions and is involved in inflammatory responses [6, 7]. VAP-1 participates in leukocyte trafficking as a leukocyte adhesion molecule [8, 9], and is also a member of the semicarbazide-sensitive amine oxidase (SSAO), which generates hydrogen peroxide, ammonia and the corresponding aldehydes [10] through oxidation of primary monoamines. Previously, using animal models we showed that VAP-1 is involved in the molecular mechanisms of acute ocular inflammation [11], inflammation-associated ocular angiogenesis [12] and leukostasis under diabetic conditions [13]. These findings indicate that, as a leukocyte adhesion molecule, VAP-1 plays a critical role not only in systemic disorders such as atherosclerosis [14], liver fibrosis [15], primary sclerosing cholangitis [16] and malignant cancer [17], but also in ocular diseases associated with inflammation such as DR.

In addition, VAP-1 exists also in a soluble form (sVAP-1) in mammals. It has been shown that, compared to healthy controls, serum SSAO activity, i.e., enzymatic activity of sVAP-1, is significantly elevated in patients with type I and type II diabetes [18]. Furthermore, we reported that, in the serum of patients with type II diabetes there was a positive correlation between the serum concentration of sVAP-1 and VEGF, whereas the soluble forms of other leukocyte adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 showed no correlation with VEGF [19]. The level of sVAP-1 also increased in the vitreous fluid samples obtained from patients with PDR [20]; therefore, in the current study we sought to investigate the link between VEGF and sVAP-1 in DR.

Material and Methods

Specimens

Undiluted vitreous samples were collected from 37 eyes of 37 patients with PDR (22 men and 15 women; mean age, 58.8±1.4y/o), who underwent pars plana vitrectomy for prolonged vitreous hemorrhage and tractional retinal detachment involving macular lesions, and were frozen rapidly at -80°C. Previously, we reported that sVAP-1 levels in the vitreous of patients with PDR increased when compared with those of patients without diabetes [20]. In the current study, we further analyzed the data points of PDR patients alone to investigate the effect of intravitreal bevacizumab (IVB) injections on vitreous concentrations of sVAP-1 in eyes with PDR. This study was conducted in accordance with the tenets of the Declaration of Helsinki and received approval from the institutional review committee of Hokkaido University Hospital (#10-0296). 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 protein levels of sVAP-1 in vitreous samples were measured using ELISA kits for human sVAP-1 (Bender MedSystems, Vienna, Austria). According to the manufacturer's protocol, the samples were processed with the reagents and the optical density was determined at 450 and 650nm using a microplate reader (Sunrise, TECAN, Männedorf, Switzerland). N epsilon-(hexanoyl) lysine (HEL) is a lipid hydroperoxide-modified lysine residue, a marker of early lipid peroxidation-derived protein modification, i.e., oxidative stress. HEL levels were measured in the vitreous samples by a competitive ELISA kit (Nikken Seil Co., Ltd., Shizuoka, Japan), according to the manufacturer's protocol. The optical density was determined at 450nm using a microplate reader (Sunrise, TECAN).

Cell Culture

Rat retinal capillary endothelial cell line TR-iBRB2, isolated from the retina of the male transgenic rats (RRID:CVCL_J379) [21], was provided by Fact, Inc., Sendai, Japan. The TR-iBRB2 cells were cultured in DMEM containing 25mM glucose and L-glutamine supplemented with 10% (v/v) FBS and 15mg/l endothelial cell growth factor (ECGF, Roche, Mannheim, Germany) in type I collagen-coated culture flasks or dishes. For VEGF stimulation, cells were cultured in serum-free DMEM and treated with rat recombinant VEGF (accession # AAL07526.1, 1 to 100ng/ml, R&D systems, Minneapolis, MN, USA).

Quantitative Real-time Polymerase Chain Reaction (PCR)

Expression levels of Vap-1 mRNA were examined by quantitative real-time PCR. Total RNA was extracted from cells using TRIZOL reagent (Life Technologies) and reverse transcribed to cDNA using GoScript™ reverse transcriptase (Promega), according to manufacturer's protocol. Analysis of mRNA level was performed on StepOnePlus™ Real-Time PCR System (Life Technologies, Delhi, India) using GoTaq® qPCR Master Mix (Promega, Tokyo, Japan). The primer sequences used for real-time PCR and the expected size of the amplification products are as follows: 5′- CGGTGCTGGCGAGAAGTTTG -3′ (forward) and 5′- TCTGCCCAGGCCCAGTTCTTC -3′ (reverse) for rat Vap-1 (NM_031582.2); 123 bp, 5′- GGGAAATCGTGCGTGACATT -3′ (forward) and 5′- GCGGCAGTGGCCATCTC -3′ (reverse) for rat Actb (NM_031144); 76 bp. PCR conditions used were 95°C, 2 min; followed by 95°C, 15 sec; 60°C, 1 min for 40 cycles. All data were calculated by the ΔΔCt method with Actb as a normalization control.

Western Blotting

For western blotting of cell culture supernatant, culture media were collected and centrifuged at 500g for 10 min at 4°C. After centrifugation, the supernatants were concentrated at 20 times using a centrifugal filter unit (Amicon ultra 3kDa, Merck, Darmstadt, Germany). The supernatants were normalized with total protein concentration measured by Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and were mixed with 5x Laemmli sample buffer and boiled at 95°C for 3 min.The samples were separated by SDS-PAGE and electroblotted to polyvinylidene fluoride (PVDF) membranes (Immobilon-PSQ, Merck). To block non-specific binding, the membranes were incubated with 5% skim milk and subsequently incubated with rabbit polyclonal antibody against VAP-1 (1:1000, ab42885, Abcam, Cambridge, UK) at 4°C overnight, then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4000; Jackson immunoResearch, West Grove, PA, USA). Signals were visualized with chemiluminescence (SuperSignal West Pico; Thermo Fisher scientific, Waltham, MA,USA), according to the manufacturer's protocol.

SSAO Activity Assay

The TR-iBRB2 cells were washed twice with serum-free DMEM and stimulated with rat recombinant VEGF (1 to 100ng/ml, R&D systems) in serum-free DMEM for 17 h and the supernatants were harvested and centrifuged at 500g for 10 min at 4°C. The supernatants were then incubated with 250μM benzylamine at 37°C for 2 h. Since benzylamine is a substrate for both SSAO and MAO-B, the supernatants 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, Fremont, CA, USA).

Zymography

The TR-iBRB2 cells were stimulated with rat recombinant VEGF (100ng/ml, R&D systems) for 17 h. The supernatants were harvested and concentrated through centrifugal ultrafiltration (Amicon ultra 3kDa), and gelatin zymoglaphy was performed according to the manufacture's protocol (Cosmobio, Tokyo, Japan). The density of negatively stained bands was measured with ImageJ software (available from https://imagej.nih.gov/ij/; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Gelatinolytic bands of approximately 92, 83, 68, and 62 kDa corresponded to proMMP-9, active MMP-9, proMMP-2, and active MMP-2, respectively. The activation ratios of MMPs were calculated by dividing the density of the band for the active form by the sum of the density of the bands for both the latent and active forms.

MMP Inhibitor Treatment

TR-iBRB2 cells were stimulated with rat recombinant VEGF (100ng/ml, R&D systems) for 17 h with or without broad spectrum MMP inhibitor Batimastat (Tocris Bioscience, Bristol, UK). The supernatants were harvested and SSAO activity was measured using a Fluoro SSAO kit (Cell technology, Fremont, CA, USA).

Hydrogen Peroxide Assay

The TR-iBRB2 cells were washed twice with serum-free DMEM and stimulated with rat recombinant VEGF (10 to 100ng/ml, R&D systems) in serum-free DMEM for 17 h and the supernatants were harvested. Since spermine is a substrate for both SSAO and spermine

oxidase (SMOX), the supernatants were pretreated with a SMOX inhibitor, MDL72527 [22] (50μM, Sigma) at 37°C for 30 min and then incubated with 200μM spermine (Sigma) at 37°C for 2 h. Hydrogen peroxide concentration in the supernatant was measured using a Fluoro hydrogen peroxide kit (Cell technology).

Reactive Oxygen Species (ROS) Detection Assay

TR-iBRB2 cells were washed twice with serum-free DMEM and stimulated with rat recombinant VEGF (100ng/ml, R&D systems) and VAP-1 inhibitor semicarbazide (100μM, Cell technology) for 17 h. The supernatants were collected and mixed with 50μM MDL72527 at 37°C for 30 min, and then incubated with 200μM spermine at 37°C for 24 h. The solution was added to freshly-seeded TR-iBRB2 cells and incubated for 24 h. Thereafter, ROS activity was measured using DCFDA-Cellular ROS Detection Assay Kit (Abcam), following the manufacturer's protocol.

Statistical Analysis

All results are presented as the mean±SEM as indicated. The student t-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 less than 0.05. Pearson correlation coefficient was used to examine correlations.

Results

VEGF neutralization reduces vitreous levels of sVAP-1/SSAO and shows diminished correlation with HEL in patients with PDR

To determine whether VEGF neutralization by IVB alters sVAP-1 concentrations in the vitreous of PDR patients, we compared the concentration of sVAP-1 in PDR patients treated with (IVB group) or without preoperative IVB injection (non-IVB group). The concentration of sVAP-1 (5.5±1.2ng/ml, n=14) was lower in the IVB group than in the non-IVB group (9.3±1.2ng/ml, n=23, $P<0.05$, Fig. 1a). Furthermore, the level of HEL, an oxidative stress marker, was also decreased in IVB group (4.5±0.6nM, n=14) compared to the non-IVB group (8.5±0.9nM, n=23, $P<0.01$, Fig. 1b).

In addition, although there was a moderate correlation between sVAP-1 and HEL in the vitreous fluid samples obtained from PDR eyes without preoperative VEGF blockade ($r=0.45$, $P<0.05$, Fig. 1c), this correlation diminished in eyes with PDR that had undergone IVB injection ($P=0.46$, Fig. 1d).

sVAP-1/SSAO is released, but not synthesized de novo, from retinal capillary endothelial cells with VEGF stimulation

To determine whether VEGF increases sVAP-1 production in retinal endothelial cells expressing the membrane form of VAP-1 on the cell surface, we investigated the effect of VEGF stimulation on both Vap-1 mRNA expression and sVAP-1 accumulation in the culture media of rat retinal capillary endothelial cell line, TR-iBRB2 cells. Real-time PCR findings showed that Vap-1 mRNA expression was not altered with VEGF stimulation in TR-iBRB2 cells (n=4, $P=0.40$, Fig. 2a). However, western blotting revealed that VEGF stimulation increased the protein level of sVAP-1 in the supernatants of cultured endothelial cells in a

dose-dependent manner (n=3 each, P<0.01, Fig. 2bc). In accordance with the increase in sVAP-1 protein levels, the enzymatic activity of SSAO was also elevated when stimulated with VEGF (n=3 each, P<0.05, Fig.. 2d). The current data indicate that sVAP-1 accumulated in the supernatant as a consequence of increased sVAP-1 secretion from retinal microvascular endothelial cells, but not de novo synthesis.

VAP-1 protein is shed by MMP-2 and MMP-9 from retinal capillary endothelial cells after VEGF stimulation

To investigate the mechanism of increased sVAP-1 secretion from retinal endothelial cells stimulated with VEGF, we sought to identify the molecules responsible for enhancing VEGF-induced sVAP-1 secretion. Previously, we reported that inflammatory cytokines such as TNF-α and IL-1β facilitated the release of sVAP-1 from retinal capillary endothelial cells through shedding of the membrane-bound form of VAP-1 by MMPs [20]. Therefore, we hypothesized that VEGF-induced secretion of sVAP-1 from retinal capillary endothelial cells is also attributed to cleavage of the membrane-bound form of VAP-1 by MMPs. Gelatin zymography revealed that proMMP-2 and proMMP-9 were increased by VEGF stimulation in TR-iBRB2 cells (n=3 each, Fig. 3ab). In addition, the activation ratio of MMP-2 (n=3 each, P<0.05) and MMP-9 (n=3 each, P<0.01) increased in the supernatant of TR-iBRB2 cells stimulated with VEGF (Fig. 3c). Furthermore, Batimastat, a MMP inhibitor, reduced enzymatic activity SSAO and thus levels of sVAP-1 in the supernatant of TR-iBRB2 cells stimulated with VEGF (n=12 each, P<0.05, Fig. 3d). Taken together, these data indicate that MMP-2 and -9 are indeed the enzymes involved in the cleavage of the membrane-bound form of VAP-1 caused by VEGF in retinal capillary endothelial cells.

VAP-1/SSAO mediates oxidative stress caused by VEGF from retinal capillary endothelial cells

To study the pathological role of increased sVAP-1 in the vitreous of PDR eyes, we measured the concentration of hydrogen peroxide, a by-product generated through amine oxidation catalyzed by VAP-1/SSAO. It is reported that spermine, one of the polyamines, was increased in the vitreous fluid of patients with PDR[23]. Therefore, we sought to determine whether VEGF increased hydrogen peroxide generation in retinal capillary endothelial cells in the presence of spermine. VEGF stimulation did elevate the levels of hydrogen peroxide ($n=5$, $P<0.01$, Fig. 4a) and ROS activity ($n=5$, $P<0.01$, Fig. 4b) in the supernatant of TR-iBRB2 cells. In the presence of spermine, ROS activity was further elevated by VEGF, and blockade of VAP-1/SSAO by VAP-1 inhibitor semicarbazide suppressed this increase ($n=5$, $P<0.01$, Fig. 4b). This indicates that VAP-1/SSAO mediates VEGF-mediated oxidative stress in retinal capillary endothelial cells.

Discussion

In the present study, we demonstrated that i) preoperative IVB injection decreased the vitreous levels of sVAP-1 and the oxidative stress marker HEL in patients with PDR, ii) IVB diminished the correlation between sVAP-1 and HEL, iii) VEGF stimulation released sVAP-1 protein from retinal capillary endothelial cells as a consequence of membrane-anchored VAP-1 shedding by MMP-2 and MMP-9, and iv) VEGF enhances oxidative stress by generating hydrogen peroxide through spermine oxidation by SSAO enzymatic cleavage of sVAP-1. The current data indicate the mechanism of sVAP-1 accumulation and the potential involvement of sVAP-1 to increase oxidative stress in eyes with DR.

In 1994, it was established that VEGF is increased in the vitreous fluid of patients with ischemic retinal disorders [24]. Subsequent analyses also revealed that VEGF is a major participant in promoting angiogenesis and vascular leakage in the pathogenesis of DR [3, 25, 26]. In the current study, it was shown that VEGF blockade by IVB injection reduced the vitreous level of sVAP-1 in eyes with PDR. Breakdown of the blood-retina-barrier by VEGF causes serum component influx into the vitreous and elevated protein concentration in PDR eyes [27, 28]. In addition, serum levels of sVAP-1 were highly elevated in patients with type I diabetes who were metabolically compromised [29]. Similarly, the serum level of sVAP-1/SSAO was also higher in type II diabetes than in normal subjects [19]. Therefore, it is possible that the increased vitreous level of sVAP-1 in eyes with PDR is in part due to hyperpermeability of retinal vessels caused by VEGF.

However, the current study also demonstrates that VEGF increases VAP-1 shedding by MMPs from retinal capillary endothelial cells, while VEGF had no effect on Vap-1 mRNA expression. Of the various MMPs, MMP-2 and MMP-9 are known as gelatinases that degrade type IV collagen, laminin and fibronectin, all of which are the main constituents of the

basement membrane. As such, much attention has been focused on the role of MMPs in neovascularization in eyes with PDR [30, 31]. Notably, accumulated lines of evidence have also revealed that MMP-2 and MMP-9 cleave cell surface molecules such as ICAM-1 [32] and P-cadherin [33]. In addition, VEGF is known to induce the production and activation of MMP-2 and MMP-9. It is reported that VEGF upregulated transcription of MMP-2 [34] and membrane type1-MMP(MT1-MMP), the activator of MMP-2 [35]. It was also demonstrated that VEGF induced expression of ETS-1 factor, which bound to MMP-9 promoter, resulting the MMP-9 upregulation [36]. In accord with previous studies, the current data also demonstrates that VEGF induced production and activation ratios of MMP-2 and MMP-9, and that VEGF also increased SSAO activity in the supernatant of cultured microvascular endothelial cells, which was reduced by the MMP inhibitor Batimastat, indicating that MMP-2 and MMP-9 mediate the shedding of the membrane form of VAP-1 and its release into the supernatant from cultured endothelial cells. Previously, we reported that the inflammatory cytokines TNF-α and IL-1β, both increased in the vitreous of PDR patients [37, 38], also induced VAP-1 shedding from retinal capillary endothelial cells [20]. The previous and current data reveal that inflammatory cytokines accelerate sVAP-1 accumulation in the vitreous of PDR patients via a common pathway.

It is reported that the soluble form of adhesion molecules, e.g., ICAM-1 and VCAM-1, was increased in eyes with DR [39-41]. However, the biological and/or physiological roles of such soluble adhesion molecules remain unclear. In contrast, sVAP-1 is known as an enzyme that converts primary amines into their corresponding aldehydes, releasing hydrogen peroxide and ammonia [42]. In the current study, it was shown that preoperative IVB injections decreased the vitreous level of oxidative stress marker HEL in patients with PDR. In addition, VEGF stimulation generated hydrogen peroxide in the presence of spermine, one of the representative polyamine increased in eyes with PDR [23]. VEGF is reported to induce

the formation of ROS via induction of NADPH oxidase activity [43]. However, the current data showed a different mechanism of ROS elevation by VEGF. Interestingly, ROS activity induced by VEGF further increased in the presence of spermine and was abrogated by SSAO inhibitor semicarbazide. Recently, we reported that sVAP-1 generated hydrogen peroxide and toxic aldehyde acrolein, both associated with oxidative stress, as a consequence of spermine oxidation [44]. The previous and current data indicate that VEGF contributes to increase of oxidative stress via induction of sVAP-1 shedding by MMPs and that anti-VEGF therapy is beneficial to reduce not only angiogenesis and vascular leakage, but also oxidative stress in PDR eyes.

The limitations of the current study are as follows. First, the effect of VEGF inhibition by IVB on sVAP-1 level was assessed in human samples, while in vitro experiments revealed the effect of VEGF stimulation on sVAP-1 level. Second, the cultured cells used in this study were an immortalized rodent cell line TR-iBRB, and the TR-iBRB cells possess endothelial properties [45]. Additional research is needed to validate the current data.

In summary, we demonstrated that proangiogenic factor VEGF facilitates shedding of membrane-bound VAP-1 from retinal capillary endothelial cells through MMP-2 and MMP-9, and that released sVAP-1 enhances oxidative stress. An important role of oxidative stress in the pathogenesis of DR has been well studied by experimental and clinical studies. These results provide a novel insight into the roles of VEGF and sVAP-1 in eyes with DR.

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Figure Legends

Fig. 1

sVAP-1/SSAO and Oxidative Stress Marker N Epsilon-(hexanoyl) Lysine (HEL) in Eyes with PDR (a) Levels of sVAP-1/SSAO in the vitreous fluid samples obtained from PDR patients with (n=14) or without (n=23) preoperative intravitreal bevacizumab injection (IVB). *, $P<0.05$. (b) Levels of HEL in the vitreous samples obtained from PDR patients with (n=14) or without (n=23) IVB. **, $P<0.01$. (c) Correlation between sVAP-1/SSAO and HEL in the vitreous fluid samples obtained from PDR patients without IVB. The equation of the regression line is $y=0.34x+5.3$. $P<0.05$, $r=0.45$, $n=23$. (d) Correlation between sVAP-1/SSAO and HEL in the vitreous fluid samples obtained from PDR patients without IVB. $P=0.46$, $n=14$.

Fig. 2

Impact of VEGF Stimulation on Vap-1 Expression and sVAP-1/SSAO Production in Retinal Capillary Endothelial Cells (a) Real-time PCR analysis of Vap-1 mRNA in retinal capillary endothelial cells at 3, 6 and 17 hours after VEGF stimulation (n=4 in each group). N.S., not significant. (b) Representative image of western blotting of sVAP-1 in the supernatant from TR-iBRB2 cells at 17 hs after VEGF stimulation. (c) Densitometric analysis (n=3 in each group). **, $P<0.01$. (d) SSAO activity, enzymatic activity of VAP-1, in the supernatant collected from TR-iBRB2 cells at 17 h after VEGF stimulation (n=6 in each group). *, $P<0.05$, **, $P<0.01$.

Fig. 3

Impact of VEGF Stimulation in Production and Activation of MMPs Proteins in the

Supernatants of Retinal Capillary Endothelial Cells (a) Representative image of gelatin zymography. The supernatants from cultured retinal capillary endothelial cells stimulated with VEGF (100ng/ml) or PBS for 17 h were loaded. Lane 1, Marker; Lane 2 to 4, PBS; Lane 5 to 7, VEGF (100ng/ml). (b) Densitometric analysis of band intensity corresponding to proMMP-2 and proMMP-9 (n=3 in each group). *, $P<0.05$. (c) Activation ratios of MMP-2 and MMP-9 (n=3 in each group). *, $P<0.05$, **, $P<0.01$. (d) SSAO activity, enzymatic activity of VAP-1, in the supernatant of cultured retinal capillary endothelial cells treated with VEGF (100ng/ml) and broad MMP inhibitor Batimastat for 17 h (n=12 in each group). *, $P<0.05$, **, $P<0.01$.

Fig. 4

Impact of VEGF Stimulation on Oxidative Stress Increase in the Supernatants of Retinal Capillary Endothelial Cells (a) Hydrogen peroxide production via spermine oxidation in the supernatant collected from cultured retinal capillary endothelial cells stimulated with VEGF (100ng/ml) for 17 hours (n=6 in each group). **, $P<0.01$. (b) Level of reactive oxygen species in the cultured retinal capillary endothelial cells stimulated with the mixture of spermine and conditioned medium harvested from endothelial cells treated with VEGF (100ng/ml) and VAP-1 inhibitor semicarbazide (100μM) for 17 hours (n=5 in each group). **, $P<0.01$.

Figure 1

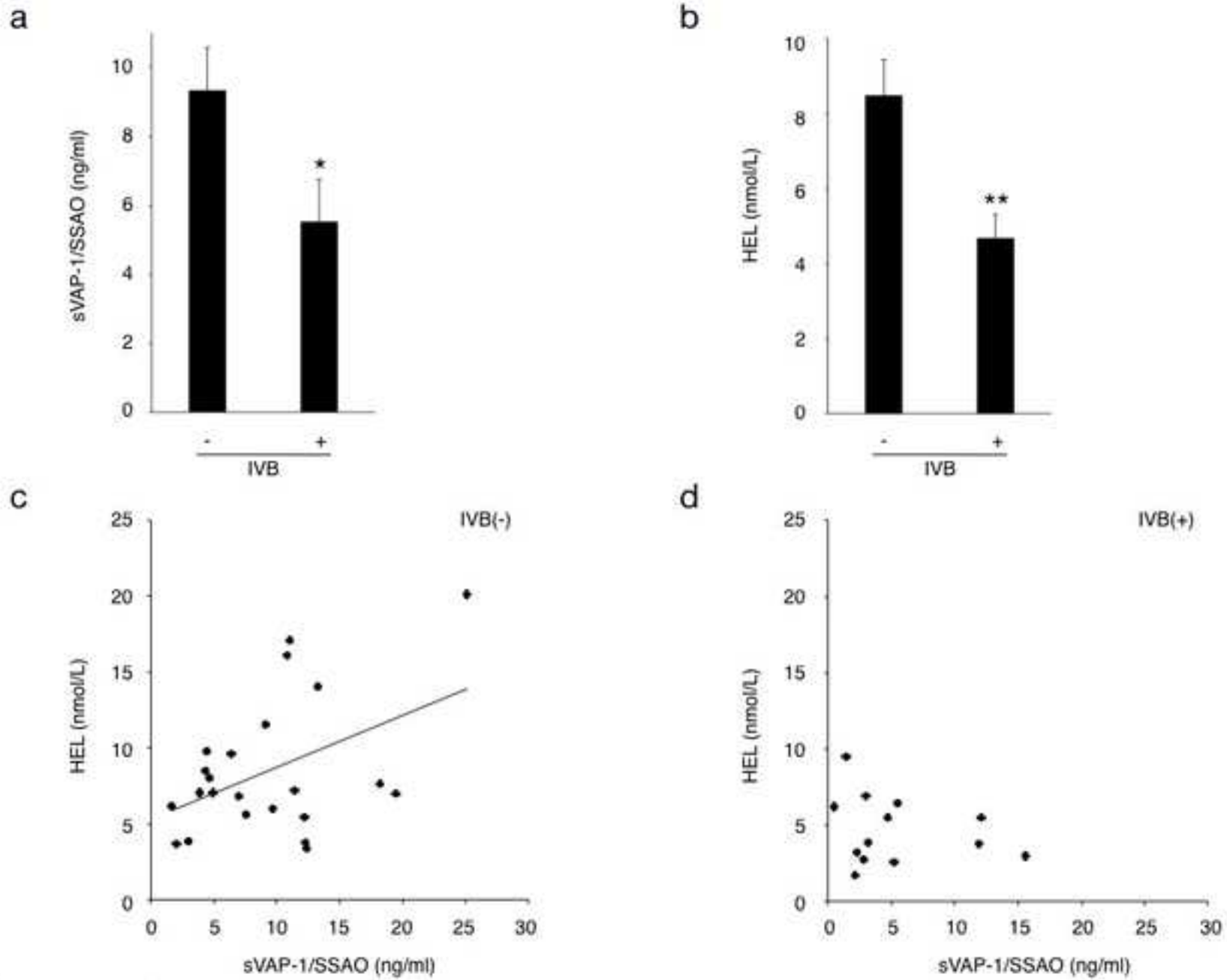

Figure 1

Figure 2

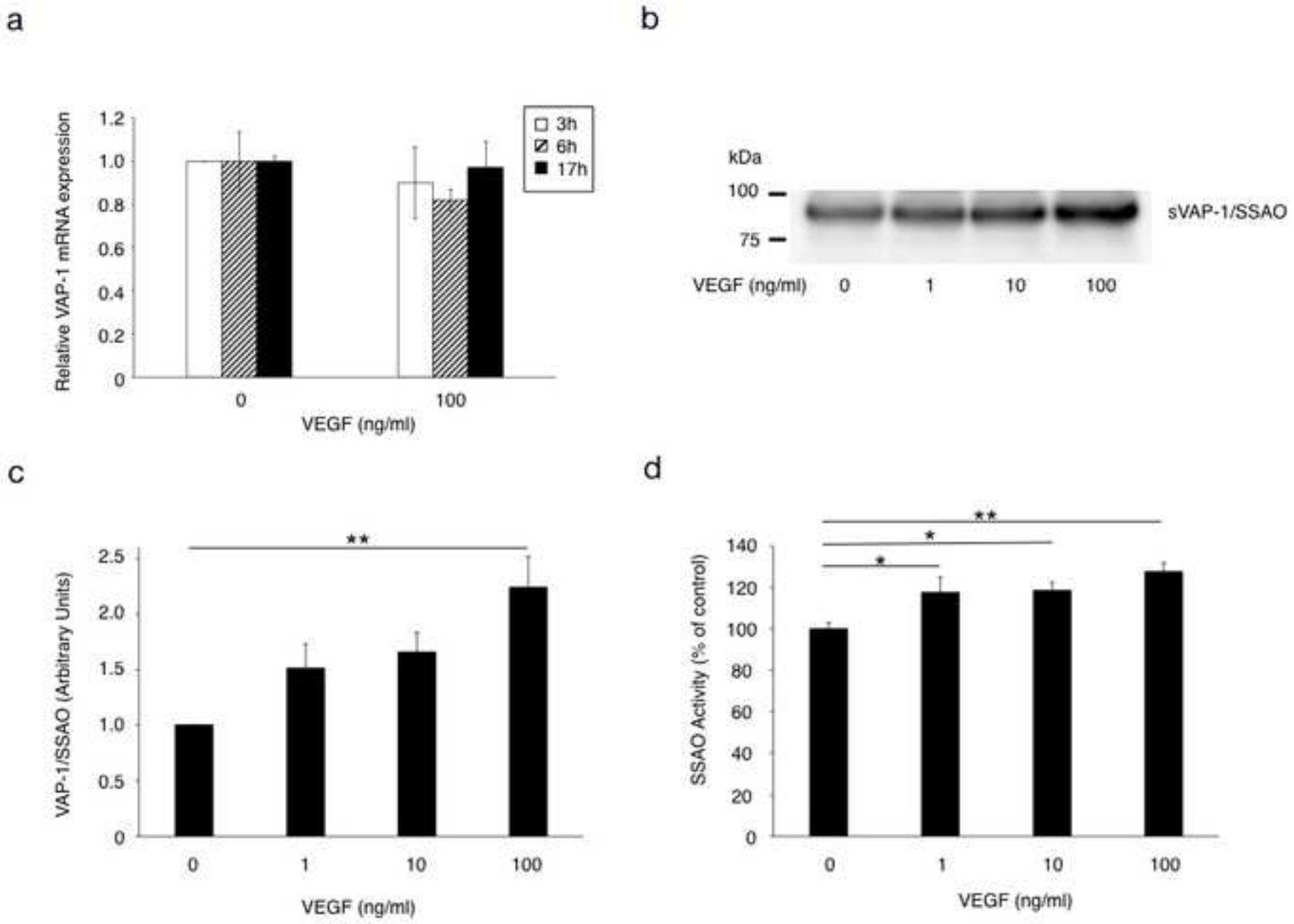

Figure 2

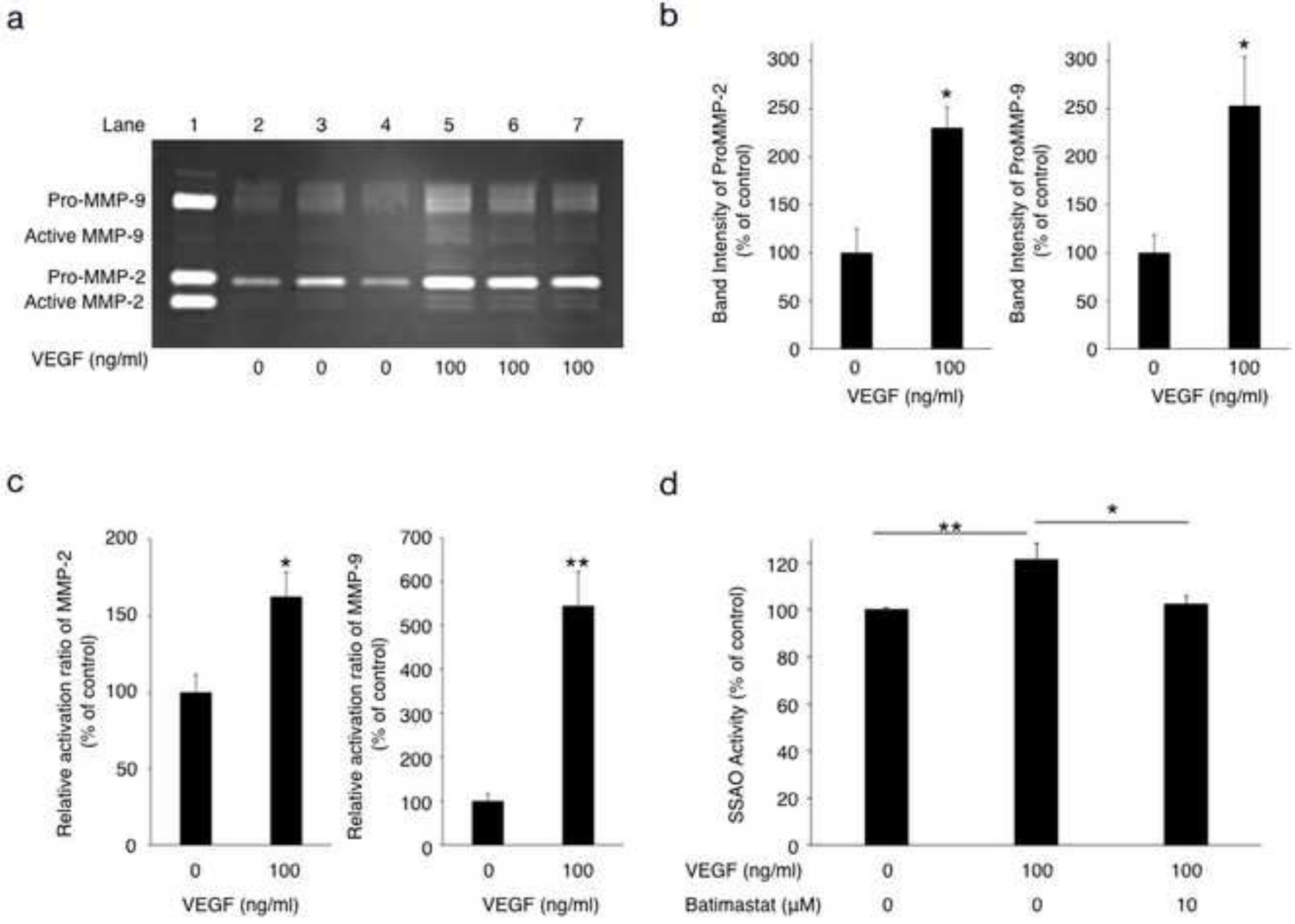
a
Lane 1 2 3 4 5 6 7
Pro-MMP-9
Active MMP-9
Pro-MMP-2
Active MMP-2
VEGF (ng/ml) 0 0 0 100 100 100
b
Band Intensity of ProMMP-2 (% of control)
300
250
200
150
100
50
0
0 100
VEGF (ng/ml)
Band Intensity of ProMMP-9 (% of control)
300
250
200
150
100
50
0
0 100
VEGF (ng/ml)
c
Relative activation ratio of MMP-2 (% of control)
200
150
100
50
0
0 100
VEGF (ng/ml)
Relative activation ratio of MMP-9 (% of control)
700
600
500
400
300
200
100
0
0 100
VEGF (ng/ml)
d
SSAO Activity (% of control)
120
100
80
60
40
20
0
VEGF (ng/ml) 0 100 100
Batimastat (μM) 0 0 10

Figure 3

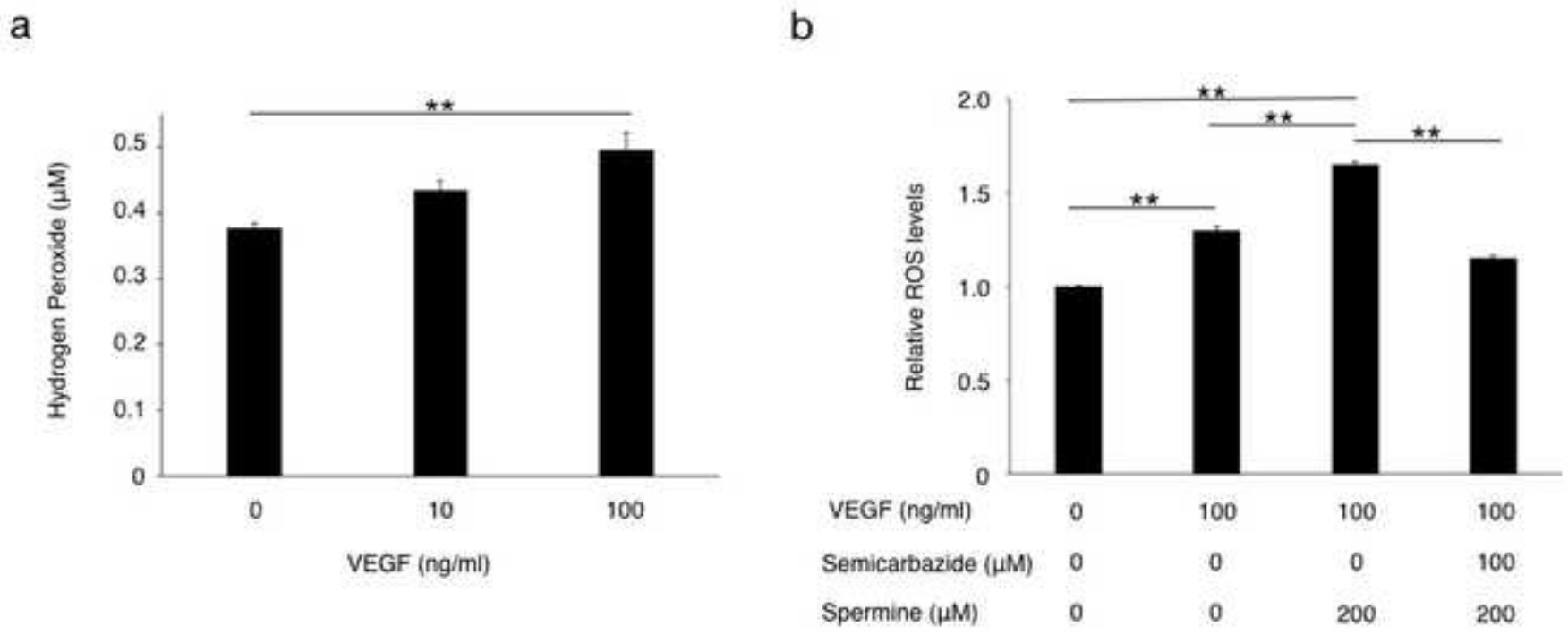
a
Hydrogen Peroxide (μM)
0
0.1
0.2
0.3
0.4
0.5
**
0
10
100
VEGF (ng/ml)
b
Relative ROS levels
0
0.5
1.0
1.5
2.0
**
**
**
**
VEGF (ng/ml) 0 100 100 100
Semicarbazide (μM) 0 0 0 100
Spermine (μM) 0 0 200 200

Figure 4