Research Article

Comparative effects of fatty acids on proinflammatory gene cyclooxygenase 2 and inducible nitric oxide synthase expression in retinal pigment epithelial cells

I-Mo Fang[1,2], Chang-Hao Yang[1], Chung-May Yang[1] and Muh-Shy Chen[1]

[1] Department of Ophthalmology, National Taiwan University Hospital, Taipei, Taiwan
[2] Department of Ophthalmology, Taipei City Hospital, Zhongxiao Branch, Taipei, Taiwan

Dietary fat modification is a promising approach to prevent age-related macular degeneration (AMD). However, which types of fatty acids carry a greater risk for AMD remains unclear. In this study, we compared the effects of 18-carbon fatty acids with different degrees of unsaturation on the expression of the proinflammatory genes cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) in human retinal pigment epithelium (RPE). Additionally, we investigated whether lutein could modulate these genes induced by fatty acids in RPE. Treatment with oleic acid, linoleic acid (LA), or linolenic acid increased the expression of iNOS and COX-2 genes and the production of prostaglandin E_2 and nitric oxide (NO) in RPE, whereas the saturated fatty acid stearic acid had little effect on these genes. Of the fatty acids studied, LA had the greatest effects on the induction of these genes. Furthermore, LA also induced NF-κB transcriptional activation the most. Lutein inhibited LA-induced expression of COX-2 and iNOS in a dose-dependent manner. These data suggested that specific unsaturated fatty acids, particularly LA, can stimulate RPE cells to express proinflammatory genes, which may contribute to the pathogenesis of AMD. Lutein inhibited the expression of these genes induced by LA through blockade of NF-κB activation.

Keywords: Age-related macular degeneration / Cyclooxygenase-2 / Dietary fatty acid / Inducible nitric oxide synthase / Lutein

Received: June 5, 2008; revised: July 24, 2008; accepted: July 27, 2008

1 Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries [1]. Most severe visual loss cases in wet AMD are caused by abnormal growth of choroidal new vessels (CNV) under the retinal pigment epithelium (RPE) and the retina, with secondary exudative retinal detachment, subretinal hemorrhage, and outer retinal degeneration [2]. Dietary fat is a kind of behaviorally modifiable risk factor for AMD and thus modification of dietary fat intake could be a promising approach to prevent AMD. However, epidemiological data regarding the type of fatty acids associated with a greater risk for AMD are inconsistent. Some studies suggested that greater intake of vegetable, polyunsaturated fats increased the risk of AMD, whereas other studies have reported the harmful effects of monounsaturated and saturated fatty acids on AMD [3–5].

One commonly believed pathogenic mechanism of AMD involves age-related changes to the RPE. Previous studies of the roles of fatty acids in developing AMD have primarily focused on their atherogenic effects [6]. However, one of the cardinal functions of the RPE is phagocytosis and intracellular lysosomal degradation of the aged photoreceptor outer segment membrane [7]. Since the photoreceptor outer segment membrane is rich in polyunsaturated fatty acids, this phagocytosis function directly exposes the RPE to various kinds of fatty acids. Furthermore, retinal concentrations of fatty acid are dependent on and modifiable by diet [8], indicating that the composition of fatty acids consumed in

Correspondence: Dr. Chang-Hao Yang, Department of Ophthalmology, National Taiwan University Hospital, 7 Chung-Shan S. Road, Taipei, Taiwan
E-mail: DAH75@tpech.gov.tw

Abbreviations: **AMD**, age-related macular degeneration; **CNV,** choroidal new vessels; **COX-2**, cyclooxygenase-2; **iNOS**, inducible NO synthase; **LA**, linoleic acid; **LnA**, linolenic acid; **NO**, nitric oxide; **OA**, oleic acid; **PDTC**, pyrrolidine dithiocarbamate; **PGE^2**, prostaglandin E_2; **RPE**, retinal pigment epithelium; **SA**, stearic acid

the diet may influence the concentrations of fatty acids exposure to RPE.

CNV development is a complex multistep process that involves a variety of biologically active substances. Of them, prostaglandin E_2 (PGE_2) and nitric oxide (NO), synthesized by cyclooxygenase (COX) and NO synthase (NOS), respectively, are not only inflammatory mediators, but also angiogenic modulators [9, 10], and are thought to correlate with neovascular formation in AMD. COX exists in two isoforms, COX-1 and COX-2, whereas NOS exists in three isoforms: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Both COX-2 and iNOS are identifiable only in the presence of certain stimuli. In an animal model, selective inhibition of COX-2 effectively suppressed the growth of CNV [11]. Similarly, selective deficiency of iNOS resulted in a significant decrease in CNV development in a mouse model [12]. These studies indicated that COX-2 and iNOS are actively involved in CNV formation in AMD.

Dietary fatty acids can be divided into four major groups: saturated fatty acids, monounsaturated fatty acids, ω-6 polyunsaturated fatty acids, and ω-3 polyunsaturated fatty acids [13]. Several lines of evidence indicated that dietary fatty acids can modulate inflammation and signal transduction pathways related to cell proliferation, apoptosis, and angiogenesis [14, 15]. In an *in vivo* model, subretinal injections of linoleic acid (LA) hyperoxide induced a cascade of angiogenic processes and resulted in CNV [16]. We have previously shown that LA induces the activation of NF-κB and increases the expression of COX-2 and iNOS in human RPE, which may contribute to CNV in AMD [17]. However, the effects of other types of fatty acids, especially different degrees of unsaturation, on the expression of these proinflammatory genes remain unclear.

Lutein is a member of the xanthophylls family of carotenoids, which are present in abundance in dark, leafy green vegetables [18]. Currently, lutein is widely used as a supplement for eye-protective nutrition, and epidemiological studies have demonstrated its beneficial role in the prevention of AMD [19, 20]. It has been hypothesized that lutein protects the macula against photo-oxidative damage by functioning as an antioxidant and optical filter [21]. Lutein also has anti-inflammatory effects [22]; however, the molecular mechanism of its anti-inflammatory effects remains unknown.

In this study, we attempted to investigate the influence of different unsaturated degrees of dietary fatty acids on the expression of proinflammatory genes that may contribute to AMD. Therefore, we compared the effects of fatty acids of 18-carbon in length with different degree of unsaturation on the expression of COX-2 and iNOS genes as well as their transcriptional activation in human RPE. Furthermore, we investigated the effects of lutein on fatty acids-induced proinflammatory events in RPE, and whether these effects are mediated through the regulation of NF-κB activation.

2 Materials and methods

2.1 Reagents

Stearic acid (SA) (18:0), oleic acid (OA) (18:1n-9), LA (18:2n-6), linolenic acid (LnA) (18:3n-3), vitamin E (α-tocopherol) and lutein were obtained from Sigma-Aldrich (St. Louis, MO, USA). The human anti-iNOS mAb was purchased from Upstate (Charlottesville, VA, USA), and human anti-COX-2 mAb was obtained from Calbiochem (San Diego, CA, USA).

2.2 Cell culture and treatment

ARPE-19 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium/F-12 human amniotic membrane nutrient mixture (DMEM/F-12; Sigma-Aldrich) with 10% fetal bovine serum (Sigma-Aldrich) in a humidified incubator at 37°C in an atmosphere of 5% CO_2. Cells were used at the third to fifth passages. Fatty acids and lutein were diluted in ethanol and the concentration of ethanol was always <0.5% according to the methods described previously [23]. Cells were stimulated with different concentrations (0, 10, 25, or 50 μM) of fatty acids for 12 h. To determine the effects of pyrrolidine dithiocarbamate (PDTC) or vitamin E, cells were pretreated with different doses of PDTC (0, 1, 5, or 10 μM) for 1 h or with vitamin E (25 μM) for 24 h, and then exposed to 50 μM of various fatty acids for an additional 12 h. For the effects of lutein, cells were incubated for 24 h with different doses of lutein (1–100 μg/mL) before addition of LA (50 μg/mL) for 12 h.

2.3 Assessment of fatty acid uptake

The uptake of fatty acids by cells was determined in 12-h incubations in the 50 μM fatty acids. The disappearance of the fatty acids from the medium was used as a measure of fatty acid uptake. After incubation, the medium fatty acids were extracted by a method adapted from that developed by Bligh and Dyer [24]. In brief, 4 vol of the dichloromethane/methanol mixture (1:2) was added to 1 vol of medium, and well mixed. Dichloromethane (1.2 mL) was added to this mixture followed by 1.6 mL water. The dichloromethane phase containing lipids was dried under flowing nitrogen. The fatty acids were methylated and assayed by gas chromatography.

2.4 Cell viability

Cell viability was determined using the MTT assay after a 12-hour exposure to various concentrations (0–50 μM) of fatty acids. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Chemicon, Temecula, CA, USA) (5 mg/mL) was added to 0.1 mL of cell suspension for 4 h,

and the formazan formed was then dissolved in isopropanol. OD was measured with a plate reader at 570 nm.

2.5 Total RNA extraction and RT-PCR analysis

Total RNA (1 μg) was prepared from ARPE-19 cells, and first-strand cDNAs were synthesized with an oligo dT-primed Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen-Gibco, Carlsbad, CA, USA). For PCR, 1 μL cDNA mixture was added to a 50-μL PCR reaction mixture consisting of 5 μL 106 PCR buffer, 2.5 pmol dNTP, 5 pmol paired primers, 1.25 U Taq polymerase (Promega, Madison, WI, USA), and ultrapure water. The following primers were used for amplification reaction: for COX-2, forward primer 5′-CAACTCTATATTGCTGGAACATGGA-3′, reverse primer 5′-TGGAAGCCTGTGATACTTTCTGTACT-3′; for iNOS, forward primer 5′-ACAGGAGGGGTTAAAGCTGC-3‘, reverse primer 5′-GCAGCTTTAACCCCTCCTGT-3′; and for β-actin, forward primer 5′-GAACCCTAAGGCCAACCGTG-3′, reverse primer 5′-TGGCATAGAGGTCTTTA CGG-3’. The reaction mixture was amplified in a PCR thermal cycler (Perkin-Elmer, Wellesley, MA, USA). The PCR cycling conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 3 min, ending with a 10-min extension at 72°C for all primers. PCR mixtures without cDNA were used as negative controls. PCR products were separated by gel electrophoresis on 2% agarose gels containing ethidium bromide (Sigma-Aldrich) and then analyzed under ultraviolet light against the DNA molecular markers. Semiquantitative PCR was performed according to the methods described previously [25]. The intensity of the products was analyzed using an image analyzer (Digital 1D Science; Eastman Kodak, Rochester, NY, USA), and the amount of PCR-amplifiable material in each reverse-transcribed sample was normalized to the amount of the housekeeping gene β-actin.

2.6 Western blot analysis

Cells were incubated with various fatty acids at 37°C for indicated times. Cells were harvested, washed twice with ice-cold PBS, and resuspended in PBS containing 0.1 mM PMSF. The suspension was lysed by three cycles of freezing and thawing. Cytosolic fractions were obtained after centrifugation at 12 000 × *g* for 20 min at 4°C. The protein content was determined by the BCA method (Pierce Biotechnology, Rockford, IL, USA). Samples (40 μg protein) were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk in PBS containing 0.1% Tween 20 (PBST) for 2 h, and then incubated with human COX-1 (1:200 dilution) or iNOS (1:250 dilution) mAb for 2 h. After washing three times with PBST, the membranes were hybridized with the horseradish peroxidase-conjugated secondary antibody for 1 h. Peroxidase activity on the membrane sheet was visualized on X-ray films by standard enhanced chemiluminescence (ECL) (Pierce Biotechnology). The blots were scanned with an image-analysis software (Photoshop, version 7.0; Adobe Systems, San Jose, CA, USA), and the ODs of the bands were calculated.

2.7 Measurement of NO and PGE_2 production by RPE cells

Nitrite concentration was determined in the supernatant of cells and used as an index of NO synthesis. Nitrite was quantified colorimetrically with Griess reagent using sodium nitrite as standard. For measuring nitrite concentration in cell medium, an equal volume of Griess reagent was added to the cell medium (0.5 mL), and absorbance of the mixture was measure at 580 nm using a Beckman DU 640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA).

The concentration of PGE_2 in culture supernatants was detected using a PGE_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA).

2.8 Nuclear protein extracts and electrophoretic mobility shift assay

ARPE-19 cells were trypsinized, resuspended and homogenized in buffer A (10 mM HEPES pH 7.8, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 1 mM PMSF). Nuclei and cytosolic fractions were separated by centrifugation at 1000 × *g* for 20 min. The nuclear fractions were washed and resuspended in the same buffer. Nuclei were extracted for 1 h at 4°C and centrifuged at 100 000 × *g* for 45 min. Supernatants (nuclear extracts) were dialyzed in buffer C (50 mM HEPES pH 7.8, 50 mM KCl, 10% glycerol, 1 mM PMSF, 0.1% mM EDTA, and 1 mM DTT). Protein concentration was determined by the BCA method, as described earlier.

Electrophoretic mobility shift assay was performed using the NF-κB DNA binding protein detection system kit (Pierce Biotechnology). Briefly, NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3) was end-labeled with biotin. Nuclear protein (10 μg) was incubated with an NF-κB consensus oligonucleotide for 30 min in a binding buffer that consisted of 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 4% glycerol, and 2 μg poly-deoxyinosinic deoxycytidylic acid (Pharmacia Biotech, Piscataway, NJ, USA). The reaction was stopped by adding 1 μL gel loading buffer and subjecting the mixture to non-denaturing PAGE on a 4% gel in 0.56 TBE buffer.

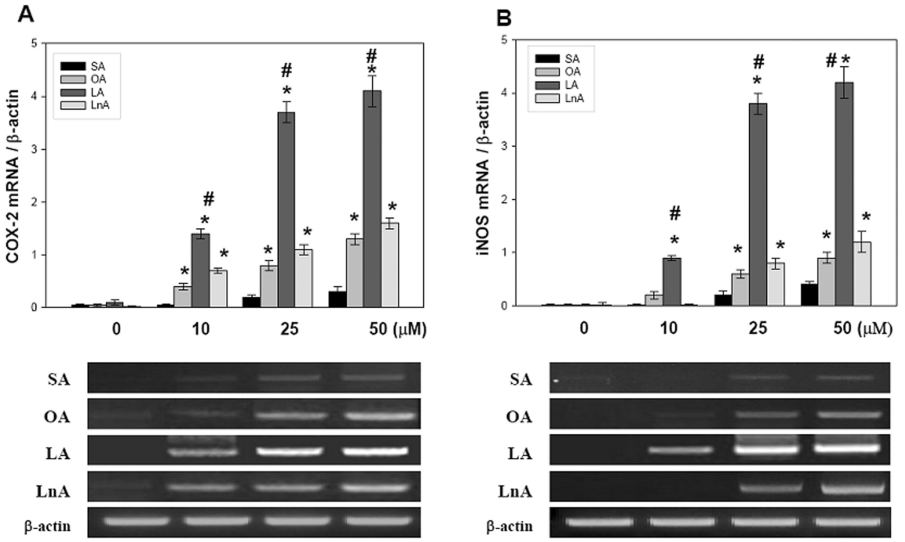

Figure 1. Effects of SA, OA, LA, and LnA on COX-2 (A) and iNOS mRNA (B) expressions in ARPE-19 cells. ARPE-19 cells were treated with increasing concentrations (10, 25, and 50 μM) of various fatty acids for 12 h. The mRNA levels of COX-2 were determined by RT-PCR analysis. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using photoimaging. Similar results were obtained in three independent experiments. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$, as compared with cells without treated with fatty acids; # $p < 0.05$, among different fatty acids at concentrations of 10, 25 or 50 μM.

2.9 Transient transfection and luciferase assays

For transient transfections, 2×10^5–4×10^5 ARPE-19 cells were seeded per well on a 24-well tissue culture dish 1 day prior to transient transfection. Cell were transfected with serum-free DMEM containing 25 μL/mL Lipofectamine 2000 reagent (Invitrogen-Gibco) and 10 μg/mL plasmid DNA including an NF-κB luciferase promoter construct or the empty vector (Clontech, San Diego, CA, USA). At 4 h after transfection, cells were washed with PBS and incubated with DMEM/F-12 (with 10% serum) for 24 h. The cells were stimulated with 50 μM SA, OA, LA, and LnA for 12 h. Luciferase activity was measured using the Luciferase Reporter Assay System (Promega) using a luminometer.

2.10 Statistical analysis

Data were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison. A probability value of 0.05 or less was considered statistically significant.

3 Results

3.1 Fatty acid uptake

The average cellular uptake of fatty acids during the 12-h incubation was 62.2 ± 8.4, 67.5 ± 6.8, 58.6 ± 6.2, and 54.3 ± 7.2 nmol/mg cell proteins for SA, OA, LA, and LnA, respectively. No statistical significance was noted concerning the differences in uptake rate between each fatty acid ($p < 0.05$, one-way ANOVA).

3.2 Effects of fatty acids on COX-2 and iNOS expression at the mRNA and protein levels in ARPE-19 cells

Exposure of cells to OA, LA, and LnA for 12 h increased the expression of COX-2 and iNOS mRNA in a dose-dependent manner (Fig. 1), whereas SA (0–50 μM) had little effect on the activation of these genes. Incubation of cells with 50 μM SA, OA, LA, or LnA for 12 h did not decrease the viability of ARPE-19 cells (data not shown). At concentrations of 10, 25, or 50 μM, LA stimulated the expression of iNOS and COX-2 genes most markedly compared with SA, OA, and LnA ($p < 0.05$ for iNOS and COX-2, one-way ANOVA and Bonferroni's multiple comparison test).

Similar to the effects on COX-2 and iNOS mRNA level, exposure of cells to OA, LA, and LnA for 12 h increased the synthesis of COX-2 and iNOS proteins in a dose-dependent manner (Fig. 2), whereas exposure to SA had little effects on the expression of these proteins. LA also stimulated the synthesis of the COX-2 and iNOS proteins most markedly at concentrations of 10, 25 or 50 μM compared with SA, OA, and LnA ($p<0.05$ for iNOS and COX-2 protein, one-way ANOVA and Bonferroni's multiple comparison test).

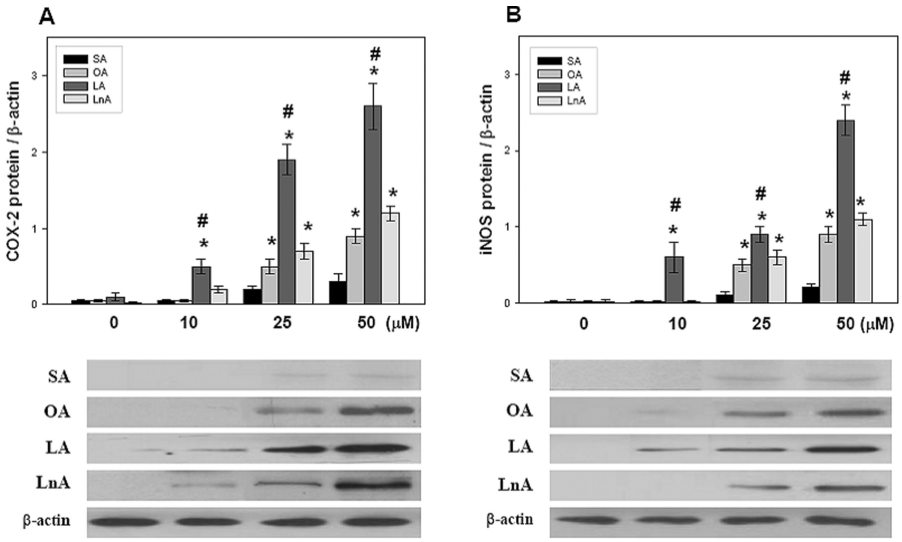

Figure 2. Immunoblot analysis of the effects of SA, OA, LA, and LnA on levels of COX-2 (A) and iNOS (B) protein in cultured ARPE-19 cells. Cells were treated with increasing concentrations (10, 25, and 50 μM) of various fatty acids for 12 h. After incubation, the cell lysates were subjected to SDS-PAGE on 10% gels and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with COX-2 or an iNOS polyclonal antibody. Bands were visualized by an ECL method. Similar results were obtained in three independent experiments. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with cells without treatment with fatty acids; # $p < 0.05$, among different fatty acids at concentrations of 10, 25 or 50 μM.

3.3 Effects of fatty acids on PGE_2 and NO production in ARPE-19 cells

Exposure of cells to OA, LA, or LnA also resulted in increased PGE_2 and NO concentrations in a dose-dependent manner. Among the fatty acids studied, LA had the highest stimulatory effects on the production of PGE_2 and NO at concentrations of 10, 25 or 50 mM (Fig. 3; $p < 0.05$ for PGE_2 and NO, one-way ANOVA and Bonferroni's multiple comparison test).

3.4 Effects of fatty acids on NF-κB activation in ARPE-19 cells

Because NF-κB is the central regulator of COX-2 and iNOS gene expression, we examined the effect of 18-carbon fatty acids on NF-κB by measuring both NF-κB DNA binding and NF-κB-dependent transcriptional activity. Exposure of cells to OA, LA, and LnA for 12 h resulted in increased NF-κB binding activity. In particular, LA activated the transcription factor NF-κB most markedly. SA had a less pronounced effect on the activation of NF-κB (Fig. 4; $p < 0.05$, one-way ANOVA and Bonferroni's multiple comparison test).

We next investigated whether the increase in NF-κB DNA binding mediated by 18-carbon fatty acids corresponded with an increase in NF-κB-dependent gene transcription. To this end, ARPE-19 cells were transiently transfected with a NF-κB-luciferase reporter construct or an empty vector. We found that exposure of ARPE-19 cells to OA, LA, or LnA for 12 h induced an increase in luciferase activity in the cells transfected with the NF-κB-luciferase reporter construct but not the empty vector (Fig. 4B). Cells exposed to LA had the highest and SA had the least increase in luciferase activity. These results indicated that 18-carbon unsaturated fatty acids OA, LA, and LnA activate NF-κB-dependent transcriptional activity in ARPE-19 cells.

3.5 Effects of the NF-κB inhibitor PDTC on COX-2 and iNOS mRNA and protein expression in ARPE-19 cells induced by unsaturated fatty acids

To determine whether the activation of NF-κB by 18-carbon unsaturated fatty acids was involved in the up-regulation of iNOS and COX-2, we studied the effects of the NF-κB inhibitor PDTC on the expression of iNOS and COX-2 in ARPE-19 cells exposed to 50 μM of various unsaturated fatty acids. Pretreatment with increasing doses of PDTC decreased OA-, LA-, and LnA-mediated stimulation of COX-2 and iNOS mRNA (Fig. 5) and protein (Fig. 6) expression in a dose-dependent manner.

3.6 Effects of the NF-κB inhibitor PDTC on PGE_2 and NO production in ARPE-19 cells induced by unsaturated fatty acids

We further determined whether the decrease in iNOS and COX-2 mRNA and protein expression by PDTC results in a

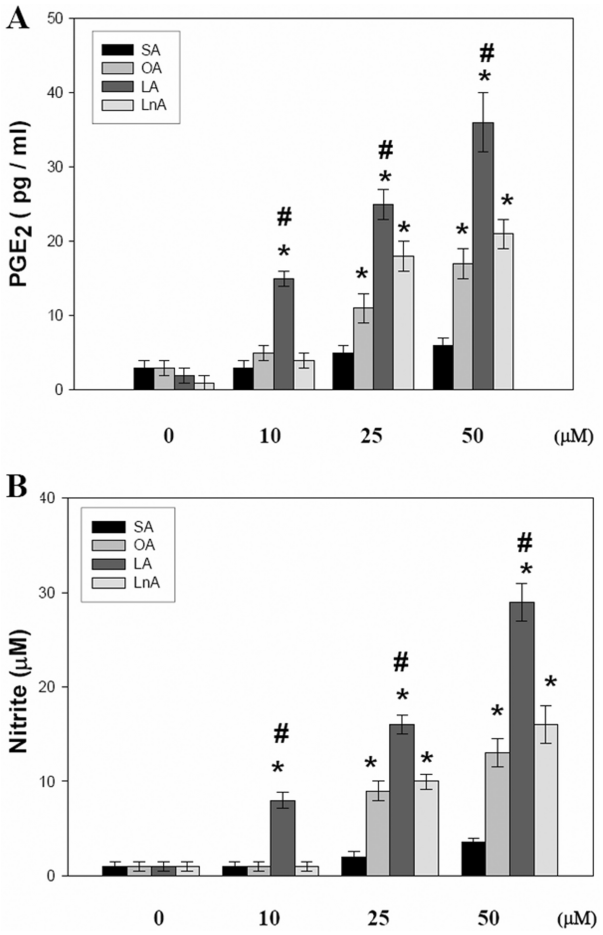

Figure 3. Effects of SA, OA, LA, and LnA on the production of PGE_2 (A) and NO (B) in ARPE-19 cells. Cells were treated without or with increasing concentrations (10, 25, and 50 μM) of various fatty acids for 12 h. The culture medium in each group was collected and analyzed by enzyme-linked immunosorbent assay kit. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with cells without treatment with fatty acids; # $p < 0.05$, among different fatty acids at concentrations of 10, 25 or 50 μM.

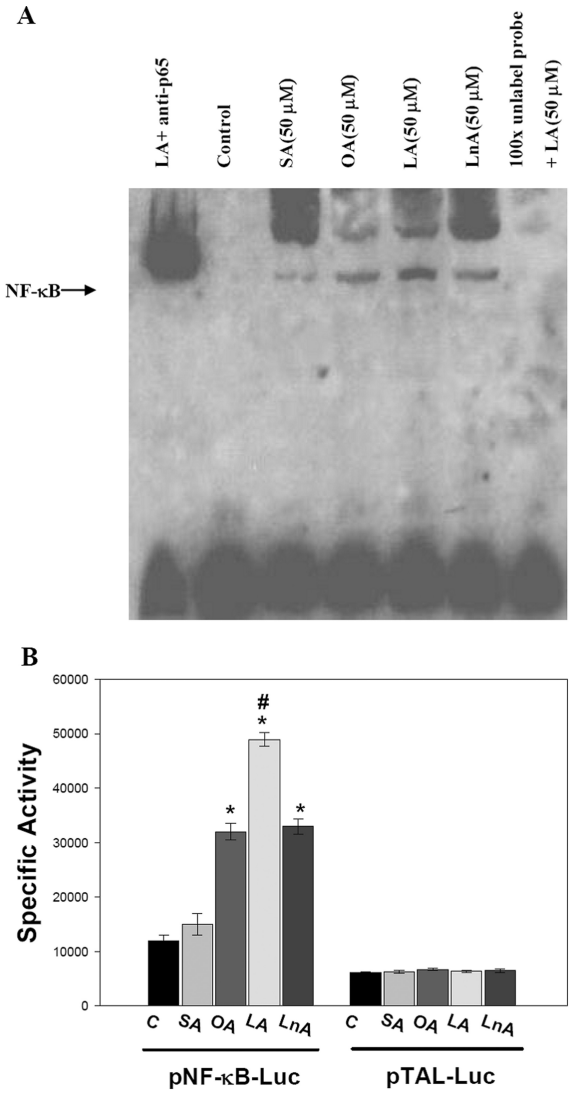

Figure 4. The effects of SA, OA, LA, and LnA on NF-κB binding ability (A) and NF-κB-dependent transcriptional activity (B) in ARPE-19 cells. NF-κB binding ability was analyzed by electrophoretic mobility shift assay. ARPE-19 cells were untreated or treated for 12 h with 50 μM of various fatty acids. Lane 1, supershift with anti-p65 antibody; lane 2, control, cells treated with PBS; lane 3, stimulation with SA (50 μM); lane 4, stimulation with OA (50 μM); lane 5, stimulation with LA (50 μM); lane 6, stimulation with LnA (50 μM); lane 7, competition with 1006 unlabeled NF-κB probe. The NF-κB-dependent transcriptional activity was measured by transient transfected ARPE-19 cells with an NF-κB-luciferase promoter construct, following which the cells were incubated with 50 μM of various fatty acids for 12 h. NF-κB-dependent transcriptional activity was determined using the luciferase assay (pNF-κB-Luc). The cells without treatment with fatty acid served control. This figure shows that fatty acids do not influence luciferase activity in cells transfected with an enhancerless construct (pTAL-luc). Specific activity is expressed as units/microgram of protein. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with control cells; # $p < 0.05$, among different fatty acids.

decreased NO and PGE_2 release from ARPE-19 cells. The results are shown in Fig. 7. PDTC caused a dose-dependent decrease in PGE_2 and NO release from OA-, LA-, and LnA-stimulated ARPE-19 cells.

3.7 Effects of vitamin E on fatty acid-induced expression of COX-2 and iNOS mRNA and activation of NF-κB

We evaluated the influence of vitamin E on fatty acid-induced expression of COX-2 and iNOS and activation of NF-kB. Vitamin E significantly reduced the OA-, LA-, and LnA-induced increase in COX-2 and iNOS mRNA levels in ARPE-19 cells (Fig. 8A). Furthermore, similar to its effect on COX-2 and iNOS mRNA expression, vitamin E effec-

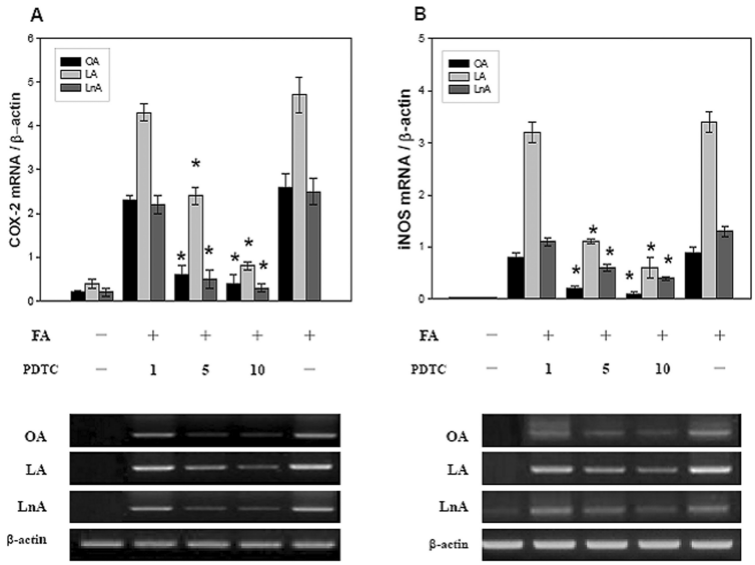

Figure 5. Pretreatment with PDTC reduced induction of COX-2 (A) and iNOS (B) mRNA expression in ARPE-19 cells treated with OA, LA, and LnA. Cells were pretreated for 1 h with different doses of PDTC (0, 1, 5, or 10 μM). After changing the medium, these cells were treated with 50 μM fatty acids for 12 h and assayed for iNOS and COX-2 mRNA expression by RT-PCR. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using photoimaging. Similar results were obtained in three independent experiments. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with cells treated with fatty acid only. FA, fatty acids.

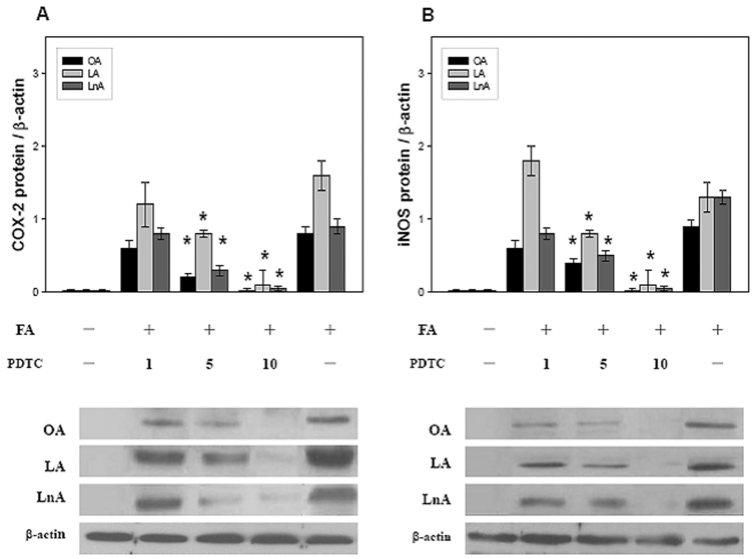

Figure 6. Pretreatment with PDTC reduced the levels of COX-2 (A) and iNOS (B) protein expression in ARPE-19 cells treated with OA, LA, and LnA. Cells were pretreated for 1 h with different doses of PDTC (0, 1, 5, or 10 μM). After changing the medium, these cells were treated with 50 μM fatty acids for 12 h and assayed for iNOS and COX-2 protein expression by Western blot analysis. Bands were visualized by an ECL method. Similar results were obtained in three independent experiments. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with cells treated with fatty acid only.

tively decreased OA-, LA-, and LnA-induced increase in luciferase activity in cells transfected with NF-κB–luciferase reporter construct, but not the empty vector (Fig. 8B).

3.8 Effects of lutein on LA-induced COX-2 and iNOS expression in ARPE-19 cells

We next determined the effects of lutein on LA-induced COX-2 and iNOS expression in ARPE-19 cells. Lutein inhibited the expression of both iNOS and COX-2 at the mRNA and protein levels in a dose-dependent fashion (Fig. 9). It did not decrease the viability of ARPE-19 cells when these cells were incubated with 100 μg/mL lutein for 24 h (data not shown). To further examine whether the inhibitory effects of lutein are attributable to the blockade of NF-κB activation, we examined the effects of lutein on both the NF-κB DNA binding ability and NF-κB-dependent transcriptional activity. Stimulation of ARPE-19 cells with LA induced an increase in NF-κB binding activity. Lutein at 100 μg/mL caused a nearly complete disappearance of LA-induced NF-κB DNA binding complex (Fig. 10A). Furthermore, lutein reduced LA-induced increase in luciferase activity in a dose-dependent manner in ARPE-19 cells transfected with the NF-κB–luciferase reporter construct, but not the empty vector lutein (Fig. 10B). These results indicated that lutein effectively reduced LA-induced activation of NF-κB and COX-2 and iNOS gene expression in ARPE-19 cells.

4 Discussion

In this study, we demonstrated that exposure of ARPE-19 cells to unsaturated fatty acids such as OA, LA, and LnA

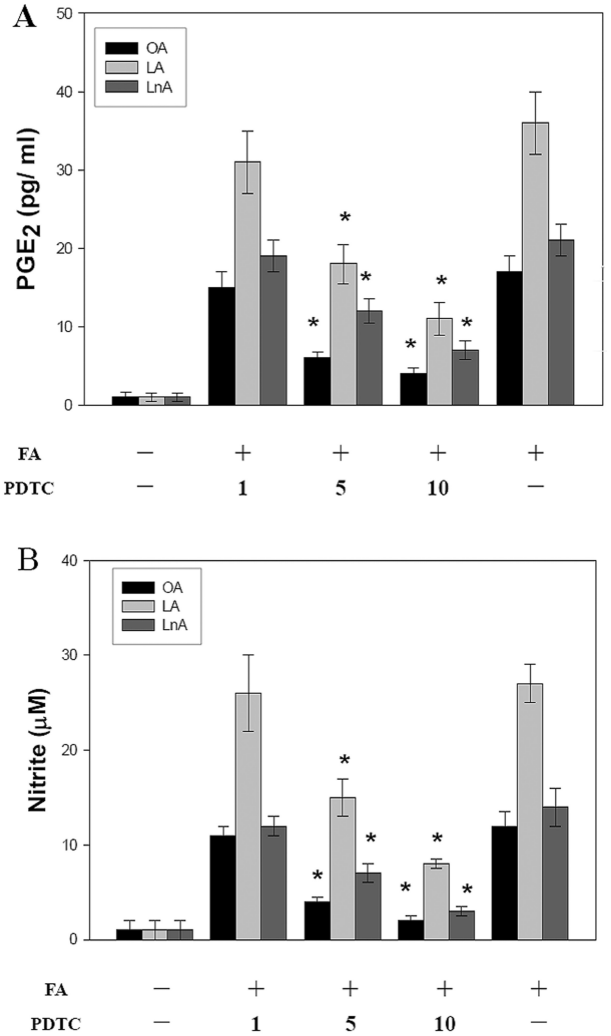

Figure 7. Pretreatment with PDTC reduced the production of PGE_2 (A) and NO (B) in ARPE-19 cells treated with OA, LA, and LnA. Cells were pretreated for 1 h with different doses of PDTC (0, 1, 5, or 10 μM). After changing the medium, these cells were treated with 50 μM fatty acids for 12 h and assayed for concentrations of PGE_2 and NO in the culture medium. Similar results were obtained in three independent experiments. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with cells treated with fatty acid only.

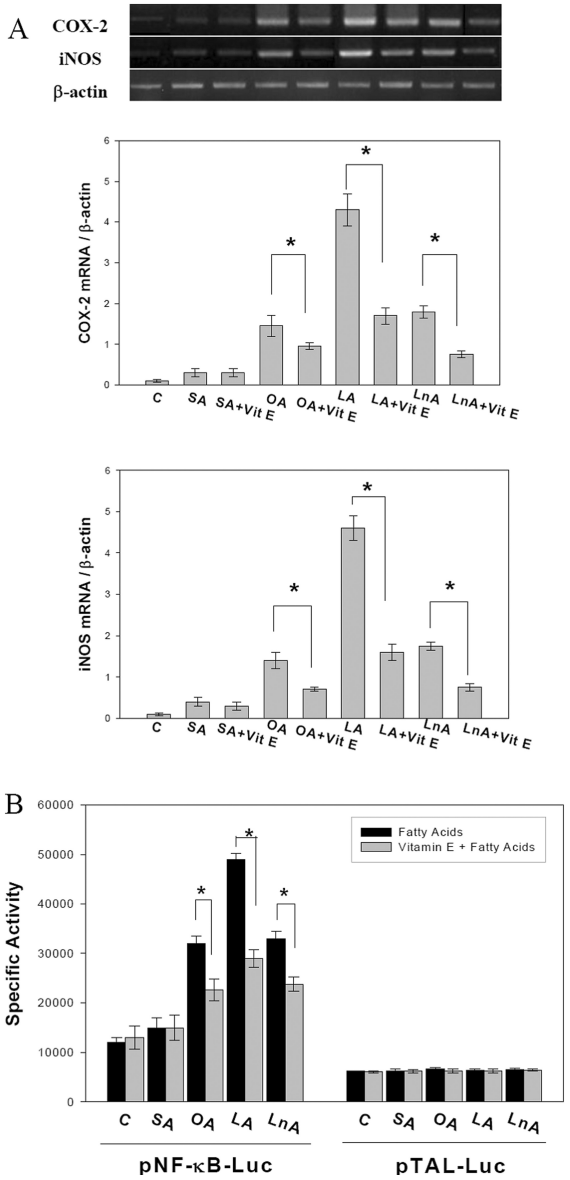

Figure 8. Inhibition of vitamin E on COX-2 and iNOS mRNA expression (A) and NF-κB-dependent transcriptional activity (B) in ARPE-19 cells induced by fatty acids. ARPE-19 cells were pretreated with 25 μM vitamin E or PBS for 24 h. After changing the medium, these cells subsequently incubated with 50 μM of various fatty acids for an additional 12 h. The mRNA levels of COX-2 and iNOS were determined by RT-PCR analysis. The NF-κB-dependent transcriptional activity was measured by transient transfected ARPE-19 cells with an NF-κB-luciferase promoter construct, following by treating with vitamin E (25 μM) and various fatty acids. NF-κB-dependent transcriptional activity was determined using the luciferase assay (pNF-κB-Luc). This figure shows that fatty acids do not influence luciferase activity in cells transfected with an enhancerless construct (pTAL-luc). Specific activity is expressed as units/microgram of protein. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with cells without treatment with vitamin E. C, control.

resulted in a dose-dependent increase in COX-2 and iNOS expression and subsequent PGE_2 and NO production, whereas exposure to the saturated fatty acid SA had much smaller effect on the activation of these genes. In particular, among the fatty acids studied, LA stimulated NF-κB transcriptional activation and induction of COX-2 and iNOS expression most markedly. These results indicated that components of dietary fats can influence gene expression patterns in human RPE. LA was the most potent fatty acid to stimulate proinflammatory genes COX-2 and iNOS expression in RPE. Moreover, lutein inhibited the expres-

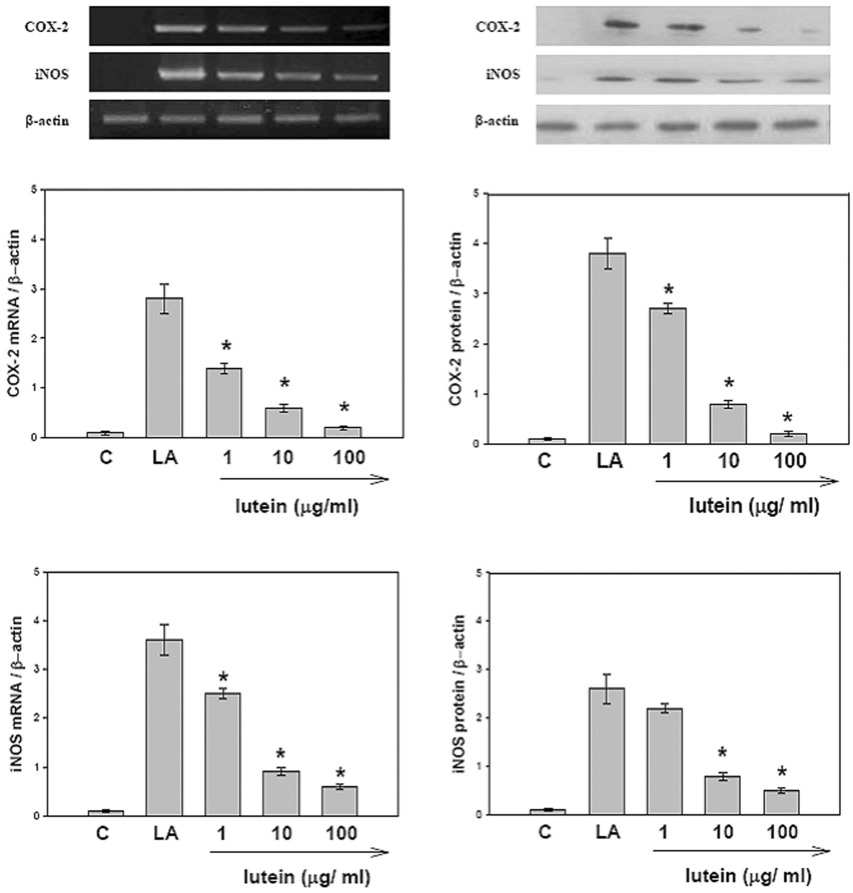

Figure 9. Inhibition of lutein on LA-induced COX-2 and iNOS expression at the mRNA (A) and protein (B) levels in ARPE-19 cells. ARPE-19 cells were pretreated with 1, 10 or 100 μg/mL lutein, or no pretreatment as a control, for 24 h. After changing the medium, these cells subsequently incubated with 50 μM LA for 12 h. The mRNA levels of COX-2 and iNOS were determined by RT-PCR analysis. The protein levels of COX-2 and iNOS wee measured by Western blot analysis. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with cells without treatment with lutein.

sion and synthesis of COX-2 and iNOS induced by LA in a dose-dependent manner. The inhibitory effect of lutein was achieved through blockade of NF-κB activation.

The association between specific types of dietary fatty acids and the development of AMD could be attributed to several mechanisms: (1) atherosclerosis of the blood vessels supplying the choroids and retina may increase the risk for AMD [26]. According to this hypothesis, the antithrombotic effects of unsaturated fatty acids could exert a beneficial effect on the vasculature of the choroid and thus may inversely relate to AMD. (2) Polyunsaturated fatty acid may increase the degree of unsaturation in the macula and thus increase the susceptibility to oxidative stress and AMD [27]. (3) ω-6 Fatty acids may compete with ω-3 fatty acids for shared enzymes to form eicosanoids [28]. Therefore, high intake of w-6 fatty acids such as LA may contribute to AMD.

In this study, we compared the causative effects of fatty acids based on the mechanism that fatty acids in the retina could directly stimulate RPE cells to express proinflammatory mediators that may subsequently induce CNV. We found that LA was the most potent 18-carbon fatty acid in inducing COX-2 and iNOS expression, which may contribute to the formation of CNV in AMD. This finding is consistent with epidemiological studies [5, 27] that showed that higher consumption of LA was associated with a higher risk for AMD. Our results may provide some evidence on the molecular biological level to support the significance of high LA intake in the pathogenesis of AMD.

The fatty acids used in this study have same carbon length but varying in degrees of saturation, which allows us to study the cellular effects of fatty acids that differs in saturation independent of carbon length. In addition, these fatty acids are the major dietary fatty acids consumed in the typical North European diet. SA (18:0) is the main saturated fatty acid, and is abundant in animal products including meat fat, milk fat, and chocolate. OA (18:1) is a monounsaturated fatty acid mainly found in non-animal sources of olive and canola oils, nuts, and avocados, and in animal sources such as beef and lamb. LA (18:2) is a ω-6 polyunsaturated fatty acid found mainly in vegetable oils, such as corn, soybean, sunflower, canola, and peanut. LnA (18:3) is a ω-3 polyunsaturated fatty acid found in flaxseed, canola, soybean, and walnut oils [29].

It is believed that COX-2 is involved in angiogenesis by directly modulating the expression of vascular endothelial growth factor ligand and receptor or by inducing the synthesis of PGE_2 to further stimulate the expression of proangiogenic factors [30]. Several *in vivo* animal studies have demonstrated that inhibition of the expression of COX-2 effectively suppresses the development of CNV, which is implied by the crucial roles of COX-2 in CNV in AMD [31]. Addi-

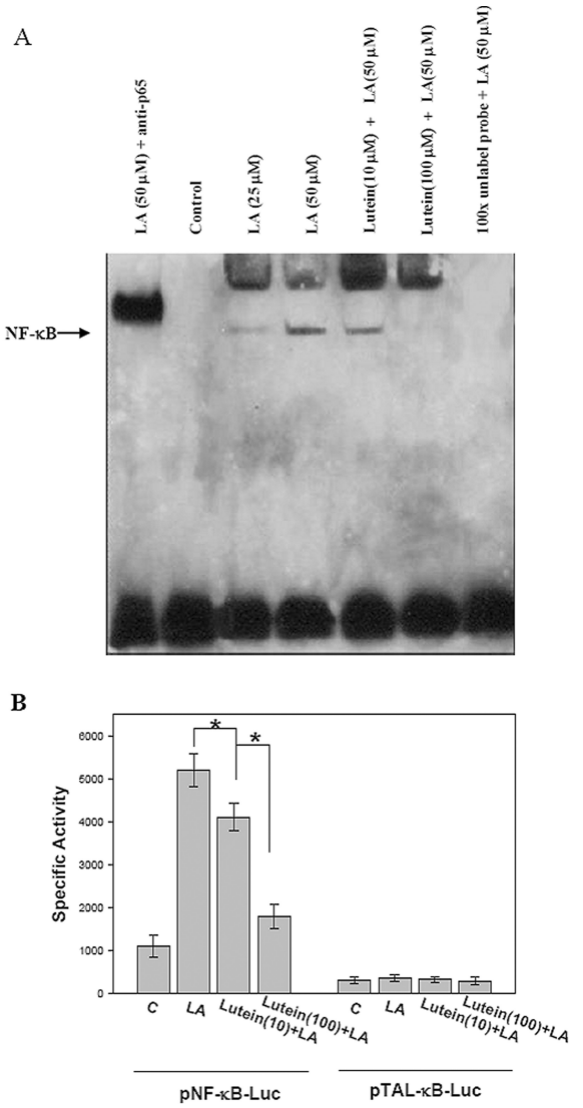

Figure 10. Inhibition of lutein on the NF-κB binding ability (A) and NF-κB-dependent transcriptional activity (B) in LA-stimulated ARPE-19 cells. ARPE-19 cells were pretreated with 10 or 100 μg/mL lutein for 24 h. After changing the medium, these cells subsequently incubated with 50 μM of LA for another 12 h. (A) Lane 1, supershift with an anti-p65 antibody; lane 2, control, cells treated with PBS only; lane 3, stimulation with LA (25 μM) alone; lane 4: stimulation with LA (50 μM) alone; lane 5, pretreatment with lutein (10 μg/mL) and then stimulation with LA (50 μM); lane 6, pretreatment with lutein (100 μg/mL) and then stimulation with LA (50 μM); lane 7, competition with 1006 unlabeled NF-κB probe. (B). The NF-κB-dependent transcriptional activity was measured by transient transfected ARPE-19 cells with an NF-κB-luciferase promoter construct or enhancerless construct (pTAL-luc), following by treating with 10 or 100 μg/mL lutein for 24 h and 50 μM LA for additional 12 h. The NF-κB-dependent transcriptional activity was determined using the luciferase assay (pNF-κB-Luc). Specific activity is expressed as units/microgram of protein. Data are expressed as the mean ± SEM of three independent experiments (bar graph). * $p < 0.05$ compared with cells treated with LA only.

tionally, the mechanisms of NO in angiogenesis appear to be related to the promotion of vasodilation and vascular permeability as well as to the vascular endothelial growth factor signaling pathway [32]. Immunohistochemical staining of surgically excised human CNV membrane revealed high expression of iNOS in RPE cells and macrophages [33], implicating iNOS in CNV formation. In this study, LA most markedly enhanced the production of iNOS and COX-2 in RPE, followed by NO and PGE_2, suggesting that LA is possibly the 18-carbon fatty acid most likely to induce AMD.

The present study demonstrated that unsaturated fatty acids, especially LA, could induce the expression of iNOS and COX-2 genes in RPE cells. Similar results have been shown in a mammary epithelial cell line in which unsaturated fatty acids, including LA, enhanced the expression of COX-2 [34]. Toborek *et al.* [35] reported that exposure of endothelial cells to LA, and to a lesser extent, LnA, stimulated the development of a proinflammatory environment within the vascular endothelium. In contrast, another study reported that saturated fatty acids induce the expression of COX-2 in macrophages, whereas unsaturated fatty acids inhibit it [36]. This discrepancy probably results from cell type-specific effects of unsaturated fatty acids on COX-2 and iNOS expression.

Transcription factor NF-κB has been shown to control the transcription of COX-2 and iNOS genes in many cell types [37, 38]. In this study, exposure of ARPE-19 cells to 18-carbon unsaturated fatty acids, including OA, LA, and LnA, resulted in increased NF-κB binding activity and enhanced NF-κB-dependent gene transcription. In addition, the NF-κB inhibitor PDTC effectively inhibited the induction of iNOS and COX genes, indicating that activation of NF-κB was involved in 18-carbon unsaturated fatty acid-induced expression of iNOS and COX-2 in ARPE-19 cells. NF-κB may be activated by numerous factors, including cytokine, mitogens, and lipopolysaccharide. In this study, pre-enrichment of cultures with the antioxidant vitamin E effectively reduced the activation of NF-κB induced by unsaturated fatty acids, indicating that oxidative stress may be responsible for unsaturated fatty acid-mediated activation of NF-κB. If, however, oxidative stress is the only factor involved in unsaturated fatty acid-induced activation of NF-κB, we would expect that a higher degree of unsaturated fatty acid would easily induce lipid peroxidation and thus induce a higher degree of oxidative stress and NF-κB activation [39]. Our results showed no relationship between the degree of unsaturation of fatty acids and activation of NF-κB. In fact, LA (18:2) appears to activate NF-κB more markedly than LnA (18:3). Similar observation was reported by Hennig *et al.* [40] who showed that LA induced the activation of endothelial cells more markedly than LnA. This finding indicated that increased lipid peroxidation alone is not sufficient to explain the fatty acid-induced activation of NF-κB. Recent evidence has demonstrated that

fatty acids can modulate the expression of COX-2 and iNOS genes through Toll-like receptors [41, 42]. Further studies are warranted to clarify the signaling pathways of fatty acid-induced expression of COX-2 and iNOS.

Lutein has become a popular nutritional supplement in AMD prevention and treatment. In the present study, we demonstrated that lutein inhibits NF-κB binding ability, which is related to the decrease in both the expression and synthesis of COX-2 and iNOS in ARPE-19 cells induced with LA. Recent studies by Jin *et al.* [43] have shown that lutein suppresses the activation of NF-κB as well as iNOS and COX-2 expression in lipopolysaccharide-induced RAW cells and in the iris-ciliary body of rats with endotoxin-induced uveitis. Lutein is a potent antioxidant that scavenges toxic oxygen species *in vitro* and protects against oxidant-induced damage in cultured liver cells [44, 45]. Because oxidative stress was, at least in part, responsible for LA activation of the COX-2 and iNOS genes, it is possible that the antioxidant property of lutein may account for the suppression of NF-κB and these inflammatory genes.

One noteworthy caveat of the present study is that we investigated the effects of dietary fatty acids individually added to cell cultures. *In vivo* situations are far more complex than this: many additional antioxidant and pro-oxidant compounds that are present *in vivo* could influence the effects of dietary fatty acids. Moreover, the cells are removed from their natural surroundings, resulting in difficulties in the estimation of the effect of local tissue factor on the metabolism of dietary fatty acids or on the expression of inflammatory genes in RPE. Despite these limitations, this study provided useful information about the effects of individual fatty acid on the expression of proinflammatory genes in RPE under careful control of the cell conditions.

In conclusion, the present study showed that unsaturated dietary fatty acids induced the expression of COX-2 and iNOS in RPE, with LA having a stronger stimulatory effect than OA or LnA. Lutein suppresses the expression of these genes induced by LA through the blockade of NF-κB activation. These data support the concept that modification of the diet by decreasing LA intake and taking vitamin/lutein supplements may be a useful prevention of AMD.

This research was funded by the National Science Council, Executive Yuan, Republic of China (NSC 96-2314-B532-005-MY3) and the Department of Health, Taipei City Government (95002-62-054).

The authors have declared no conflict of interest.

5 References

[1] Friedman, D.-S., O'Colmain, B.-J., Munoz, B., Tomany, S.-C. *et al.*, Prevalence of age-related macular degeneration in the United States. *Arch. Ophthalmol.* 2004, *122*, 564 – 572.

[2] Vingerling, J.-R., Dielemans, I., Hofman, A., Grobbee, D.-E. *et al.*, The prevalence of age-related maculopathy in the Roterdam Study. *Ophthalmology* 1995, *102*, 205 – 210.

[3] Chua, B., Flood, V., Rochtchina; E., Rochtchina, E. *et al.*, Dietary fatty acids and the 5-year incidence of age-related maculopathy. *Arch. Ophthalmol.* 2006, *124*, 981 – 986.

[4] SanGiovanni, J.-P., Chew, E.-Y., Clemons, T.-E., Davis, M.-D. *et al.*, Age-Related Eye Disease Study Research Group. The relationship of dietary lipid intake and age-related macular degeneration in a case-control study. AREDS Report No. 20. *Arch. Ophthalmol.* 2007, *125*, 671 – 679.

[5] Seddon, J.-M., Rosner, B., Sperduto, R.-D., Yannuzzi, L. *et al.*, Dietary fat and risk for advanced age-related macular degeneration. *Arch. Ophthalmol.* 2001, *119*, 1191 – 1199.

[6] Snow, K., Seddon, J., Do age-related macular degeneration and cardiovascular diseases share common antecedents? *Ophthalmic Epidemiol.* 1999, *6*, 125 – 143.

[7] Bok, D., Retinal photoreceptor-pigment epithelium interaction. *Invest. Ophthalmol. Vis. Sci.* 1985, *26*, 1659 – 1694.

[8] Su, H.-M., Keswich, L.-A., Brenna, J.-T., Increasing dietary linoleic acid in young rats increases and then decreases docosahexaenoic acid in retina but not in brain. *Lipids* 1996, *31*, 1289 – 1298.

[9] Chiou, G.-C., Review: Effects of Nitric oxide on eye diseases and their treatment. *J. Ocul. Pharmacol. Ther.* 2001, *17*, 189 – 198.

[10] Iniguez, M.-A., Rodriguez, A., Volpert, O.-V., Fresno, M., Redondo, J.-M., Cyclooxygenase-2: A therapeutic target in angiogenesis. *Trends Mol. Med.* 2003, *9*, 73 – 78.

[11] Hu, W., Criswell, M.-H., Ottlecz, A., Cornell, T.-L. *et al.*, Oral administration of lumiracoxib reduces choroidal neovascular membrane development in the rat laser-trauma model. *Retina* 2005, *25*, 1054 – 1064.

[12] Ando, A., Yang, A., Mori, K., Yamada, H. *et al.*, Nitric oxide is proangiogenic in the retina and choroids. *J. Cell. Physiol.* 2002, *191*, 116 – 124.

[13] Tapiero, H., Ba, G.-N., Couvreur, P., Tew, K.-D., Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Biomed. Pharmacother.* 2002, *56*, 215 – 222.

[14] SanGiovanni, J.-P., Chew, E.-T., The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. *Prog. Retin. Eye Res.* 2005, *24*, 87 – 138.

[15] Larsson, S., Kumlin, M., Ingelman-Sunberg, M., Wolk, A., Dietary long-chain n-3 fatty acids for prevention of cancer: a review of potential mechanisms. *Am. J. Clin. Nutr.* 2004, *79*, 935 – 945.

[16] Tamai, K., Spaide, R.-F., Ellis, E.-A., Iwabuchi, S. *et al.*, Lipid hydroperoxide stimulates subretinal choroidal neovascularization in the rabbit. *Exp. Eye Res.* 2002, *74*, 301 – 308.

[17] Fang, I-M., Yang, C.-H., Yang, C.-M., Chen, M.-S., Linoleic acid-induced expression of inducible nitric oxide synthase and cyclooxygenase II *via* p42/44 mitogen-activated protein kinase and nuclear factor-κB pathway in retinal pigment epithelial cells. *Exp. Eye Res.* 2007, *85*, 667 – 677.

[18] Ahmed, S.-S., Lott, M.-N., Marcus, D-M., The macular xanthophylls. *Surv. Ophthalmol.* 2005, *50*, 183 – 193.

[19] Coleman, H., Chew, E., Nutritional supplementation in age-related macular degeneration. *Curr. Opin. Ophthalmol.* 2007, *18*, 220 – 223.

[20] Seddon, J.-M., Multivitamin-multimineral supplements and eye disease: Age-related macular degeneration and cataract. *Am. J. Clin. Nutr.* 2007, *85*, 304S – 307S.

[21] Junghans, A., Sies, H., Stahl, W., Macular pigments lutein and zeaxanthin as blue light filters studied in liposomes. *Arch. Biochem. Biophys.* 2001, *391*, 160 – 164.

[22] Lee, E.-H., Faulhaber, D., Hanson, K.-M., Ding, W. *et al.*, Dietary lutein reduces ultraviolet radiation-induced inflammation and immunosuppression. *J. Invest. Dermatol.* 2004, *122*, 510 – 517.

[23] Siddiqui, R-A., Jenski, L.-J., Neff, K., Harvey, K. *et al.*, Docosahexaenoic acid induces apoptosis in Jurkat cells by a protein phosphatase-mediated process. *Biochim. Biophys. Acta* 2001, *1499*, 265 – 275.

[24] Bligh, E.-G., Dyer, W.-J., A rapid method of total lipid extraction and purification. *Can. J. Biochem. Biophys.* 1959, *37*, 911 – 917.

[25] Fang, I.-M., Yang, C.-H., Lin, C.-P., Yang, C.-M., Chen, M.-S., Expression of chemokine and receptors in Lewis rats with experimental autoimmune anterior uveitis. *Exp. Eye Res.* 2004, *78*, 1043 – 1055.

[26] Cho, E., Hung, S., Walter, W.-C., Siegelman, D. *et al.*, Prospective study of dietary fat and the risk of age-related macular degeneration. *Am. J. Clin. Nutr.* 2001, *73*, 209 – 218.

[27] Seddon, J.-M., Cotc, J., Rosner, B., Progression of age-related macular degeneration-association with dietary fat, transunsaturated fat, nuts and fish intake. *Arch. Ophthalmol.* 2003, *121*, 1728 – 1737.

[28] Escrich, E., Moral, R., Grau, L., Costa, I., Solanas, M., Molecular mechansims of the effects of olive oil and other dietary lipids on cancer. *Mol. Nutr. Food Res.* 2007, *51*, 1279 – 1292.

[29] Chong, E.-W., Sinclair, A. J., Guymer, R. H., Facts on fats. *Clin. Exp. Ophthalmol.* 2006, *34*, 464 – 471.

[30] Sköld, M., Culheim, S., Hammarberg, H., Piehl, F. *et al.*, Induction of VEGF and VEGF receptors in the spinal cord after mechanical spinal injury and prostaglandin administration. *Eur. J. Neurosci.* 2000, *12*, 3675 – 3686.

[31] Takahashi, H., Yanagi, Y., Tamaki, Y., Uchida, S., Muranaka, K., COX-2-selective inhibitor, etodolac, suppresses choroidal neovascularization in a mice model. *Biochem. Biophys. Res. Commun.* 2004, *325*, 461 – 466.

[32] Fischer, S., Clauss, M., Wiesnet, M., Dieter, R. *et al.*, Hypoxia induces permeability in brain microvessel endothelial cells *via* VEGF and NO. *Am. J. Physiol.* 1999, *276*, C812 – C820.

[33] Hattenbach, L.-O., Falk, B., Nurnberger, F., Koch, F.-H., Ohrloff, C., Detection of inducible nitric oxide synthase and vascular endothelial growth factor in choroidal neovascular membranes. *Ophthalmologica* 2002, *216*, 209 – 214.

[34] Meade, E.-A., McIntyre, T.-M., Zimmerman, G.-A., Prescott, S.-M., Peroxisome perliferators enhance cyclooxygenase-2 expression in epithelial cells. *J. Biol. Chem.* 1999, *274*, 8328 – 8334.

[35] Toborek, M., Lee, Y.-W., Garrido, R., Kaiser, S., Hennig, B., Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells. *Am. J. Clin. Nutr.* 2002, *75*, 119 – 125.

[36] Lee, J.-Y., Sohn, K.-H., Rehh, S.-H., Hwang, D., Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J. Biol. Chem.* 2001, *276*, 16683 – 16689.

[37] Chen, C.-J., Ou, Y.-C., Lin, S.-Y., Liao, S.-L., Chen, S.-Y., Manganese modulates pro-inflammatory gene expression in activated glia. *Neurochem. Int.* 2006, *49*, 62 – 71.

[38] Hsieh, H.-L., Wu, C.-B., Sun, C.-C., Liao, C.-H. *et al.*, Sphingosine-1- phosphate induces COX-2 expression *via* PI3K/Akt and p42/p44 MAPK pathways in rat vascular smooth muscle cells. *J. Cell. Physiol.* 2006, *207*, 757 – 766.

[39] Dontsov, A.-E., Glickman, R.-D., Ostrovsky, M.-A., Retinal pigment epithelium pigment granules stimulate the photooxidation of unsaturated fatty acids. *Free Radic. Biol. Med.* 1999, *26*, 1436 – 1446.

[40] Hennig, B., Meerarani, P., Ramadass, P., Watkins, B.-A., Toborek, M., Fatty acid-mediated activation of vascular endothelial cells. *Metabolism* 2000, *49*, 1006 – 1013.

[41] Lee, J.-Y., Hwang, D.-H., The modulation of inflammatory gene expression by lipids: Mediation through toll-like receptors. *Mol. Cells* 2006, *21*, 174 – 185.

[42] Lee, J.-Y., Plakidas, A., Lee, W.-H., Heikkinen, A. *et al.*, Differential modulation of toll-like receptors by fatty acids: Preferential inhibition by n-3 polyunsaturated fatty acids. *J. Lipid Res.* 2003, *44*, 479 – 486.

[43] Jin, X.-H., Ohgami, K., Shiratori, K., Suzuki, Y. *et al.*, Inhibitory effects of lutein on endotoxin-induced uveitis in Lewis rats. *Invest. Ophthalmol. Vis. Sci.* 2006, *47*, 2562 – 2568.

[44] Chopra, M., Wilson, R.-L., Thurnham, D.-I., Free radical scavenging of lutein *in vitro*. *Ann. N. Y. Acad. Sci.* 1993, *691*, 246 – 249.

[45] Martin, K.-R., Failla, M.-L., Smith, J.-C. Jr., Beta-carotene and lutein protect HepG2 human liver cells against oxidant-induced damage. *J. Nutr.* 1996, *126*, 2098 – 2106.

