Research Article

Dietary lutein modulates inducible nitric oxide synthase (iNOS) gene and protein expression in mouse macrophage cells (RAW 264.7)

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Lutein is an oxycarotenoid primarily found in dark-green leafy vegetables such as spinach and kale. Other dietary sources which contain moderate amounts of lutein include corn, egg yolks, and fruits like oranges and kiwi. Although a number of *in vivo* studies have demonstrated the anti-inflammatory effect of lutein, its *in vitro* anti-inflammatory molecular mechanism of action is unknown. In this study, we have investigated the *in vitro* anti-inflammatory effect of lutein using LPS-stimulated mouse macrophage cell line (RAW 264.7). The inhibition of LPS-stimulated nitric oxide (NO) was measured and the expression of inducible NO synthase (iNOS) was assessed at the mRNA and protein levels in mouse macrophage cells after treatment with lutein. Lutein decreased the LPS-induced NO production by 50% compared to LPS alone. Real-time PCR analysis showed a 1.9-fold reduction in iNOS expression at the mRNA level. Western blotting revealed that lutein decreased LPS-induced iNOS expression at the protein level by 72.5%. The results of this study suggest the anti-inflammatory properties of lutein demonstrated by the decrease in the expression of iNOS at the mRNA and protein levels in RAW 264.7 mouse macrophage cells.

Keywords: Anti-inflammatory / iNOS / Lutein / Macrophage / Nitric oxide

Received: September 8, 2006; revised: November 27, 2006; accepted: November 29, 2006

1 Introduction

Carotenoids are plant pigments that contribute to the color of a variety of fruits and vegetables [1]. Lutein ((3R,3′R, 6′R)-β,ε-carotene-3,3′-diol) is an oxycarotenoid that belongs to the xanthophyll family of carotenoids [2, 3] and is found in several dark-green leafy vegetables such as kale and spinach [1, 4] (Fig. 1). Other dietary sources which contain moderate amounts of lutein include corn, egg yolks, broccoli, water cress, fresh parsley, green peas, and fruits like orange and kiwi [1, 2].

Lutein and zeaxanthin are not synthesized by the human body and therefore must be obtained by the consumption of foods containing sufficient amounts of lutein and zeaxanthin [2, 5]. Both these xanthophylls are distributed in different tissues of the human body. The highest concentration of lutein and zeaxanthin has been reported in the ocular tissue especially in the macular region of the retina [6, 7]. It has been found that liver, adrenal, adipose, pancreas, kidney, and breast tissues also contain high concentrations of lutein [6].

Several epidemiological studies have shown that there is an inverse association between the intake of vegetables that are high in lutein and the incidence of ocular diseases including age-related macular degeneration (AMD) and cataracts [8 – 10]. The anti-inflammatory activity of lutein has been demonstrated in a number of *in vivo* studies. The *in vivo* anti-inflammatory and immunosuppression properties of lutein were reported in a study conducted by Lee *et al*. [11] where a lutein enriched diet reduced UV radiation-induced inflammation in the ears of mice. In another study, Gonzalez *et al*. [12] have shown that ultraviolet B (UVB)-induced skin inflammation in the ears of mice was suppressed with a diet supplemented with lutein and zeaxanthin.

LPS, a bacterial endotoxin found in the cell wall of Gram negative bacteria, plays a major role in the onset of an

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Abbreviations: iNOS, inducible nitric oxide synthase; **MTT,** 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide; **NO,** nitric oxide

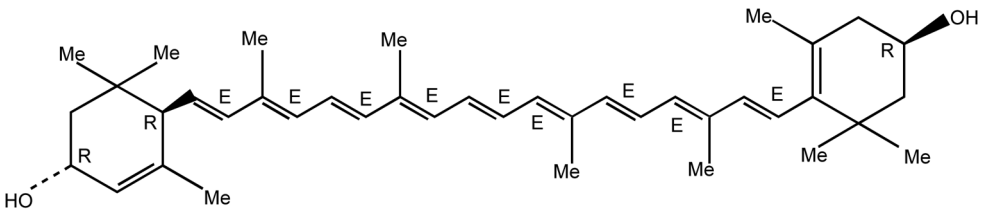

Figure 1. The chemical structure of lutein.

inflammatory response by the activation of immune cells. LPS activates monocytes and macrophages of the immune system by forming a complex with LPS-binding protein, which binds to the membrane glycophosphatidylinositol-anchored protein CD14. Activation of monocytes and macrophages by LPS leads to the activation of protein kinases, subsequently activating signaling pathways, resulting in the expression of the inflammatory gene inducible nitric oxide synthase (iNOS) [13].

The LPS-induced nitric oxide (NO) response in macrophages is an important aspect in inflammation. NO is synthesized from L-arginine by iNOS as a result of the activation of immune cells in response to LPS and cytokines. NO is a short-lived biological mediator involved in various physiological and pathophysiological processes in the human body [14]. NO also plays a role in neurotransmission, vasodilation, and in modulating the immune response [14]. The production of iNOS by macrophage cells plays a role in the onset of a number of acute and chronic inflammatory diseases [15]. iNOS produces NO at high concentrations during inflammatory response and results in the activation of a number of transcription factors such as the NF-κB pathway [16]. This prolonged expression of NO ultimately results in the increased expression of pro-inflammatory genes iNOS and cyclooxygenase-2 (COX-2) [17]. High concentrations of NO have been shown to cause DNA damage *in vitro* and *in vivo* that can ultimately lead to genetic diseases and cancer [18]. Therefore, inhibiting NO production by blocking iNOS activity at the mRNA and protein levels may be a useful strategy for the treatment of chronic inflammatory disorders.

Many studies have also investigated the *in vitro* anti-inflammatory effect of different nutraceuticals such as β-carotene, genistein, apigenin, quercetin, wogonin, luteolin, and epigallocatechin gallate (EGCG) [19–23]. Although a number of *in vivo* studies have demonstrated the anti-inflammatory effect of lutein [11, 12], its *in vitro* anti-inflammatory molecular mechanism of action is unknown. Therefore, in this study we investigated the *in vitro* anti-inflammatory molecular mechanism of action of dietary lutein using mouse macrophage cell line (RAW 264.7).

2 Materials and methods

2.1 Reagents and cells

RAW 264.7 mouse macrophage cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) and held at 37°C with 7.5% carbon dioxide. DMEM, LPS, Tri-Reagent, Griess reagent, monoclonal anti-β-actin antibody, lutein, and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The mouse monoclonal anti-iNOS antibody was purchased from BD Biosciences Pharmingen (San Diego, CA, USA), and anti-mouse IgG immunoglobulin/HRP was purchased from DAKO (Carpinteria, CA, USA). RAW 264.7 mouse macrophage cells were treated with LPS alone (positive control) or together with various concentrations of lutein. Lutein (97% purity, Sigma-Aldrich) was dissolved in ethanol to prepare a stock solution of 100 mM which was further diluted to make various concentrations of lutein (1.25–10 mM) and cells were incubated at 37°C for 18 h in 10 mL of DMEM media to reach a final concentration of 1.25–10 μM.

2.2 MTT assay for cell viability

MTT is a pale yellow substrate that is reduced by the living cells to yield a dark blue formazan product. This process requires active mitochondria and even freshly dead cells do not reduce significant amounts of MTT. RAW 264.7 mouse macrophage cells (2.5×10^5) were cultured in a 96-well flat-bottom plate for 12 h and subsequently treated with various concentrations of lutein (1.25–30 μM) for 18 h. Thereafter, the culture medium was aspirated and 100 μL of MTT dye (1 mg/mL in PBS) was added into each well and incubated at 37°C for 4 h. Acidified isopropanol (0.1 N HCl) was added to each well to solubilize the blue formazan crystals [24]. The index of cell viability was determined by measuring the OD of color produced by MTT dye reduction at 570 nm and compared with that of the untreated control cells.

2.3 NO measurement

RAW 264.7 mouse macrophage cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) and held at 37°C with 7.5% carbon dioxide in a 96-well flat-bottom tissue culture plate for 12 h. Cells were then treated with LPS (1 μg/mL) alone (positive control) or LPS with various concentrations of lutein (1.25 – 10 μM) for 18 h. The cell supernatants were collected at the end of the culture for nitrite, which were used as a measure of NO production [25]. Equal volumes of Griess reagent (100 μL; Sigma-Aldrich) were added to each cell supernatant (100 μL), and the absorbance was measured at 570 nm. The concentration of nitrite (μM) was calculated from a standard curve drawn with a known concentration of sodium nitrite dissolved in DMEM. The results are presented as the mean ± SD of 4 replicates of one representative experiment and this experiment has been repeated three times with similar results.

2.4 Real-time PCR

Quantitative gene expression of iNOS was performed on the iCycler MYIQ real-time PCR detection system (BioRad, Hercules, CA, USA) using one step Real-Time PCR (Applied Biosystems, Foster City, CA, USA). Total RNA was isolated using Tri-reagent (Sigma-Aldrich). Primers and probes (Taqman™) for iNOS were purchased from Applied Biosystems. TaqMan reaction master mix was prepared using 2 μL of RNA (100 ng/μL), 19.25 μL of PCR-grade RNase-DNase free water, 25 μL of TaqMan™ One-step RT-PCR Master Mix (Applied Biosystems), 2.5 μL of TaqMan primers and probes, and 1.25 μL of TaqMan enzyme with a total volume of 50 μL. Amplification was carried out with the following parameters: one cycle at 55°C for 15 min, one cycle at 95°C for 3 min, and 40 cycles at 60°C for 30 s each. The expression of iNOS genes after treatment with lutein (10 μM) compared to positive control (LPS only) was estimated using the comparative C_T method ($\Delta\Delta C_T$) [26]. The threshold cycles (C_T) for iNOS and β-actin were determined for lutein (10 μM) and LPS treated samples. In this experiment, a comparison was made between the expression levels of iNOS and β-actin in the lutein (10 μM) treated and LPS samples. The mean C_T value of each sample was calculated and SDs were determined for each mean C_T value. The ΔC_T values were calculated by subtracting the average C_T value of β-actin from the average C_T value of iNOS for each sample. The $\Delta\Delta C_T$ value is then calculated by subtracting the ΔC_T of lutein (10 μM) from the ΔC_T value of LPS alone using manufacturer's instructions (Applied Biosystems).

The relative expressions of the target gene (iNOS) in the lutein (10 μM) treated and LPS treated samples were calculated using $2^{-\Delta\Delta C_T}$. The values of treated samples were expressed as *n*-fold difference relative to the expression of control samples (Applied Biosystems).

2.5 Preparation of total protein lysate for immunoblot

The RAW 264.7 mouse macrophage cells were cultured with LPS alone or with various concentrations of lutein (1.25 – 10 μM) for 18 h. At the end of the incubation period, the cells were washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 5 mM PMSF, 2 mM sodium orthovanadate, 10 μg/mL of leupeptin, 25 μg/mL of aprotinin, 1 mM sodium pyrophosphate, and 20% glycerol. After incubation for 30 min on ice, lysates were centrifuged (12 500 rpm, 15 min) and supernatants were collected and protein concentration in samples was estimated by BioRad protein assay reagent (BioRad), following manufacturer's instructions.

2.6 Immunoblot (Western blot)

Equal amounts of protein (100 μg) from each sample (control and treatment) were resolved on an SDS-polyacrylamide electrophoresis gel (7.5% separating gel) for 1 h. The proteins were then transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was then blocked in blocking buffer containing 20 mM sodium phosphate buffer, pH 7.6, 150 mM NaCl, 0.1% Tween-20, and 5% nonfat dry milk for 1 h at room temperature. Thereafter, the membrane was incubated with primary iNOS antibody at 4°C overnight. The membrane was then washed every 10 min for 40 min with PBS–Tween-20. Afterwards, the membrane was incubated with the secondary antibody at room temperature for 1 h. Specific bands were detected using enhanced chemiluminescence detection system (Amersham Biosciences) by exposing the membrane to X-ray film [24]. The density of the protein bands were analyzed using BioRad Quantity One® 1-D Analysis Software (Molecular Imager ChemiDoc XRS System, BioRad, Richmond, CA, USA).

2.7 Statistical analysis

Data were expressed as mean ± SD of indicated experiments. Statistical significance between two groups was determined by Student's *t*-test. The significance level was set at $p < 0.05$.

3 Results

3.1 Inhibition of LPS-induced NO production from RAW 264.7 mouse macrophage cells by lutein

LPS was used to stimulate NO production in RAW 264.7 mouse macrophage cells. Since NO has a very short half-life, the concentration of nitrite was considered as an indicator of NO production. Griess reagent was used to determine the concentration of nitrites (μM) in cell supernatants

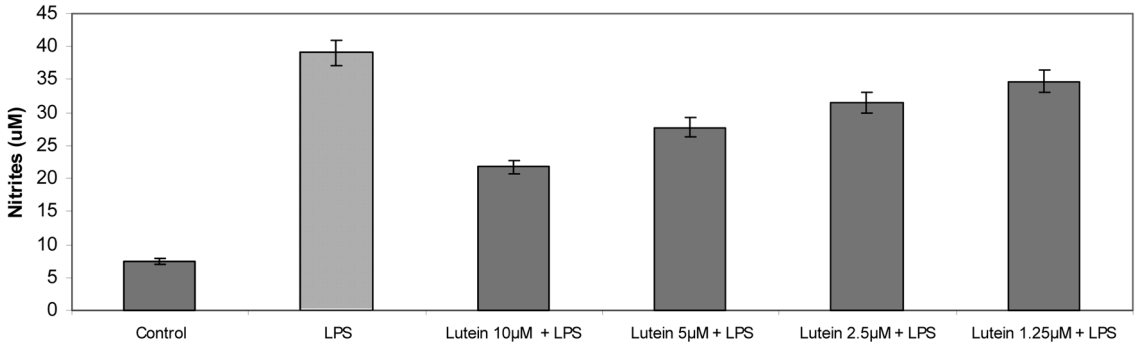

Figure 2. Effect of lutein on NO production. RAW 264.7 mouse macrophage cells were treated with 1 μg/mL of LPS alone (positive control) or with various concentrations of lutein (1.25–10 μM) with LPS for 18 h. The concentration of nitrite in the supernatant was measured using Griess reagent. Each bar represents mean ± SD of four replicates of one representative experiment. This experiment has been repeated three times with similar results. *Represents statistical significance of the difference between LPS (positive control) and lutein groups + LPS (*$p < 0.05$).

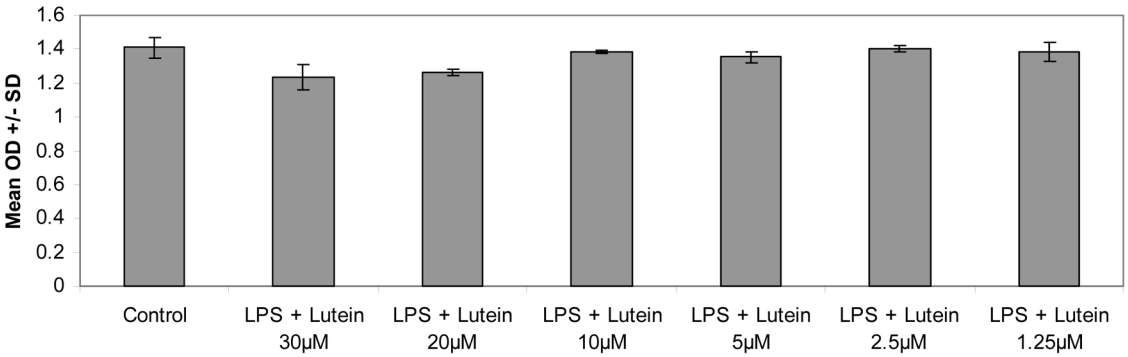

Figure 3. Effect of lutein on RAW 264.7 mouse macrophage cell viability. RAW 264.7 mouse macrophage cells were treated with various concentrations of lutein (1.25–30 μM) for 18 h and cell viability assay was performed using MTT dye, as mentioned in Section 2. Each bar represents mean ± SD of four replicates of one representative experiment. This experiment has been repeated three times with similar results.

after 18 h of treatment with LPS (1 μg/mL) alone (positive control) or with different concentrations of lutein. Upon treatment with lutein (1.25–30 μM), the nitrite production by RAW 264.7 mouse macrophage cells was inhibited in a dose-dependent manner. LPS-induced production of NO is significantly reduced after treatment with lutein at the 10 μM concentration ($p < 0.05$; Fig. 2).

3.2 Effect of lutein 10 μM on RAW 264.7 cell viability

To determine if lutein is cytotoxic to the cells, RAW 264.7 mouse macrophage cells were treated with various concentrations of lutein (1.25–30 μM) for 18 h, and cell viability assay was performed using MTT dye. It was observed that lutein displayed a dose-dependent response in cell viability and lutein at 10 μM was observed to be the highest nontoxic dose. Treatments at 1.25, 2.5, and 5 μM showed no significant decrease in cell viability. However, treatments with concentrations higher than 10 μM (20 and 30 μM) showed more than 20% decrease in the viability of cells in comparison with control cells (Fig. 3).

3.3 Inhibition of LPS-induced iNOS gene expression by lutein

In order to investigate whether the decrease in NO production is due to a down-regulation of iNOS gene expression at the mRNA level a real-time PCR assay was carried out. Extracted RNA was reverse transcribed to yield cDNA followed by sequential PCR reactions, as described in Section 2. The real-time PCR results indicated that lutein (10 μM) down-regulates the expression of LPS-induced iNOS at the mRNA level by 1.9-fold (47% down-regulation in gene expression) (Fig. 4). β-Actin was used as the housekeeping gene that had a constant expression for the lutein treated and LPS treated (positive control) RNA samples.

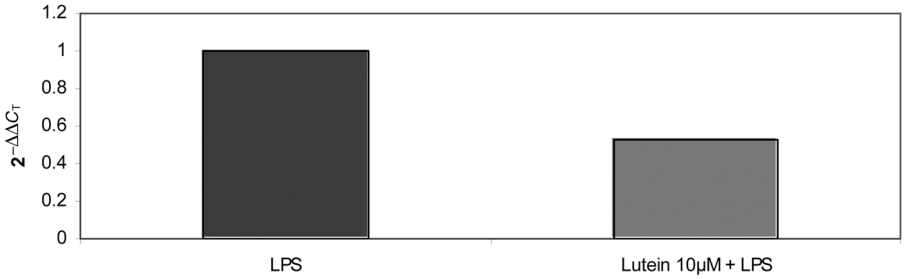

Figure 4. Effect of lutein (10 μM) on iNOS mRNA expression detected by real-time PCR. RAW 264.7 mouse macrophage cells were treated with 1 μg/mL of LPS alone (positive control) or with lutein (10 μM) for 18 h. Quantitative gene expression of iNOS was performed on the iCycler MYIQ real-time PCR detection system (BioRad) using one step real-time PCR (Applied Biosystems). The relative expressions of the target gene (iNOS) in the lutein (10 μM) treated and LPS treated samples were calculated using $2^{-\Delta\Delta C_T}$, according to the manufacturer's instructions (Applied Biosystems). The values of treated samples were expressed as *n*-fold difference relative to the expression of control samples.

3.4 Inhibition of LPS-induced iNOS protein expression by lutein (10 μM)

In order to investigate whether the inhibition of NO production was due to a decreased protein expression of iNOS, the effect of lutein on iNOS protein expression was studied by immunoblot. Equal amounts of protein (100 μg) were resolved in SDS-PAGE and then transferred to a nitrocellulose membrane and the expression of iNOS was then detected using specific antibodies. The results showed that incubation with lutein (10 μM) after 18 h inhibited iNOS protein expression in RAW 264.7 mouse macrophage cells. Treatment with 5 μM lutein for 18 h also showed a down-regulation in iNOS protein expression. However, the results indicate that lutein decreases the expression of iNOS at the protein level in a dose-dependent manner. The detection of β-actin was also performed in the same blot as an internal control. The intensity of protein bands were analyzed using BioRad Quantity One software (Molecular Imager ChemiDoc XRS System, BioRad) in three independent experiments and showed an average of 72.5% down-regulation of iNOS protein after treatment with lutein 10 μM compared to positive control (LPS) (Fig. 5).

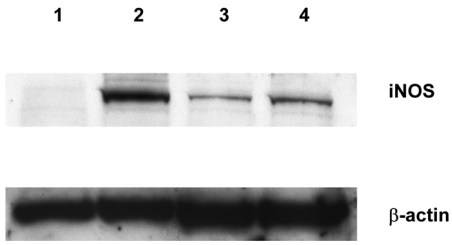

Figure 5. Inhibition of LPS-induced iNOS protein expression by lutein detected by immunoblot. RAW 264.7 mouse macrophage cells were treated with 1 μg/mL of LPS alone (positive control) or with lutein (5 – 10 μM) for 18 h. Western blot analysis was performed by resolving 100 μg of the total cell lysates in 8% SDS-PAGE and then transferred to a nitrocellulose membrane and protein was detected with specific antibodies, as mentioned in Section 2. This experiment was repeated twice with similar results. The detection of β-actin was done in the same blot as control. Lane 1, control (without any treatment); Lane 2, LPS (1 μg/mL); Lane 3, lutein (10 μM) + LPS; and Lane 4, lutein (5 μM) + LPS. The density of the protein bands were analyzed using BioRad Quantity One 1-D Analysis Software (Molecular Imager ChemiDoc XRS System, BioRad).

4 Discussion

Lutein is an oxycarotenoid that belongs to the xanthophyll family of carotenoids and is found primarly in dark-green leafy vegetables such as spinach and kale [1, 2, 4]. Lutein is not synthesized by the human body and therefore must be consumed in the diet [2, 5]. The consumption of lutein is considered safe as stated by the FDA who has recognized lutein as one of the GRAS (generally recognized as safe) supplements for food applications [27].

Carotenoids have been implicated to be important dietary nutrients due to their antioxidant activity that protects against oxidative damage by inactivating the reactive oxygen species (ROS) [3]. Carotenoids are involved in scavenging singlet oxygen species and peroxyradicals and this effect has been shown to be associated with the presence of conjugated double bonds in carotenoid molecules [9]. Wang *et al*. [28] have investigated the antioxidant activity of lutein by photochemiluminescence (PCL) assay and the β-carotene-linoleic acid model system (B-CLAMS). Lutein was reported as a more potent antioxidant than β-carotene and lycopene.

Chronic inflammation is a complex process and involves the production of a number of proinflammatory mediators

such as NO and cytokines that are involved in the development of inflammatory human diseases [29, 30]. Nuclear factor kappa B (NF-κB) is a transcription factor that is involved in the activation of several chronic inflammatory diseases [31, 32]. Bai *et al.* [20] have investigated the anti-inflammatory properties of β-carotene and it was demonstrated that treatment of LPS-induced mouse macrophage cells (RAW 264.7) with β-carotene inhibited the production of NO, prostaglandins, and cytokines as well as the expression of inflammatory genes such as iNOS and COX-2 by suppressing the activation of transcription factor NF-κB.

Lutein has been shown as an anti-inflammatory compound in several *in vivo* studies. The *in vivo* anti-inflammatory and immunosuppression properties of lutein were reported in a study conducted by Lee *et al*. [11] where a lutein enriched diet reduced UV radiation-induced inflammation in the ears of mice. In another study, Gonzalez *et al*. [12] have shown that UVB-induced skin inflammation in the ears of mice was suppressed with a diet supplemented with lutein and zeaxanthin.

LPS activates macrophages that play a role in the early immune response in reaction to tissue injury and the presence of invaders such as bacterial endotoxin [13]. In this study, LPS was used to induce an inflammatory response in RAW 264.7 mouse macrophage cells, followed by incubation with lutein. NO production was measured as the proinflammatory mediator involved during inflammatory response with LPS. We have demonstrated that lutein (10 μM) inhibited the production of NO in a dose-dependent manner (Fig. 2). Our findings are consistent with studies on the effect of β-carotene on the inhibition of NO production studied by Bai *et al.* [20]. Their results also indicate the potential anti-inflammatory effect of β-carotene as demonstrated by an inhibition of NO production in a dose-dependent manner. However, the IC_{50} for the inhibition of NO by β-carotene was 30 μM [20] in contrast to our current study which indicates a higher IC_{50} value with lutein (data not shown). Our study suggests that lutein may be a more potent anti-inflammatory molecule compared to β-carotene. Figure 3 clearly indicates that the 1.25 to 10 μM concentrations of lutein did not inhibit cell viability. However, 20 and 30 μM lutein concentrations did exhibit a significant decrease in cell viability. Therefore, a 10 μM lutein concentration was found to be the highest nontoxic dose and was selected for further studies.

NO is implicated in many physiological and pathological conditions. NO production was studied in patients with rheumatoid arthritis (RA) and it was found that NO production was markedly increased in RA patients compared to healthy controls [33]. Furthermore, increased iNOS expression has been demonstrated in ulcerative colitis patient samples [34] and in a variety of human malignant tumors including breast [35], lung [36], prostate [37], bladder [38], colorectal [39], and melanoma [40]. High expression of iNOS was observed in each of these tumors compared to the adjacent normal tissue [41]. Therefore, inhibiting the overexpression of iNOS may serve as a therapeutic target for preventing chronic inflammatory disorders.

Much attention has been drawn to the anti-inflammatory effect of different nutraceuticals. We have chosen to investigate the anti-inflammatory potential of lutein in LPS-induced NO production using a mouse macrophage cell line (RAW 264.7). Several studies using mouse macrophage cells (RAW 264.7) have investigated the *in vitro* anti-inflammatory properties of nutraceuticals including several flavonoids such as apigenin, wogonin, luteolin, tectorigenin, quercetin [21], (−)-epigallocatechin-3-gallate (EGCG), apigenin, genistein, kaempferol [22], and garcinol [42]. In each of these studies the expression of iNOS was reduced, suggesting a decrease in NO production and the potential use of these nutraceuticals as natural anti-inflammatory compounds. Further research to determine whether nonprovitamin A carotenoids like lutein may also provide therapeutic benefits in the suppression and prevention of inflammatory diseases is warranted.

Since lutein demonstrated the inhibition of LPS-induced NO production in mouse macrophage cells (RAW 264.7), we further investigated whether the inhibition of NO production was at the mRNA or at the protein level. The real-time PCR results indicated that the incubation of cells with lutein down-regulated the expression of LPS-induced iNOS at the mRNA level (Fig. 4). This finding correlates with the findings of Bai *et al.* [20] where β-carotene was shown to reduce the expression of iNOS at the mRNA level. Furthermore, we investigated whether the inhibition of iNOS was also exhibited at the protein level. Our results indicate that the incubation of mouse macrophage cells (RAW 264.7) with lutein (10 μM) decreased the expression of iNOS protein in a dose-dependent manner (Fig. 5). Bai *et al.* [20] have also shown that β-carotene inhibits iNOS protein expression in LPS-induced mouse macrophage cells using immunoblot.

The data from this study suggest the anti-inflammatory properties of lutein demonstrated by the decrease in the expression of iNOS at the mRNA and protein levels in RAW 264.7 mouse macrophage cells. More extensive studies are currently in progress to further establish how lutein inhibits iNOS expression at the mRNA and protein levels by possibly modulating signaling cascades and transcription factors.

We are grateful to Professor Paul Lachance and Marynell Reyes for reading and editing the manuscript. This work was supported by the New Jersey Agricultural Experiment Stations Hatch Project (Cook College, Rutgers, The State University of New Jersey) and the Center for Advanced Food Technology (New Brunswick, New Jersey).

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