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Induction of vascular endothelial growth factor by 4-hydroxynonenal and its prevention by glutathione precursors in retinal pigment epithelial cells

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Abstract

Although 4-hydroxynonenal, a highly reactive lipid peroxidation product, is implicated in several age-related disorders such as Alzheimer's and Parkinson's diseases, its role in age-related macular degeneration is not known. The purpose of this study was to determine whether 4-hydroxynonenal increases vascular endothelial growth factor (VEGF) expression in human retinal pigment epithelial cells (ARPE-19), a source of VEGF in choroidal neovascularization observed in age-related macular degeneration. In addition, it was the purpose of this study to assess whether glutathione (GSH) and GSH precursors can inhibit the effects of 4-hydroxynonenal. At 1 μM, 4-hydroxynonenal did not alter cell viability, but elevated VEGF secretion and mRNA expression by 35% ($p < 0.05$) and 1.9-fold ($p < 0.05$), respectively. However, at concentrations 5 μM and above, 4-hydroxynonenal reduced VEGF secretion as well as cell viability. At 1 and 10 μM, 4-hydroxynonenal did not induce apoptosis in ARPE-19 cells. 4-Hydroxynonenal (1 μM) reduced intracellular GSH by 25% ($p < 0.05$) and increased oxidative stress by 50% ($p < 0.05$). GSH precursor pretreatment for 1 h, which increased intracellular GSH levels by 50% ($p < 0.05$), as well as GSH co-treatment, inhibited the VEGF-inductive and cytotoxic effects of 4-hydroxynonenal. Thus, 4-hydroxynonenal (1 μM) induces VEGF expression and secretion in ARPE-19 cells. This effect is likely due to GSH depletion and an associated increase in intracellular oxidative stress, resulting in increased VEGF mRNA levels. 4-Hydroxynonenal-mediated VEGF secretion as well as cytotoxicity can be reversed with GSH precursor pretreatment or GSH co-treatment.

Keywords: ARPE-19 cell; GSH precursor; 4-Hydroxynonenal; Oxidative stress; VEGF (Vascular endothelial growth factor)

1. Introduction

Vascular endothelial growth factor (VEGF), a homodimeric 34–45-kDa protein, is now emerging as a risk factor for age-related macular degeneration, wherein vascular hyperpermeability and neovascularization are observed (Macular Photocoagulation Study Group, 1991). The expression of VEGF is significantly increased in the retinas (Frank, 1997; Kliffen et al., 1997) and plasma (Lip et al., 2001) of age-related macular degeneration subjects. VEGF is capable of inducing vascular permeability by binding to Flt-1 receptor (K_d: 10–20 pM) (Park et al., 1994) and angiogenic effect by binding to Flk-1 receptor (K_d: 75–125 pM) (Shalaby et al., 1995). Of the known five isoforms of VEGF (with 206, 189, 165, 145, and 121 amino acids) (Park et al., 1993; Whittle et al., 1999), human retinas predominantly express $VEGF_{165}$ and $VEGF_{121}$, the secretory forms of VEGF, and relatively low levels of $VEGF_{189}$, the non-secretory isoform (Gerhardinger et al., 1998). Interestingly, retinal pigment epithelial cells (RPE) contribute significantly to the constitutive retinal VEGF expression (Adamis et al., 1993). Furthermore, RPE cells secrete more VEGF towards basolateral or choroid side (Blaauwgeers et al., 1999), possibly facilitating its action on choriocapillaris. In addition, overexpression of VEGF in RPE cells of the retina might be a responsible factor in the development of choroidal neovascularization in vivo (Yi et al., 1997; Husain et al., 1997). In age-related macular degeneration subjects, VEGF expression is induced in

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RPE cells (Kliffen et al., 1997) and elevated levels of lipid peroxidation products are observed adjacent to RPE cells before the development of choroidal neovascularization (Ishibashi et al., 1998).

In the plasma of age-related macular degeneration patients, reactive oxygen species are elevated (Gottsch et al., 1990) and glutathione (GSH), an endogenous antioxidant, is depleted (Samiec et al., 1998), possibly leading to elevated oxidative stress in age-related macular degeneration (Beatty et al., 2000). Interestingly, oxidative stress induces VEGF expression (Kuroki et al., 1996), suggesting a role for oxidative stress in neovascular age-related macular degeneration (Hammes et al., 1999). The plasma levels of oxidative stress marker, 4-hydroxynonenal, a highly reactive lipid peroxidation product, are elevated progressively with aging (Chiarpotto et al., 1995). Such an elevation in 4-hydroxynonenal levels is now implicated in age-related pathologies such as Parkinson's disease (Selley, 1998) and Alzheimer's disease (Lovell et al., 1997). However, the role of 4-hydroxynonenal in age-related macular degeneration is not known.

The retinal 4-hydroxynonenal levels are elevated from 0.64 to 7.7 nmol/g under oxidative stress (Van Kuijk, 1988). 4-Hydroxynonenal can penetrate cells (Parola et al., 1998) and stimulates several cellular functions such as adenyl cyclase activity, phospholipase C activity, activator protein (AP)-1 activity, heat-shock protein transcription factor activity and protein synthesis at 1 μM, and at higher concentrations (>10 μM), it inhibits these functions (Dianzani et al., 1999). Also, 4-hydroxynonenal depletes cellular GSH, thereby elevating oxidative stress (Uchida et al., 1999). 4-Hydroxynonenal reduces cellular GSH levels either by inhibiting glutathione reductase (Vander Jagt et al., 1997) and the associated conversion of oxidized glutathione (GSSG) to GSH or by spontaneously forming 4-hydroxynonenal–GSH conjugates (Srivastava et al., 1994). Interestingly, reduced plasma GSH (Gottsch et al., 1990) and glutathione reductase activity (Cohen et al., 1994) and elevated retinal oxidative stress (Ohia et al., 1994) were observed with aging. Thus, there is a possibility that 4-hydroxynonenal may deplete GSH in the aging retinas, thereby increasing the oxidative stress and the expression of VEGF.

Since GSH levels are depleted and oxidative stress, lipid peroxidation products, and VEGF levels are elevated in age-related macular degeneration, one objective of this study was to determine whether 4-hydroxynonenal depletes GSH and induces oxidative stress and VEGF expression in ARPE-19 cells, a spontaneously formed cell line from human RPE cells (Dunn et al., 1996). To this end, we determined the effect of 4-hydroxynonenal on VEGF mRNA expression and protein secretion in ARPE-19 cell line. Also, the effect of 4-hydroxynonenal on intracellular GSH levels, oxidative stress, and cell viability was determined. The second objective of this study was to determine whether the effects of 4-hydroxynonenal could be reversed with GSH supplements. To this end, the ability of GSH precursor pretreatment and GSH co-treatment in inhibiting the effect of 4-hydroxynonenal on VEGF secretion and cell viability was assessed.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM-F/12), fetal bovine serum, penicillin–streptomycin and L-glutamine were obtained from Gibco-BRL (Gibco, Grand Island, NY). Cell culture flasks (T-75 cm^2) and 96-well plates were purchased from Becton Dickinson Labware (Franklin Lakes, NJ). 4-Hydroxynonenal was purchased from Cayman Chemicals (Ann Arbor, MI). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), GSH, GSSG, *O*-phthalaldehyde, and *N*-ethylmaleimide were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

ARPE-19 cells, obtained from American Type Culture Collection (ATCC, Rockville, MD), were cultured in DMEM/F-12 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. The medium was changed every 2 days and all studies were conducted by using confluent cells of passages 20–40 following a 12-h quiescence in serum-free medium. All the experiments were repeated two to three times with $n=8$ in each experiment for VEGF secretion and cell viability studies, and $n=4$ for intracellular oxidative stress, GSH, and GSSG estimations.

2.3. Estimation of VEGF

ARPE-19 cells grown in 96-well plates were incubated with 4-hydroxynonenal (0–25 μM) dissolved in serum-free medium. At the end of 12 h, supernatants were collected to estimate secreted VEGF using an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's recommendations (Research Diagnostics, Flanders, NJ). Briefly, supernatant samples of both cell types, standards, and blank samples were added to an ELISA plate, which was pre-coated overnight with the coating antibody (goat anti-human VEGF antibody), which recognizes VEGF121 and VEGF165. All the samples were allowed to incubate for 2 h with the coating antibody. Subsequently, the plate was washed three times with wash buffer and the wells were incubated with the detection antibody (biotin-conjugated goat anti-human VEGF) for 2 h. Following secondary antibody incubation, the plate was incubated with poly-HRP80–streptavidin for 20 min. Subsequently, the plate was washed three times and allowed to incubate in the presence of substrate solution 3,3′,5,5′-tetramethylbenzi-

dine (TMB-S) for 20 min until the reaction was stopped with sulfuric acid (0.5 M). All the absorbances were measured using a microtiter plate reader (Fischer Scientific, PA) with a test wavelength of 450 nm and a reference wavelength set at 550 nm.

2.4. Semi-quantitative analysis of VEGF mRNA

VEGF mRNA expression was determined using the access reverse transcriptase-polymerase chain reaction (RT-PCR) system (Promega, Madison, WI). Briefly, confluent ARPE-19 cells grown in T-75 flasks were treated with 1 μM 4-hydroxynonenal. At the end of 0, 1, 2, 4, and 6 h, 4-hydroxynonenal exposure was discontinued and the total RNA was extracted using RNA STAT-60™ RNA isolation kit (TEL-TEST, Friendswood, TX). The RNA pellet was dissolved in deionized autoclaved water, quantified for RNA, and a volume equivalent to 5 μg was taken to amplify VEGF mRNA as described earlier (Bandi and Kompella, 2001). The primers used can detect all the splice variants of VEGF. However, only $VEGF_{121}$ and $VEGF_{165}$ are seen amplified in ARPE-19 cells. The VEGF mRNA expression was normalized with GAPDH expression, a housekeeping gene whose levels are not altered under hypoxic stress (Grimshaw and Mason, 2001). The products were separated by electrophoresis on a 2% agarose gel, and $VEGF_{165}$ mRNA (584 bp) and $VEGF_{121}$ mRNA (452 bp) were identified and quantified using densitometric analysis (Nucleovision™ Imaging System, Nucleotech, San Mateo, CA).

2.5. Intracellular GSH and GSSG estimation

ARPE-19 cells grown in 24-well plates were treated with 1 μM 4-hydroxynonenal for 0.1, 0.25, 0.5, 1, 2, 4, 6, 8, and 12 h. The cells were washed with ice-cold phosphate buffered saline (PBS, pH: 7.4) and lysed with 500 μl of lysis buffer containing 3.33% trichloroacetic acid. The samples were centrifuged at $12{,}500 \times g$ for 10 min at 4 °C and 50 μl of the supernatant was taken to estimate GSH and GSSG using a spectrofluoremetric method described earlier (Hissin and Hilf, 1976).

2.6. Measurement of oxidative stress

The oxidative stress within the cells was determined using 2′,7′-dichlorofluorescein diacetate (DCF-DA) as described earlier (Carter et al., 1994). Briefly, confluent ARPE-19 cells grown in T-75 flasks were incubated with 20 μM DCF-DA for 1 h at 37 °C. These cells were trypsinized and suspended in PBS to obtain a final concentration of 1×10^6 cells/ml. To 0.5 ml of this cell suspension, 4-hydroxynonenal solution with or without GSH was added and incubated for 30 min at 37 °C and the fluorescence was measured using flow cytometry (FACS Calibur, Becton Dickinson).

2.7. Cell viability assay

ARPE-19 cells grown to confluency in 96-well plates were exposed to 4-hydroxynonenal (0–25 μM) with or without GSH co-treatment or GSH precursor pretreatment. At the end of 12 h incubation, the supernatants were removed and the cells were washed with ice-cold PBS three times and incubated with 30 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) for 1 h at 37 °C. Viable cells reduce MTT to formazan, which was dissolved in 100 μl of dimethylsulfoxide. The absorbance of this solution was measured at a dual wavelength of 550/650 nm using a micro-plate reader (Fisher Scientific).

2.8. Cell apoptosis assay

To determine the effect of 4-hydroxynonenal on induction of apoptosis, flow cytometric analysis was performed by staining the cells with propidium iodide (Mangipudy and Vishwanatha, 1999). Briefly, ARPE-19 cells were treated for 12 h with 4-hydroxynonenal (1 and 10 μM) and 1×10^6 cells/ml were aliquoted following culture. The aliquoted cells were pelleted by centrifugation at 2000 rpm for 5 min. The pellet was washed twice with PBS and the cells were

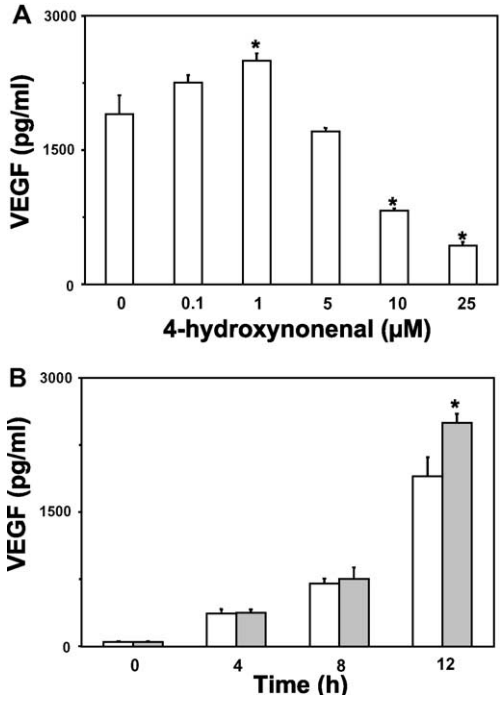

Fig. 1. 4-Hydroxynonenal induces VEGF secretion and VEGF mRNA expression in ARPE-19 cells in a dose-and time-dependent manner. (A) Dose-dependent effect of 4-hydroxynonenal on VEGF secretion at the end of 12 h. Data are expressed as mean ± S.D. for $n = 8$, $^*P < 0.05$. (B) Time-course of VEGF secretion in controls (□) and 1 μM 4-hydroxynonenal treated (▨) cells at the end of 0, 4, 8, and 12 h. Cumulative VEGF secretion is expressed as mean ± S.D. for $n = 8$, $^*P < 0.05$.

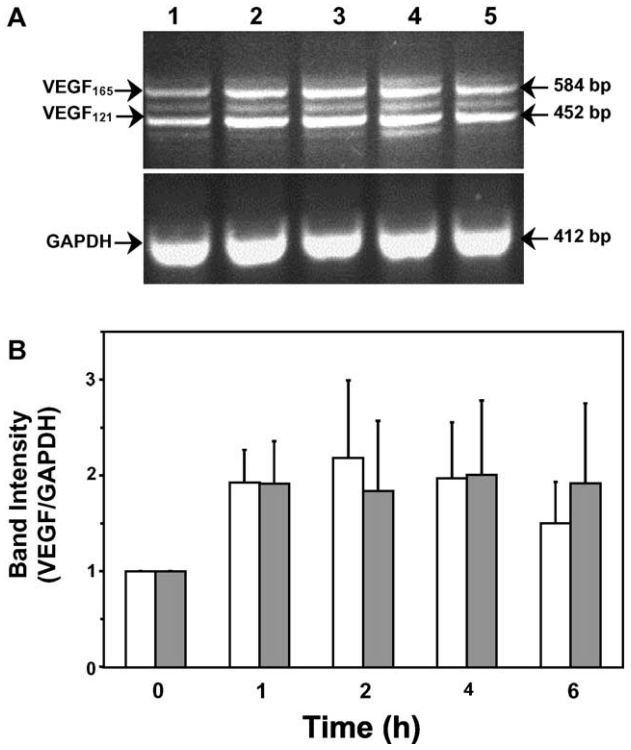

Fig. 2. (A) Time-dependent effect of 1 μM 4-hydroxynonenal on VEGF mRNA expression. At each time-point, 5 μg RNA was taken for RT-PCR analysis to amplify VEGF and GAPDH mRNAs. Key—Lane 1: untreated ARPE-19 cells; Lanes 2, 3, 4, and 5: ARPE-19 cells treated with 4-hydroxynonenal for 1, 2, 4, and 6 h, respectively. (B) Time dependency of the ratios of $VEGF_{165}$ (□) and $VEGF_{121}$ (▩) mRNAs to GAPDH band intensities. Data are expressed as mean ± S.D. for $n=3$.

fixed with 1 ml of 70% ethanol, and the cells were dispersed and incubated for 2 h at 4 °C. The cells were then spun down at 2000 rpm for 5 min and ethanol was decanted. After this, the cells were again washed twice with PBS and spun down. Finally, 1 ml of Telford reagent (DNA stain for ethanol fixed cells; containing RNAase A and propidium iodide) was added and incubated overnight. The fluorescence in cells was analyzed for apoptosis using a flow cytometer (Becton Dickinson).

2.9. Statistical analysis

Data are expressed as mean ± S.D. Comparison of mean values was done using a paired Student's *t*-test. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. 4-Hydroxynonenal induces VEGF secretion and VEGF mRNA expression

The effect of 4-hydroxynonenal on VEGF secretion from ARPE-19 cells was determined by estimating VEGF levels in the supernatants of the cells treated with or without 4-hydroxynonenal. Secreted VEGF levels at the end of 12 h in response 4-hydroxynonenal treatments (0, 0.1, 1, 5, 10, and 25 μM) are shown in Fig. 1A. The observed VEGF levels in controls are similar to those reported for primary human RPE cells (Mousa et al., 1999). The maximum induction was observed with 1 μM 4-hydroxynonenal with an increase in VEGF secretion from 1899 ± 199 pg/ml (50 ± 5.6 pM) to 2498 ± 99 pg/ml (90 ± 2.8 pM). However, higher concentrations of 4-hydroxynonenal reduced VEGF secretion. A time-course indicated that ARPE-19 cells continuously secrete VEGF, suggesting that VEGF secretion is constitutive in ARPE-19 cells. The cumulative VEGF secretion is significantly higher at 12 h after 4-hydroxynonenal treatment (Fig. 1B). Also, 1 μM 4-hydroxynonenal significantly increased the expression of $VEGF_{165}$ and $VEGF_{121}$ mRNAs (Fig. 2A) by 1.9-fold as early as 1 h post-exposure in ARPE-19 cells (Fig. 2B).

3.2. 4-Hydroxynonenal depletes intracellular GSH and induces oxidative stress

Since 4-hydroxynonenal-induced biological effects are linked to depletion of intracellular GSH and oxidative stress

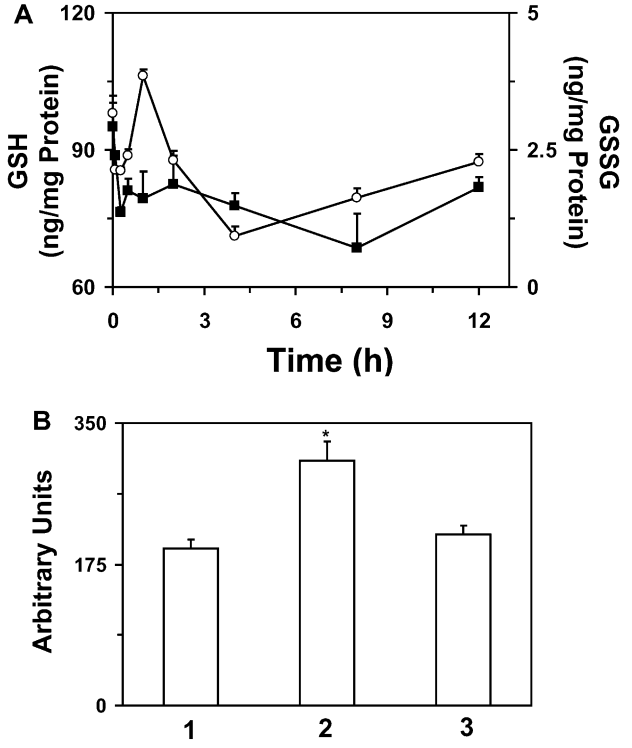

Fig. 3. 4-Hydroxynonenal depletes intracellular GSH and induces oxidative stress in ARPE-19 cells. (A) Effect of 1 μM 4-hydroxynonenal on intracellular GSH (■) and GSSG (○) at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, and 12 h post-exposure. Data are expressed as mean ± S.D. for $n=4$. (B) Effect of 1 μM 4-hydroxynonenal on oxidative stress measured using flowcytometer. Key—1: Control ARPE-19 cells; 2: ARPE-19 cells treated with 1 μM 4-hydroxynonenal; and 3: ARPE-19 cells treated with 1 μM 4-hydroxynonenal and 3 mM GSH. The fluorescence intensity is expressed as mean ± S.D. for $n=4$, * $P<0.05$.

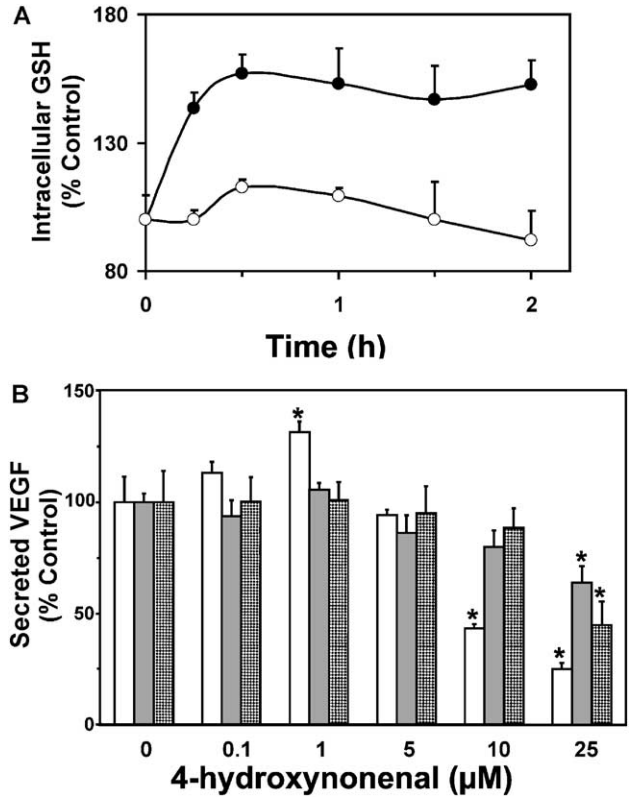

Fig. 4. Effect of GSH treatments on intracellular GSH levels and 4-hydroxynonenal-induced VEGF secretion in ARPE-19 cells. (A) Effect of 1 mM each of GSH precursors (glycine, glutamine, and cystein) (●) and 3 mM GSH (○) on intracellular GSH. Data are expressed as mean ± S.D. for $n=4$. (B) Effect of no treatment (□), GSH co-treatment (▩), and pre-treatment with GSH precursors for 1 h (◈) on 4-hydroxynonenal-induced VEGF secretion at the end of 12 h. Data are expressed as mean ± S.D. for $n=8$, $^{*}P<0.05$.

(Dianzani, 1999), intracellular GSH levels and oxidative stress were estimated after treating ARPE-19 cells with 1 μM 4-hydroxynonenal. We observed that 1 μM 4-hydroxynonenal treatment significantly decreased intracellular levels of GSH from 95 ± 5 to 76 ± 1 nmol/mg protein within 15 min of incubation and sustained these levels up to 12 h (Fig. 3A).

Table 1
Effects of GSH treatments on 4-hydroxynonenal-induced cytotoxicity in ARPE-19 cells

4-Hydroxynonenal (μM)	Control	GSH co-treatment	Precursors pre-treatment
0	100 ± 3.14	100 ± 3.8	100 ± 12.7
0.1	95 ± 7.5	97.6 ± 1.5	90.7 ± 10.8
1	95.5 ± 3.5	91.2 ± 2.7	81.2 ± 6.8
5	49.5 ± 2.8[a]	94.4 ± 16.6	80.8 ± 1.4[a]
10	39.3 ± 8.4[a]	91.7 ± 11.9	73.5 ± 2.1[a]
25	23.1 ± 5.9[a]	102 ± 9.3	77.2 ± 16[a]

The % absorbance of formazan from MTT assay is expressed as mean ± S.D. for $n=8$.

[a] Indicates statistically significant difference from 0 μM 4-hydroxynonenal-treated control.

Furthermore, GSSG levels were decreased from 3.2 ± 0.3 to 2.1 ± 0.06 nmol/mg protein within 15 min. Interestingly, GSSG levels increased sharply to 3.8 nmol/mg protein at the end of 1 h, beyond which they remained below baseline up to 8 h. By the end of 12 h, GSSG levels returned to control levels. Furthermore, 4-hydroxynonenal (1 μM) treatment of DCF-DA-loaded ARPE-19 cells significantly increased the intracellular mean fluorescence intensity from 210 ± 12 to 316 ± 23 (Fig. 3B), suggesting the induction of oxidative stress. Co-treatment of ARPE-19 cells with 3 mM GSH inhibited 4-hydroxynonenal-induced oxidative stress.

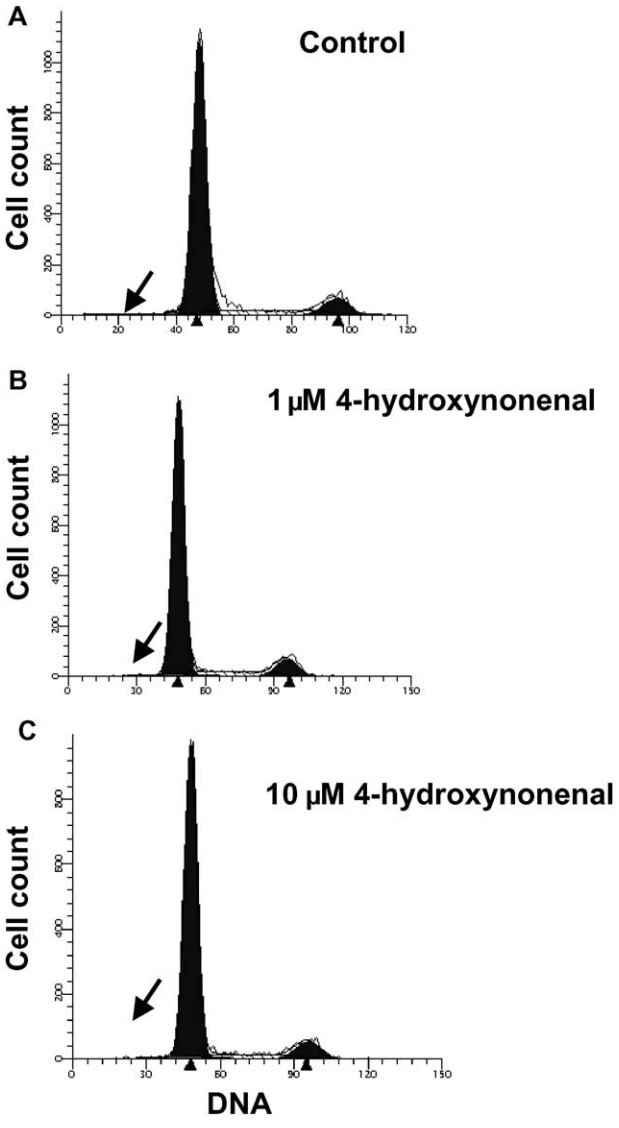

Fig. 5. Effect of 4-hydroxynonenal on apoptosis in ARPE-19 cells. Representative DNA histograms of ARPE-19 cells treated for 12 h with A: Control, B: 1 μM 4-hydroxynonenal, and C: 10 μM 4-hydroxynonenal. After 12 h treatment with/without 4-hydroxynonenal, the cells were subjected to FACS (fluorescence activated cell sorter) analysis after staining with propidium iodide. The arrows point to the apoptotic peak (A_0) and values on the x-axis denote DNA content and the values on y-axis denote cell counts.

3.3. GSH co-treatment and GSH precursor pretreatment inhibit 4-hydroxynonenal-induced VEGF secretion and cytotoxicity

4-Hydroxynonenal-mediated oxidative stress and therefore VEGF secretion can potentially be countered by either blocking 4-hydroxynonenal entry into the cells or by increasing intracellular antioxidant capacity. One way to minimize the entry of 4-hydroxynonenal into cells is to co-treat with GSH, as GSH readily forms conjugates with 4-hydroxynonenal (Esterbauer et al., 1975). An approach to elevate cellular antioxidant capacity is to elevate intracellular GSH levels by pre-incubating cells with GSH precursors (1 mM each of glycine, glutamine, and cysteine). Indeed, we observed that pretreatment with GSH precursors elevated intracellular GSH levels by 50% within 15 min (Fig. 4A). Consistent with a previous report (Davidson et al., 1994), exogenous GSH (3 mM) failed to elevate intracellular GSH significantly. GSH (3 mM) co-treatment as well as GSH precursor-treatment completely inhibited VEGF secretion induced by 1 μM 4-hydroxynonenal (Fig. 4B). Also, these treatments inhibited the reduction in VEGF secretion observed at ≥ 5 μM 4-hydroxynonenal. However, these treatments were not effective in countering the effect of 25 μM 4-hydroxynonenal.

The protective effect of glutathione against 4-hydroxynonenal-induced cytotoxicity was assessed using MTT assay. At the end of 12 h exposure, 1 μM 4-hydroxynonenal did not significantly inhibit ARPE-19 cell viability. On the other hand, 4-hydroxynonenal at 5 μM and above significantly reduced cell viability (Table 1). GSH co-treatment as well as GSH precursor pretreatment inhibited this 4-hydroxynonenal-induced cytotoxicity. From propidium staining, it is evident that 4-hydroxynonenal at tested concentrations (1 and 10 μM) did not change the A_0 peak, suggesting no induction of apoptosis by 4-hydroxynonenal at the end of 12 h (Fig. 5).

4. Discussion

As the retinal lipid peroxidation is elevated with aging (Zhao et al., 1996) and RPE cell VEGF expression is increased in age-related macular degeneration (Lopez et al., 1996), we tested the hypothesis that 4-hydroxynonenal, a lipid peroxidation product, induces VEGF expression and secretion in a human retinal pigmented epithelial cell line (ARPE-19). The results of this study indicate that 4-hydroxynonenal elevates VEGF secretion from ARPE-19 cells at 1 μM and that it inhibits VEGF secretion at >5 μM. These effects of 4-hydroxynonenal could be inhibited by increasing intracellular GSH or by co-incubating cells with GSH. The secretion of VEGF might be associated with VEGF induction at the molecular level rather than burst discharge from potentially pre-existing pools, as indicated by the delayed increase in VEGF secretion (Fig. 1B). This is supported by the induction of VEGF mRNA by 1 μM 4-hydroxynonenal (Fig. 2A). Such induction may be associated with 4-hydroxynonenal-induced increase in the activator protein (AP-1) binding activity (Camandola et al., 2000), which is known to induce the expression of VEGF (Damert et al., 1997) in ocular tissues.

Oxidative stress is a potent stimulus for VEGF expression (Brown et al., 2000). An increase in oxidative insults or a decrease in cellular antioxidant capacity can induce oxidative stress. Treatment of ARPE-19 cells with 1 μM 4-hydroxynonenal depleted both oxidative (GSSG) and reduced forms of intracellular GSH (Fig. 3A). Since the GSSG is the cellular source of GSH, compensation of GSSG for the cellular GSH might be responsible for its depletion. A sharp rise in GSSG levels between 30 min and 1 h following 4-hydroxynonenal exposure is consistent with an increase in oxidative insult levels and the associated oxidation of GSH to GSSG. In addition, we observed that 1 μM 4-hydroxynonenal increased fluorescence intensity from DCF-DA, an oxidative stress probe, by 55%, suggesting that 4-hydroxynonenal induces oxidative stress in ARPE-19 cells (Fig. 3B). Another possible mechanism for decreased intracellular GSH is formation of GSH–4-hydroxynonenal conjugates within the cell (Boon et al., 1999). Active transport of these conjugates by multidrug resistance-associated protein (MRP1) (Renes et al., 2000) and multispecific organic anion transporter (Dygas et al., 1998) may also result in depleted cellular GSH. Indeed, evidence exists for the presence of MRP1 in ARPE-19 cells (Aukunuru et al., 2001).

4-Hydroxynonenal-induced oxidative stress was inhibited by 3 mM GSH co-treatment, possibly due to the formation of 4-hydroxynonenal-GSH adducts in the medium. Interestingly, anti-oxidant supplement therapy reduced the risk of vision loss in age-related macular degeneration subjects (Richer, 1996), supporting a potential involvement of oxidative stress in the development of age-related macular degeneration. Hence, we tested whether GSH treatments inhibit 4-hydroxynonenal-induced VEGF expression in ARPE-19 cells. At first, we examined intracellular GSH levels after incubating ARPE-19 cells with GSH and precursors of GSH (glycine, glutamine, and cysteine). In agreement with a previous report (Davidson et al., 1994), we observed an increase in intracellular GSH only when cells were incubated with GSH precursors. However, both co-treatment with GSH and 1 h pretreatment with GSH precursors inhibited 4-hydroxynonenal-induced VEGF secretion from ARPE-19 cells (Fig. 4B). While elevation of intracellular GSH might be responsible for the effect of GSH precursor pretreatment, formation of impermeable 4-hydroxynonenal–GSH conjugates outside the cell might be responsible for the effect of GSH co-treatment. The cell viability assay indicated that 4-hydroxynonenal induces VEGF secretion from ARPE-19 cells at 1 μM without altering cell viability (Table 1). However, consistent with the previous reports (Ruef et al., 1998),

higher concentrations (≥ 5 μM) reduced VEGF secretion as well as cell viability, suggesting that 4-hydroxynonenal is cytotoxic to ARPE-19 cells at these concentrations. The cytotoxic effect might be either due to necrosis or apoptosis. The cytotoxic effect of 4-hydroxynonenal in ARPE-19 cells might be associated with induction of necrosis rather than apoptosis as evidenced from propidium iodide staining study (Fig. 5), which indicated the absence of apoptosis. Indeed, 4-hydroxynonenal has been shown to induce necrosis in a dose-dependent manner in rat liver cells following oral administration (Nishikawa et al., 1992). Interestingly, degeneration and necrosis of RPE cells has been associated with the development of age related macular degeneration (Sarks, 1976). The findings of this study may be of relevance to age-related macular degeneration (Lopez et al., 1996; Zhao et al., 1996) and diabetic retinopathy (Verdejo et al., 1999; Obrosova et al., 2000; Armstrong et al., 1998; Toti et al., 1999), wherein retinal or vitreal levels of VEGF and lipid peroxidation products are elevated.

In conclusion, we demonstrated for the first time that 4-hydroxynonenal induces VEGF expression in RPE cells. The possible mechanism involves depletion of intracellular GSH and resulting increase in oxidative stress, leading to increased VEGF mRNA levels. 4-Hydroxynonenal-induced VEGF expression and secretion can be inhibited by co-incubating cells with GSH or by increasing intracellular GSH levels using GSH precursors.

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References

Adamis, A.P., Shima, D.T., Yeo, K.T., Yeo, T.K., Brown, L.F., Berse, B., D'Amore, P.A., Folkman, J., 1993. Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. Biochem. Biophys. Res. Commun. 193, 631–638.

Armstrong, D., Augustin, A.J., Spengler, R., Al-Jada, A., Nickola, T., Grus, F., Koch, F., 1998. Detection of vascular endothelial growth factor and tumor necrosis factor alpha in epiretinal membranes of proliferative diabetic retinopathy, proliferative vitreoretinopathy and macular pucker. Ophthalmologica 212, 410–414.

Aukunuru, J.V., Sunkara, G., Bandi, N., Thoreson, W.B., Kompella, U.B., 2001. Expression of multi-drug resistance protein (MRP)-1 in human retinal pigment epithelial cells and its interaction with BAPSG, a novel aldose reductase inhibitor. Pharm. Res. 18, 565–572.

Bandi, N., Kompella, U.B., 2001. Budesonide reduces vascular endothelial growth factor secretion and expression in airway (Calu-1) and alveolar (A549) epithelial cells. Eur. J. Pharmacol. 425, 109–116.

Beatty, S., Koh, H., Phil, M., Henson, D., Boulton, M., 2000. The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv. Ophthalmol. 45, 115–134.

Blaauwgeers, H.G., Holtkamp, G.M., Rutten, H., Witmer, A.N., Koolwijk, P., Partanen, T.A., Alitalo, K., Kroon, M.E., Kijlstra, A., van Hinsbergh, V.W., Schlingemann, R.O., 1999. Polarized vascular endothelial growth factor secretion by human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris. Evidence for a trophic paracrine relation. Am. J. Pathol. 155, 421–428.

Boon, P.J., Marinho, H.S., Oosting, R., Mulder, G.J., 1999. Glutathione conjugation of 4-hydroxy-2,3-nonenal in the rat in vivo, the isolated perfused liver and erythrocytes. Toxicol. Appl. Pharmacol. 159, 214–223.

Brown, N.S., Jones, A., Fujiyama, C., Harris, A.L., Bicknell, R., 2000. Thymidine phosphorylase induces carcinoma cell oxidative stress and promotes secretion of angiogenic factors. Cancer Res. 60, 6298–6302.

Camandola, S., Poli, G., Mattson, M.P., 2000. The lipid peroxidation product 4-hydroxy-2,3-nonenal increases AP-1-binding activity through caspase activation in neurons. J. Neurochem. 74, 159–168.

Carter, W.O., Narayanan, P.K., Robinson, J.P., 1994. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. J. Leukoc. Biol. 55, 253–258.

Chiarpotto, E., Biasi, F., Scavazza, A., Camandola, S., Dianzani, M.U., Poli, G., 1995. Metabolism of 4-hydroxy-2-nonenal and aging. Biochem. Biophys. Res. Commun. 207, 477–484.

Cohen, S.M., Olin, K.L., Feuer, W.J., Hjelmeland, L., Keen, C.L., Morse, L.S., 1994. Low glutathione reductase and peroxidase activity in age-related macular degeneration. Br. J. Ophthalmol. 78, 791–794.

Davidson, P.C., Sternberg, P., Jones, D.P., Reed, R.L., 1994. Synthesis and transport of glutathione by cultured human retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 35, 2843–2849.

Dianzani, M.U., Barrera, G., Parola, M., 1999. 4-Hydroxy-2,3-nonenal as a signal for cell function and differentiation. Acta Biochim. Pol. 46, 61–75.

Damert, A., Ikeda, E., Risau, W., 1997. Activator-protein-1 binding potentiates the hypoxia-induciblefactor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells. Biochem. J. 327, 419–423.

Dunn, K.C., Aotaki-Keen, A.E., Putkey, F.R., Hjelmeland, L.M., 1996. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Exp. Eye Res. 62, 155–169.

Dygas, A., Makowski, P., Pikula, S., 1998. Is the glutathione conjugate of *trans*-4-hydroxy-2-nonenal transported by the multispecific organic anion transporting-ATPase of human erythrocytes? Acta Biochim. Pol. 45, 59–65.

Esterbauer, H., Zollner, H., Scholz, N., 1975. Reaction of glutathione with conjugated carbonyls. Z. Naturforsch. 30, 466–473.

Frank, R.N., 1997. Growth factors in age-related macular degeneration: pathogenic and therapeutic implications. Ophthalmic Res. 29, 341–353.

Gerhardinger, C., Brown, L.F., Roy, S., Mizutani, M., Zucker, C.L., Lorenzi, M., 1998. Expression of vascular endothelial growth factor in the human retina and in nonproliferative diabetic retinopathy. Am. J. Pathol. 152, 1453–1462.

Gottsch, J.D., Pou, S., Bynoe, L.A., Rosen, G.M., 1990. Hematogenous photosensitization. A mechanism for the development of age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 31, 1674–1682.

Grimshaw, M.J., Mason, R.M., 2001. Modulation of bovine articular chondrocyte gene expression in vitro by oxygen tension. Osteoarthr. Cartil. 9, 357–364.

Hammes, H.P., Hoerauf, H., Alt, A., Schleicher, E., Clausen, J.T., Bretzel, R.G., Laqua, H., 1999. *N*(epsilon)(carboxymethyl)lysin and the AGE receptor RAGE colocalize in age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 40, 1855–1859.

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74, 214–216.

Husain, D., Ryan, A.M., Cuthbertson, R.A., Gragoudas, E.S., Adamis, A.P., Michaud, N., Miller, J.W., 1997. Vascular endothelial growth factor (VEGF) expression is correlated with choroidal neovascularization in a monkey model. Invest. Ophthalmol. Vis. Sci. 38, S501.

Ishibashi, T., Murata, T., Hangai, M., Nagai, R., Horiuchi, S., Lopez, P.F., Hinton, D.R., Ryan, S.J., 1998. Advanced glycation end products in age-related macular degeneration. Arch. Ophthalmol. 116, 1629–1632.

Kliffen, M., Sharma, H.S., Mooy, C.M., Kerkvliet, S., de Jong, P.T., 1997. Increased expression of angiogenic growth factors in age-related maculopathy. Br. J. Ophthalmol. 81, 154–162.

Kuroki, M., Voest, E.E., Amano, S., Beerepoot, L.V., Takashima, S., Tolentino, M., Kim, R.Y., Rohan, R.M., Colby, K.A., Yeo, K.T., Adamis, A.P., 1996. Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo. J. Clin. Invest. 98, 1667–1675.

Lip, P.L., Blann, A.D., Hope-Ross, M., Gibson, J.M., Lip, G.Y., 2001. Age-related macular degeneration is associated with increased vascular endothelial growth factor, hemorheology and endothelial dysfunction. Ophthalmology 108, 705–710.

Lopez, P.F., Sippy, B.D., Lambert, H.M., Thach, A.B., Hinton, D.R., 1996. Trans differentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest. Ophthalmol. Vis. Sci. 37, 855–868.

Lovell, M.A., Ehmann, W.D., Mattson, M.P., Markesbery, W.R., 1997. Elevated 4-hydroxynonenal in ventricular fluid in Alzheimer's disease. Neurobiol. Aging 18, 457–461.

Macular Photocoagulation Study Group (MPSG), 1991. Argon laser photocoagulation for neovascular maculopathy. Five year results from randomized clinical trials. Arch. Ophthalmol. 109, 1109–1114.

Mangipudy, R.S., Vishwanatha, J.K., 1999. Role of nitric oxide in the induction of apoptosis by smokeless tobacco extract. Mol. Cell. Biol. 200, 51–57.

Mousa, S.A., Lorelli, W., Campochiaro, P.A., 1999. Role of hypoxia and extracellular matrix-integrin binding in the modulation of angiogenic growth factor secretion by retinal pigmented epithelial cells. J. Cell. Biochem. 74, 135–143.

Nishikawa, A., Sodum, R., Chung, F.L., 1992. Acute toxicity of *trans*-4-hydroxy-2-nonenal in Fisher 344 rats. Lipid 27, 54–58.

Obrosova, I.G., Fathallah, L., Greene, D.A., 2000. Early changes in lipid peroxidation and antioxidative defense in diabetic rat retina: effect of DL-alpha-lipoic acid. Eur. J. Pharmacol. 398, 139–146.

Ohia, S.E., Bagchi, M., Stohs, S.J., 1994. Age-related oxidative damage in Long–Evans rat retina. Res. Commun. Mol. Pathol. Pharmacol. 85, 21–31.

Park, J.E., Keller, G.A., Ferrara, N., 1993. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol. Biol. Cell 4, 1317–1326.

Park, J.E., Chen, H.H., Winer, J., Houck, K.A., Ferrara, N., 1994. Placenta growth factor. Potentiation of vascular endotelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not Flk-1/KDR. J. Biol. Chem. 269, 25646–25654.

Parola, M., Robino, G., Marra, F., et al., 1998. HNE interacts with JNK isoforms in human hepatic stellate cells. J. Clin. Invest. 102, 1942–1950.

Renes, J., De Vries, E.E., Hooiveld, G.J., Krikken, I., Jansen, P.L., Muller, M., 2000. Multidrug resistance protein MRP1 protects against the toxicity of the major lipid peroxidation product 4-hydroxynonenal. Biochem. J. 350, 555–561.

Richer, S., 1996. Multicenter ophthalmic and nutritional age-related macular degeneration study: Part 2. Antioxidant intervention and conclusions. J. Am. Optom. Assoc. 67, 30–49.

Ruef, J., Rao, G.N., Li, F., Bode, C., Patterson, C., Bhatnagar, A., Runge, M.S., 1998. Induction of rat aortic smooth muscle cell growth by the lipid peroxidation end product 4-hydroxy-2-nonenal. Circulation 97, 1071–1078.

Samiec, P.S., Drews-Botsch, C., Flagg, E.W., Kurtz, J.C. , Sternberg Jr., P., Reed, R.L., Jones, D.P. 1998. Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes. Free Radic. Biol. Med. 24, 699–704.

Sarks, S.H., 1976. Ageing and degeneration in the macular region: a clinicopathological study. Br. J. Ophthalmol. 60, 324–341.

Selley, M.L., 1998. (*E*)-4-hydroxy-2-nonenal may be involved in the pathogenesis of Parkinson's disease. Free Radic. Biol. Med. 25, 169–174.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsentein, M., Wu, X.F., Breitman, M.L., Schuh, A.C., 1995. Failure of blood-island formation and vasculogenesis in Flk-1 deficient mice. Nature 376, 62–66.

Srivastava, S.K., Singhal, S.S., Bajpai, K.K., Chaubey, M., Ansari, N.H., Awasthi, Y.C., 1994. A group of novel glutathione S-transferase isozymes showing high activity towards 4-hydroxy-2-nonenal are present in bovine ocular tissues. Exp. Eye Res. 59, 151–159.

Toti, P., Greco, G., Motolese, E., Stumpo, M., Cardone, C., Tosi, G.M., 1999. Cell composition and immunohistochemical detection of VEGF, TGF-beta, and TNF-alpha in proliferative vitreoretinopathy. J. Submicrosc. Cytol. Pathol. 31, 363–366.

Uchida, K., Shiraishi, M., Naito, Y., Torii, Y., Nakamura, Y., Osawa, T., 1999. Activation of stress signaling pathways by the end product of lipid peroxidation. 4-Hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. J. Biol. Chem. 274, 2234–2242.

Vander Jagt, D.L., Hunsaker, L.A., Vander Jagt, T.J., Gomez, M.S., Gonzales, D.M., Deck, L.M., Royer, R.E., 1997. Inactivation of glutathione reductase by 4-hydroxynonenal and other endogenous aldehydes. Biochem. Pharmacol. 53, 1133–1140.

Van Kuijk, F.J.G.M., 1988. Detection of lipid peroxidation associated with retinal degeneration using gas chromatography mass spectroscopy. Thesis, University of Nijmegen, the Netherlands.

Verdejo, C., Marco, P., Renau-Piqueras, J., Pinazo-Duran, M.D., 1999. Lipid peroxidation in proliferative vitreoretinopathies. Eye 13, 183–188.

Whittle, C., Gillespie, K., Harrison, R., Mathieson, P.W., Harper, S.J., 1999. Heterogeneous vascular endothelial growth factor (VEGF) isoform mRNA and receptor mRNA expression in human glomeruli, and the identification of VEGF148 mRNA, a novel truncated splice variant. Clin. Sci. (Colch) 97, 303–312.

Yi, X., Ogata, N., Komada, M., Yamamoto, C., Takahashi, K., Omori, K., Uyama, M., 1997. Vascular endothelial growth factor expression in choroidal neovascularization in rats. Graefe's Arch. Clin. Exp. Ophthalmol. 235, 313–319.

Zhao, M., Zhang, C., Wu, Y., 1996. The relationship between the retinal lipid peroxidation or antioxidation and age in rats. Chung-Hua Yen Ko Tsa Chih 32, 230–232.