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Synergistic anti-inflammatory effects of low doses of curcumin in combination with polyunsaturated fatty acids: Docosahexaenoic acid or eicosapentaenoic acid

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ABSTRACT

Inflammatory response plays an important role not only in the normal physiology but also in the pathology such as cancers. As chronic inflammations are associated with malignancies, it is important to prevent inflammation-mediated neoplastic formation, promotion and/or progression. One possible intervention will be using cancer chemopreventive agents such as curcumin (CUR), a potent anti-inflammatory and anti-oxidative stress compound. Polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) are potent anti-inflammatory agents by decreasing the production of inflammatory eicosanoids, cytokines, and reactive oxygen species (ROS). The present study aims at examining whether CUR with DHA or EPA would have synergistic anti-inflammatory effects in RAW 264.7 cells. Non-toxic concentrations of single and combination of the compounds were investigated at 6, 12 and 24 h. The nitric oxide (NO) suppression effects were most prominent at 24 h. All the combinations of CUR and DHA or EPA with lower concentrations of CUR 5 μM and 25 μM of DHA or EPA were found to have synergistic effects in suppressing LPS-stimulated NO and endogenous NO levels. Importantly, very low doses of CUR 2.5 μM and DHA or EPA of 0.78 μM could synergistically suppress the LPS-induced prostaglandin E_2 (PGE_2). The combinations were also found to suppress iNOS, COX-2, 5-lipoxygenase (5-LOX) and $cPLA_2$ but induce HO-1. Taken together, the present study clearly shows the synergistic anti-inflammatory as well as anti-oxidative stress effects of CUR and PUFA.

1. Introduction

Inflammatory response plays a very important role not only in the normal physiology but also in the pathology such as cancers [1]. As chronic inflammations are associated with malignancies, it is important to prevent the inflammation before neoplasm formation, promotion and/or progression. One way of doing so is by the use of chemopreventive agents such as curcumin (CUR), a constituent of turmeric of ginger family, which is a potent anti-inflammatory [2] via inhibiting nuclear factor-kappa-B (NF-κB) pathway [3–5], induction of heat shock response [6] as well as activation of nuclear factor-erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway [7]. It is well-recognized that CUR activates Nrf2/ARE signaling pathway leading to induction of phase II genes such as glutathione S-transferase (GST) and hemeoxygenase-1 (HO-1) [8]. Recently, the effects of CUR in cancers management have been tested in clinical trials and the benefits have been reported [9–12]. Polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) are potentially potent anti-inflammatory agents by decreasing the production of inflammatory eicosanoids, cytokines, and reactive oxygen species (ROS) [13,14]. The benefits of the PUFA have also been demonstrated in clinical trials in cancer patients [15,16] and patients with ulcerative colitis [17]. Thus, it is of interest and clinical significance to examine the protective anti-inflammatory effects of CUR alone and in combination with PUFA, DHA or EPA.

The potential benefits of using combination of different chemopreventive compounds could be quite obvious. It is hopeful that a greater chemopreventive effect could be achieved by compounds targeting different signaling mechanisms [18,19]. In this context, with our present study, we hypothesized that when

Abbreviations: CUR, curcumin; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acids; Nrf2, nuclear factor-erythroid 2-related factor 2; ARE, antioxidant response element; NF-κB, nuclear factor-kappa-B; LPS, lipopolysaccharide; COX, cyclooxygenase; LOX, lipoxygenase; iNOS, inducible nitric oxide synthase; HO-1, hemeoxygenase-1; ROS, reactive oxygen species; $cPLA_2$, cytosolic phospholipase A_2; PGE_2, prostaglandin E_2; qRT-PCR, quantitative reverse-transcriptase-polymerase chain reaction; CI, combination index.

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CUR and DHA or EPA are used together, synergistic chemopreventive effects such as anti-inflammatory response could be achieved. Our study clearly demonstrates the synergistic anti-inflammatory as well as anti-oxidative stress effects of CUR and PUFA.

2. Materials and methods

2.1. Materials and cell cultures

All materials were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified including Curcumin from *Curcuma longa* (Turmeric), (CUR, catalog no. C1386, ~70%), *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, catalog no. D2534, ≥98%), *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA, catalog no. E2011, ≥99%), 2, 3-diaminonapthalene (DAN), sodium nitrate ($NaNO_3$), and lipopolysaccharide (LPS) derived from *Escherichia coli* 055:B5.

Murine leukemic monocytic macrophage cell line, RAW 264.7 cells were maintained and cultured at 37 °C under a humidified, 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, GIBCO Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin and 1.176 g/L sodium bicarbonate.

2.2. Measurement of nitrite (NO) concentration by fluorimetric assay

Biologically produced nitric oxide (NO) is rather unstable and is very rapidly oxidized to nitrite and nitrate in aqueous solution. The assay of NO can be determined by measuring the stable oxidized product of NO, nitrite. A commonly used well-known Griess assay, however lacks the sensitivity, since the detection of limit is about 1 μM, which is relatively insensitive to detect the nitrite amount. Therefore, a fluorimetric assay was modified from Misko et al. [20] and used to detect the nitrite concentrations in the supernatant of cell culture.

Nitrite standards made from nitrate sodium were diluted freshly in deionized water. 10 μL of freshly prepared DAN (0.05 mg/mL in 0.62 M HCl) was used to react with nitrite (50 μL of supernatant) to form 1-(*H*)-naphthotriazole, a fluorescent product. After a 10 min of incubation at room temperature in the dark, the reaction was terminated with 5 μL of 2.8 M NaOH. Formation of the 2,3-diaminonaphthotriazole in dark opaque 96-well plates was measured using a microplate fluorescence reader, FLx-800 from Bio-Tek Instruments Inc. (Winooski, VT, USA) with excitation at 360 and 460 nm emission with a gain setting at 75%.

2.3. Testing the ability of CUR, DHA or EPA alone or combinations of CUR + DHA and CUR + EPA for suppressing NO levels

All single compound and combinations of CUR and DHA or EPA at various concentrations were tested. 10^5 cells per wells in 96-well microplate were seeded overnight to allow the cells to adhere to the plate. On the day of experiment, the tested compounds were prepared in DMEM without FBS. The cells were first treated with 50 μL of various chemopreventive agents. One hour later, another 50 μL of the chemopreventive agents with the stimulant of inducible NO synthase (iNOS), LPS were added into the well to achieve final tested concentrations of single compound CUR 0.63, 1.25, 2.5, 5, 10, 20, 40 and 80 μM, DHA 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM, EPA 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM. In combination study, total of different 24 combinations of drugs were tested; 0.63 μM CUR + 0.78 μM DHA, 0.63 μM CUR + 0.78 μM EPA, 0.63 μM CUR + 1.56 μM DHA, 0.63 μM CUR + 1.56 μM EPA, 0.63 μM CUR + 3.13 μM DHA, 0.63 μM CUR + 3.13 μM EPA, 1.25 μM CUR + 0.78 μM DHA, 1.25 μM CUR + 0.78 μM EPA, 1.25 μM CUR + 1.56 μM DHA, 1.25 μM CUR + 1.56 μM EPA, 2.5 μM CUR + 0.78 μM DHA, 2.5 μM CUR + 0.78 μM EPA, 2.5 μM CUR + 1.56 μM DHA, 2.5 μM CUR + 1.56 μM EPA, 2.5 μM CUR + 3.13 μM DHA, 2.5 μM CUR + 3.13 μM EPA, 2.5 μM CUR + 6.25 μM DHA, 2.5 μM CUR + 6.25 μM EPA, 5 μM CUR + 6.25 μM DHA, 5 μM CUR + 6.25 μM EPA, 5 μM CUR + 12.5 μM DHA, 5 μM CUR + 12.5 μM EPA, 5 μM CUR + 25 μM DHA and 5 μM CUR + 25 μM EPA.

Induction of iNOS activity was accomplished by treatment of LPS (final concentration 10 μg/mL) in RAW 264.7 cells. Positive control cells were treated only with LPS and negative control cells were treated with 0.1% DMSO. The NO suppressing ability of single compound or combination treatments is calculated from the following equation:

$$\%\,\text{NO inhibition} = \frac{(\text{corrected nitrite content in positive control} - \text{corrected nitrite content in treated sample})}{\text{corrected nitrite content in positive control}} \times 100\%$$

In the cells, the nitrite content of negative control were subtracted from the positive and treated samples (i.e., corrected nitrite content in positive control and corrected nitrite content in treated sample) before the calculation of % NO inhibition was carried out to give an indication of the absolute % NO inhibition by the drugs, either in singly or in combination.

2.4. Cell viability test by MTS assay

The viability of the cells towards all tested compounds in various concentrations were tested using the cell viability test based on the ability of cells to convert a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] into its reduced colored formazen product. This bio-conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. All single compound and combinations of CUR and DHA or EPA tested for NO suppressing activity were also tested in this MTS assay. Similar to NO suppressing tests, 10^5 cells per wells in 96-well microplate were seeded overnight to allow the cells to adhere to the plate. 10 μL MTS CellTiter 96® Aqueous one solution cell proliferation assay (Promega, Madison, WI, USA) was added to cells 24 h after the cells were treated similar to the NO suppressing activity tests described above. The microplate was incubated for 1 h at 37 °C under a humidified, 5% CO_2 atmosphere. The microplate was measured at 490 nm by a spectrophotometer (μQuant Biomolecular Spectrophotometer, Bio-Tek Instruments Inc., Winooski, VT, USA). Control cells were treated with 0.1% DMSO and the viability of cells are calculated from this equation: absorbance in treated sample/absorbance in control × 100%.

2.5. Measurement of prostaglandin E_2 (PGE_2) concentration by ELISA assay

RAW 264.7 cells were treated in a similar fashion to the NO suppressing test, using selected concentrations of drugs and combination of compounds that were identified to be synergistic in suppressing the NO production stimulated by LPS. Following treatment, the PGE_2 content in the supernatant of cell culture was determined using a commercially available enzyme immunoassay kit (Prostaglandin EIA kit, catalog no. 514010, Cayman Chemical Company, Ann, Arbor, MI, USA) according to the manufacturer's instructions. The % suppression of PGE_2 was calculated in a similar manner as the calculation for % NO suppression above.

2.6. Investigation of synergistic effects of CUR and DHA or EPA by combination index (CI)

The investigation of synergistic effects of CUR and DHA or EPA involves plotting the dose–response effects for CUR, DHA and EPA alone and for multiple combinations of agents. The combination drug effect analysis based on Loewe additivity model was used to examine the drug interactions as reviewed by Lee et al. [21]. The interaction of drugs can be characterized by the combination index (CI) which is defined as:

$$\mathrm{CI} = \frac{d_1}{D_{x,1}} + \frac{d_2}{D_{x,2}}$$

where d_1 and d_2 are doses of drug 1 (CUR) and drug 2 (DHA or EPA) in combination, which produces an effect x, while $D_{x,1}$ and $D_{x,2}$ are the doses of drug 1 (CUR) and drug 2 (DHA or EPA) that produce the same effect x when given alone. $d_1/D_{x,1} + d_2/D_{x,2}$, is also called the interaction index at the combination doses (d_1 and d_2). When the CI is equal to, less than or greater than 1, the combination doses will be additive, synergistic or antagonistic, respectively. Although the exact mechanism of interaction may often be unknown, this test will attempt to figure out whether the suppression of inflammation is involved by reducing expression of iNOS and cyclooxygenase-2 (COX-2) as demonstrated by suppression of NO and PGE_2, respectively. It was also concluded that the Loweve additivity model is one of the best general reference models for evaluating potential drug interactions [21].

2.7. RNA isolation and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assay

RAW 264.7 cells were treated using selected concentrations that examined the compound singly or in combinations. The cells were treated similarly as described above. After 6 h of treatment, total RNA was extracted using RNeasy Micro Kit (Qiagen, Valencia, CA, USA). RNA concentrations were determined by Quant-iT™ RiboGreen® RNA Reagent and Kit (Invitrogen, Grand Island, NY, USA). From each sample, 0.1 mg of total RNA was then reverse transcribed to single-stranded cDNA by TaqMan® Reverse Transcription Reagents (catalog no. N808-0234, Applied Biosystems Inc., Foster City, CA, USA). Then qPCR analyses were performed on the aliquots of the cDNA preparations with SYBR Green PCR Master Mix (catalog no. 4309155, Applied Biosystems Inc., Foster City, CA, USA) to detect quantitatively the gene expression of COX-2, iNOS, hemeoxygenase-1 (HO-1), 5-lipoxygenase (5-LOX), cytosolic phospholipase A_2 ($cPLA_2$) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (as an internal standard) using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). The primer pairs were designed using Primer Quest Oligo Design and Analysis Tool by Integrated DNA Technologies Inc. (Coralville, IA, USA) and the sequences are listed in Table 1. All assays were confirmed using melting curves to confirm the presence of single PCR products. Minimum of two independent experiments were carried out, each experiment had at least triplicate samples for each treatment.

2.8. Protein extraction and Western blotting

20 μg of protein samples were subjected to 4–15% gradient polyacrylamide gel (Criterion Tris–HCl gel, Bio-Rad Lab, Hercules, CA, USA) electrophoresis and the resolved proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) using a semi-dry transfer system (Fisher Scientific, Pittsburgh, PA, USA). The non-specific binding of antibodies were blocked with 5% bovine serum albumin (BSA) in PBST buffer (0.1% Tween 20 in PBS). Antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Immunodetection of COX-2 (catalog no. sc-1745), iNOS (catalog no. sc-650), HO-1 (catalog no. sc-10789) and β-actin (catalog no. sc-1616) proteins was carried out using respective primary antibodies (1:1000 in 3% BSA in PBST buffer) and horseradish peroxidase (HRP) conjugated secondary antibodies (1:1000 in 3% BSA in PBST buffer). The immunocomplexes were determined by using the enhanced chemiluminescent system for detecting HRP on immunoblots (Thermo Scientific, Rockford, IL, USA) and the bands were visualized and captured by BioRad ChemiDoc XRS system (Hercules, CA, USA). The protein bands were quantified by densitometry using Image J software (version 1.37 g, National Institute of Health, USA) and represented as the protein/β-actin ratio. Minimum of three independent experiments were carried out, each experiment had at least duplicated blots for each treatment.

2.9. Data presentation and statistical analysis

The results were presented as means ± standard error of the mean (SEM) unless otherwise indicated. Data were analyzed by a Student's *t*-test or one-way ANOVA to determine statistical differences between groups using SPSS statistical software (version 16.0 for Windows, SPSS Inc., Chicago, IL, USA), the statistical significance of mean differences was based on a p value of <0.05. Mann–Whitney U tests were used to determine statistical differences between groups for the protein levels obtained from Western blots, with a p value of <0.05 is considered to be significant. Box-plots were used to present the normalized density data for the protein expression levels of iNOS, COX-2 and HO-1 as previously described [22].

3. Results

3.1. Non-toxic effects of CUR, DHA and EPA on RAW 264.7 cells treated with or without LPS

To determine the effects of single compound, CUR, DHA, EPA and various combinations of CUR with DHA and CUR with EPA on cells viability, RAW 264.7 cells were initially seeded in microplates followed by different treatments. Fig. 1 shows the results of the MTS assay after 24 h treatment. Without LPS stimulation, with the exception of the highest concentrations of the compounds, i.e., CUR 80 μM, DHA 100 μM and EPA 100 μM, all the treatments were non-toxic as compared to the non-treated cells (Fig. 1A). In the presence of LPS, the viability profiles of the treated cells are similar

Table 1
Oligonucleotide primers used for qualitative real-time RT-PCR (qRT-PCR).

Gene	Accession no.	Forward (5′) primer	Reverse (3′) primer
Cyclooxygenase-2 (COX-2)	NM_011198.3	5′-TGC CTG GTC TGA TGA TGT ATG CCA-3′	5′-AGT AGT CGC ACA CTC TGT TGT GCT-3′
Cytosolic phospholipase A_2 ($cPLA_2$)	NM_008869.3	5′-ACG TGT CTC CAC GTC AAA CCT GAT-3′	5′-TGC TTC CAA ATA GGT CAG GAG CCA-3′
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	XM_001473623.1	5′-TGA AGC AGG CAT CTG AGG G-3′	5′-CGA AGG TGG AAG AGT GGG AG-3′
Hemeoxygenase-1 (HO-1)	NM_010442.1	5′-CCT CAC TGG CAG GAA ATC ATC-3′	5′-CCT CGT GGA GAC GCT TTA CAT A-3′
Inducible nitric oxide synthase 2 (iNOS)	NM_010927.2	5′-CCT GGT ACG GGC ATT GCT-3′	5′-GCT CAT GCG GCC TCC TTT-3′
5-Lipoxygenase (5-LOX) activating protein	NM_009663.1	5′-CCA CAA GGT GGA GCA TGA AAG CAA-3′	5′-TAC GCA GTT CTG GTT GGC AGT GTA-3′

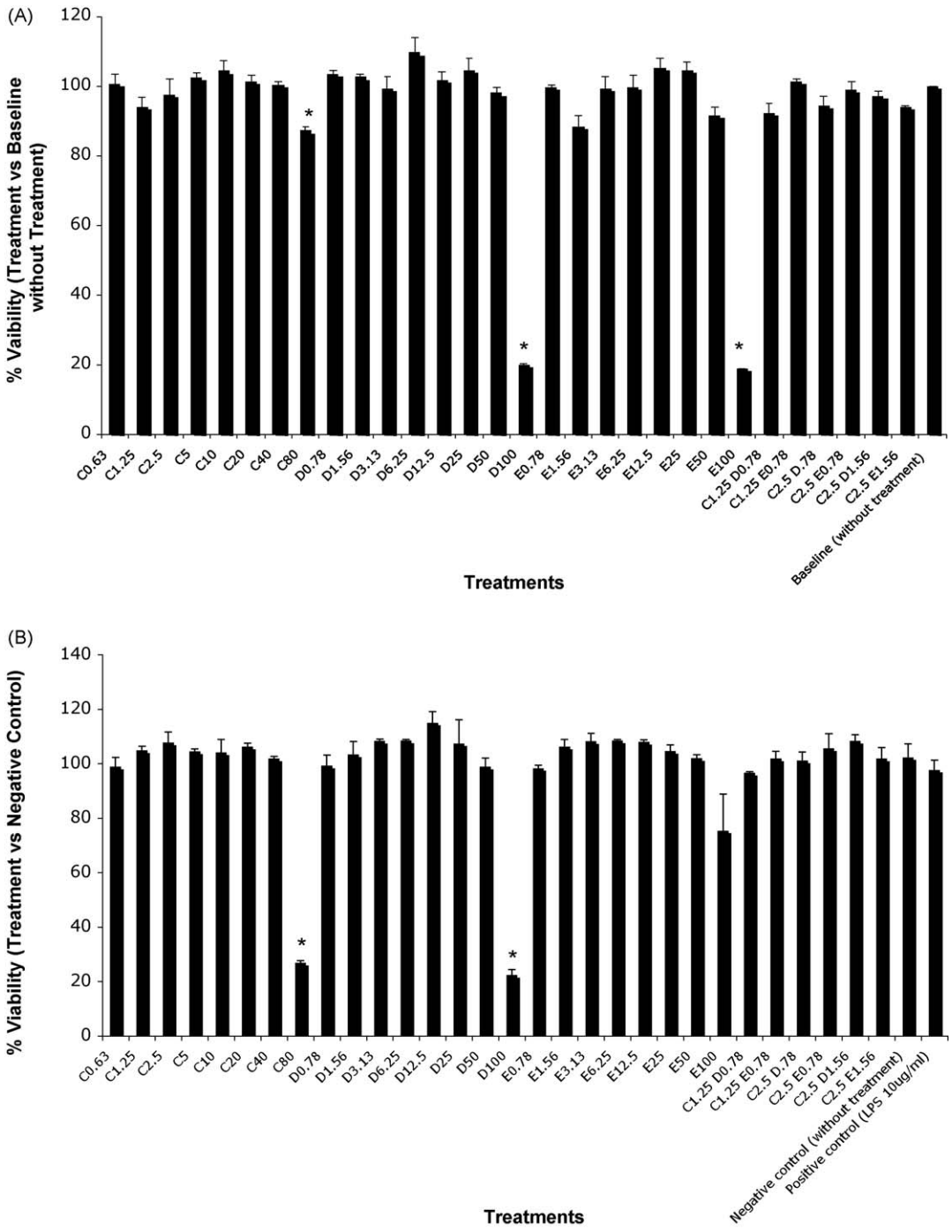

Fig. 1. (A) RAW 264.7 cells treated with drugs only. (B) The cells treated with LPS (10 μg/mL) and drugs. Results are expressed as the mean ± SEM from three samples. $^{*}p < 0.01$, compared with corresponding value for non-treated cells. C denotes CUR, D denotes DHA and E denotes EPA.

to those that are not treated with LPS (Fig. 1B). Subsequently, selected non-toxic concentrations for single and combination compounds were further examined for their chemopreventive anti-inflammatory properties.

3.2. Synergistic suppression of NO by CUR with DHA or EPA on RAW 264.7 cells treated with or without LPS

When RAW 264.7 cells were stimulated with LPS, biomarker of NO, nitrite was produced. To examine whether the combinations of CUR with DHA and CUR with EPA have synergistic effects in suppressing the NO in LPS-stimulated RAW 264.7 cells, various combinations at 6, 12 and 24 h were tested (Fig. 2A). The suppression effects were most prominent at 24 h time point (data not shown). At 6, 12 and 24 h, all the combinations of CUR with DHA or EPA at lower than 5 μM of CUR and DHA or EPA of less than 25 μM were found to have synergistic effects in suppressing NO production with CI values of lesser than 1 (Fig. 2A).

Due to such promising synergistic effects, we next examined whether the combination treatments were able to suppress the endogenous NO in RAW 264.7 cells without LPS stimulation. Similar treatments at 6, 12 and 24 h were carried out in the absence of LPS stimulation. Most of the combinations were able to suppress endogenous NO synergistically (Fig. 2B). At 6, 12 and 24 h, all the

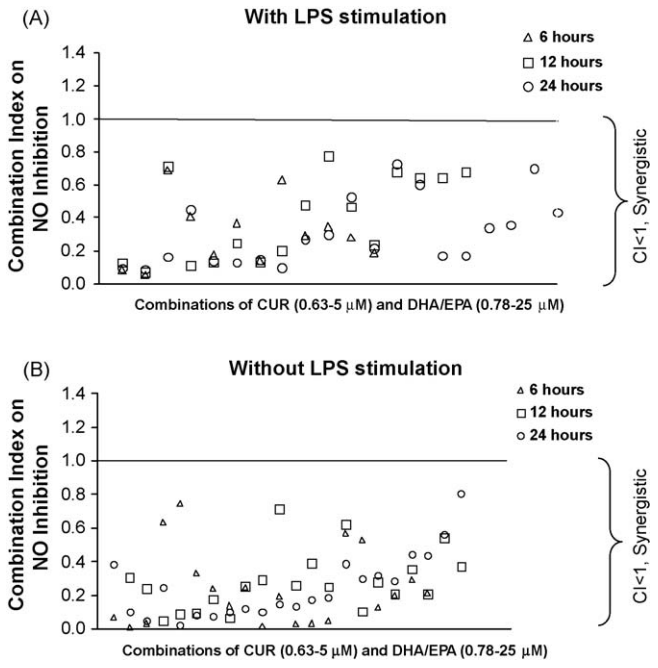

Fig. 2. All combinations as described in Section 2.3 were tested, and only those showing synergistic activity, having CI values of less than 1 are presented. (A) CI values of CUR and DHA or CUR and EPA combinations stimulated with LPS. RAW 264.7 cells were treated with different drug concentrations from 0.63 to 5 μM of CUR combined with 0.78–25 μM of DHA or EPA for 6, 12 and 24 h. The cells were stimulated by 10 μg/mL LPS 1 h after the drug treatments. All the combined concentrations displayed CI < 1 when the cells were treated for 24 h. For the 6 and 12 h treatments, the CI values are less than 1 when CUR concentrations were not more than 2.5 μM and DHA or EPA concentrations were not more than 6.25 μM. (B) When the cells were not treated by LPS. The NO synergistic suppression effect is time- and concentration-dependent, i.e., at 6 h, fewer combinations were synergistic but at 24 h, all the combinations of 0.63–5 μM CUR with 0.78–25 μM of DHA or EPA have CI values less than 1.

combinations of 0.63–5 μM of CUR with 0.78–25 μM of DHA or EPA have CIs less than 1. In both cases whether with or without LPS treatments, the NO synergistic suppression effects were time- and concentration-dependent, i.e., greater effect was seen with longer incubation time and higher concentrations of CUR (data not shown).

3.3. Synergistic suppression of PGE_2 by CUR with DHA or EPA on LPS treated RAW 264.7 cells

Without LPS stimulation, there was already a substantial amount of PGE_2 in the cells (16,656.85 ± 6185.40 pg/mL). When RAW 264.7 cells were stimulated by LPS, there were accumulations of PGE_2 (22,733.99 ± 5021.94 pg/mL). All the tested concentrations of CUR, DHA and EPA, either alone or in combinations, were able to suppress the accumulation of PGE_2 (data not shown). For instance, single compound, CUR as low as 0.63 μM, was able to suppress more than 100% of the basal non-LPS treated levels of PGE_2 negative (from 16,656.85 ± 6185.40 pg/mL to 14,937.70 ± 3139.15 pg/mL). Both CUR and EPA showed dose-dependent suppression of PGE_2. Interestingly, CUR at 2.5 μM and DHA or EPA at 0.78 μM could also synergistically suppress LPS-induced PGE_2 between 90% and 100% (data not shown).

3.4. Suppression of iNOS, COX-2, and synergistic induction of HO-1 by CUR with DHA or EPA

Since nitrite and PGE_2 production were suppressed synergistically by CUR with DHA or EPA, we next examined the protein expression levels of iNOS and COX-2. It was hypothesized that the synergism was due to the decreased production of the *de novo* synthesis iNOS and COX-2 proteins. As determined by Western blotting (Fig. 3), as compared to non-LPS treated control cells, the LPS treated cells showed an increase of iNOS (Fig. 3A) and COX-2 (Fig. 3B) protein levels. From the Western blots, the sizes of the iNOS, COX-2 and β-actin proteins were determined to be 130, 72 and 43 kDa. In addition, Fig. 3 also shows that pretreatment of the cells with CUR or DHA alone, did not inhibit iNOS protein expression, while EPA alone exhibited some degree of suppression of iNOS. All the combination treatments with CUR and DHA or EPA were able to suppress iNOS protein expression as compared to the non-treated cells. These data suggest that the observed reduction of nitrite and PGE_2 by CUR and DHA or EPA was due reduced protein expression of iNOS and COX-2 in RAW 264.7 cells.

It was also hypothesized that the combination of CUR with DHA or EPA could enhance the Nrf2/ARE-mediated antioxidant enzyme HO-1 therefore Western blotting for the expression of HO-1 was also performed. Interestingly, while LPS treatment attenuated HO-1 protein expression, all the synergistic combinations of CUR with DHA or EPA were able to restore HO-1 protein expression, and the effects were dose-dependent with the combination treatments (Fig. 3C). The synergistic induction of HO-1 protein was observed at 2.5 and 5 μM of CUR, with HO-1 induced 1.5–3 folds (Fig. 3C and D). CUR alone, as low as 2.5 and 5 μM, was able to restore HO-1 in a dose-dependent manner. The levels of mRNA expression for iNOS and COX-2 treated with the combinations of CUR with DHA or EPA (Fig. 4A and B) were also consistent with their protein expression levels (Fig. 3), indicating that the observed decrease in the production of nitrite and PGE_2 was a result of reduced transcription of iNOS and COX-2 genes and increased expression of HO-1 gene (data not shown).

3.5. Modulation of 5-LOX and $cPLA_2$ mRNA by CUR, DHA and EPA

The mRNA expression levels of 5-LOX and $cPLA_2$ were also quantified by qRT-PCR in the cells treated with single compound or combinations. CUR alone did not suppress 5-LOX, but EPA alone appeared to suppress 5-LOX with DHA to a lesser extend (Fig. 4C). Interestingly low doses of EPA were able to suppress 5-LOX after cells were treated with LPS (Fig. 4C). Pretreatments with CUR, DHA and EPA alone or their combinations were able to suppress the expression of $cPLA_2$ (Fig. 4D). In all the cases, the combinations of CUR with DHA or EPA at lower concentration, 2.5 and 3.13 μM, respectively, showed greater suppression effects as compared to the treatments when CUR or DHA alone at their corresponding concentrations.

4. Discussion

Drug synergism is dependent in part on the concentrations of the combined drugs, at too high concentrations, one may not see synergism. Synergism will be of clinical significance when one uses both drugs at lower concentrations to produce better efficacy with lower toxicity than the single drug alone. The present study aims to investigate the potential synergistic anti-inflammatory effects of low doses of CUR in combination with PUFA such as DHA or EPA in a LPS-stimulated inflammatory model using RAW 264.7 cells. Very low doses of CUR, as low as 0.63 μM and very low doses of DHA and EPA, as low as 0.78 μM were tested to mimic the low dietary consumption levels, in the context of potential cancer chemoprevention. It has been reported from a Phase I clinical trial that after taking 4000, 6000 and 8000 mg of CUR orally, the average peak serum concentrations were 0.51, 0.63 and 1.77 μM, respectively [9]. A very recent pharmacokinetic study of DHA/EPA with 48

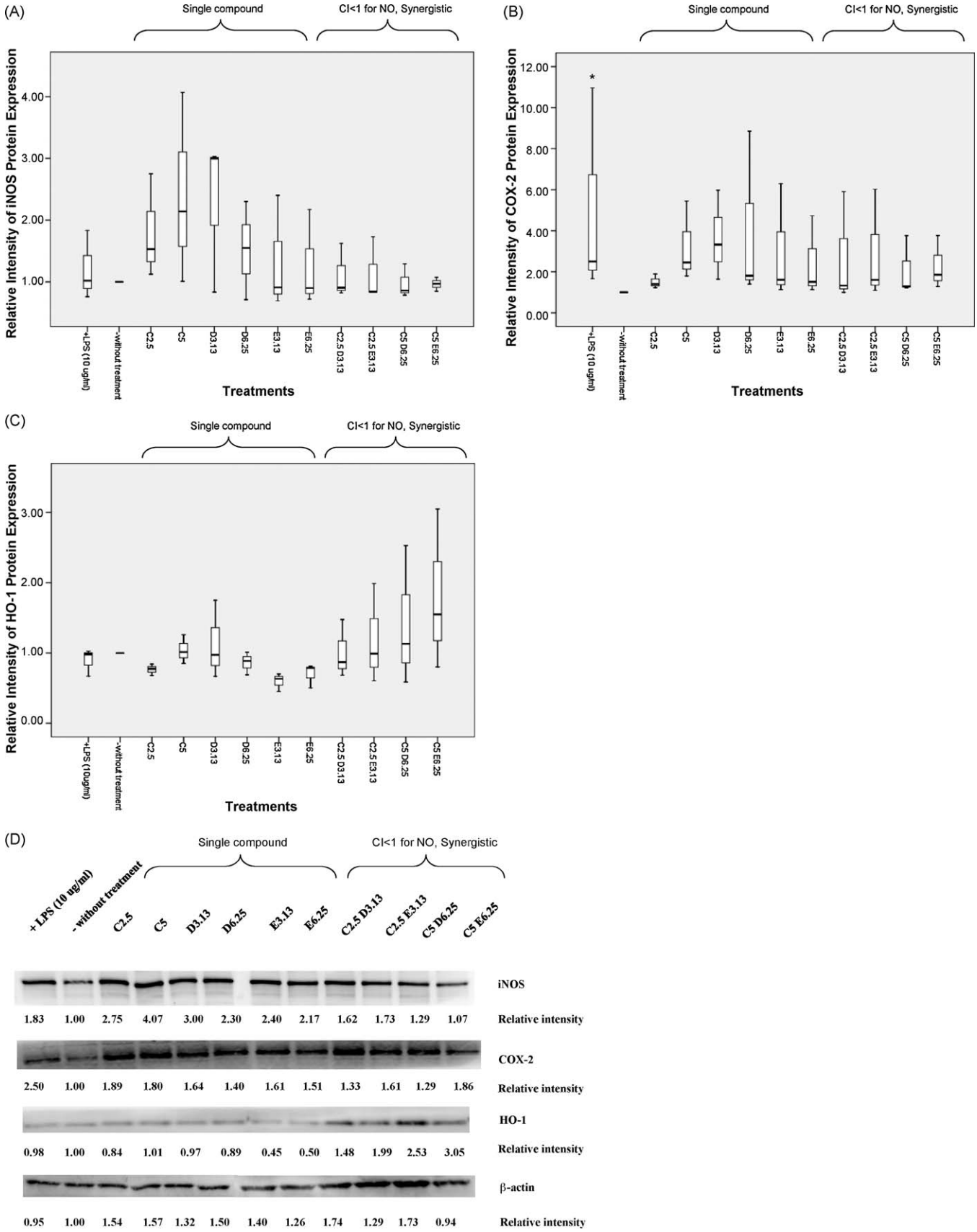

Fig. 3. Effects of CUR, DHA, EPA and their combinations on RAW 264.7 cells by Western blotting. The protein bands were quantified by densitometry and represented as the protein over the β-actin, ratio. Normalized density data for iNOS, COX-2 and HO-1 are shown as box-plots in (A), (B) and (C), respectively. The median is marked by a line within the box. The two vertical lines outside the box extend to the smallest and largest normalized density value. (D) Shows the representative images of three independent experiments. Each marker is normalized to its own control (negative without treatment). C denotes CUR, D denotes DHA and E denotes EPA. $^{*}p < 0.05$, compared with the LPS treated cells.

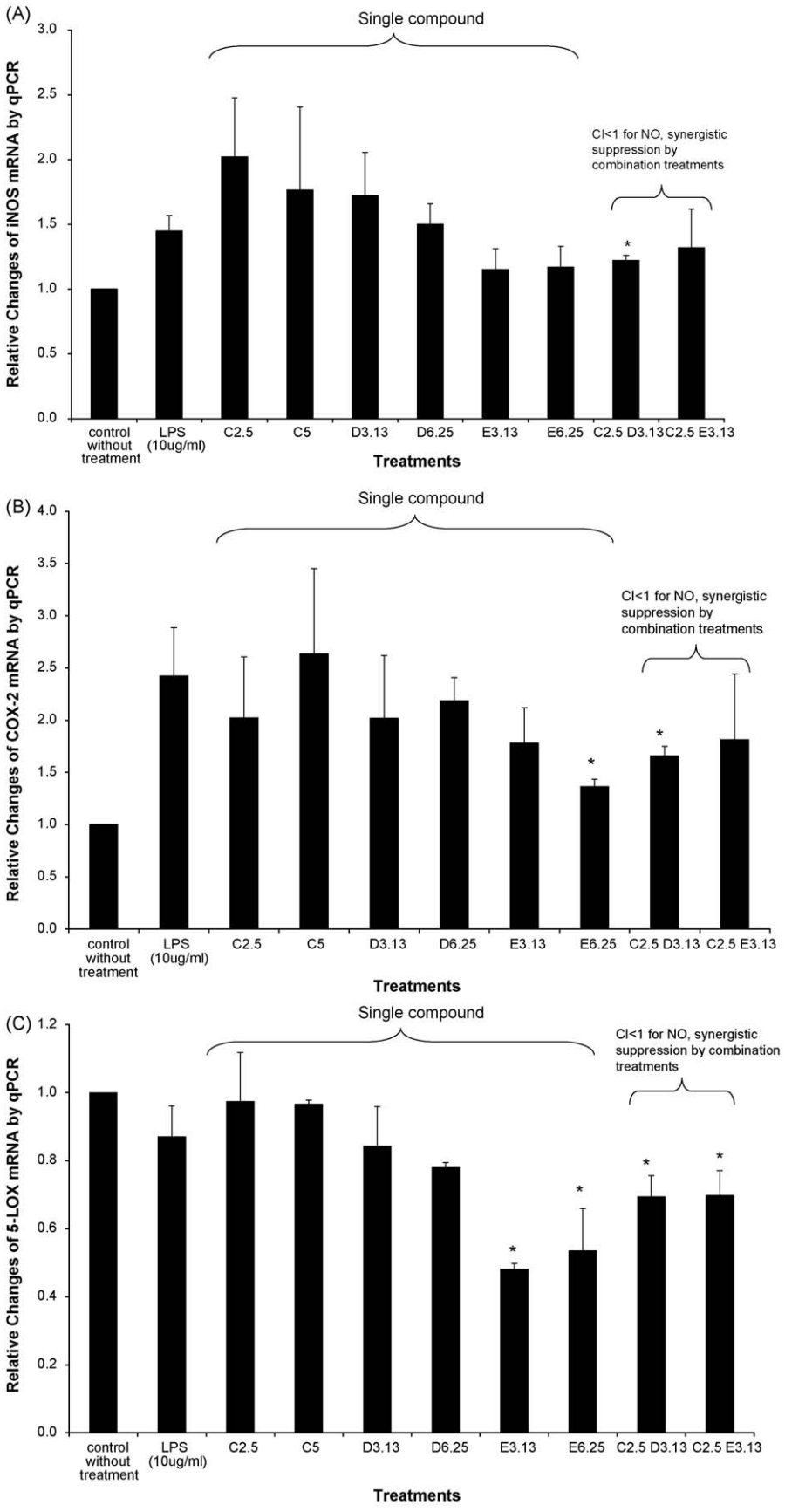

Fig. 4. Real-time PCR (qRT-PCR) results expressed in fold changes of mRNA over the control, using GAPDH as endogenous housekeeping gene. (A) Shows the relative expression level of iNOS. (B) Shows the relative expression level of COX-2. (C) Shows the relative expression level of 5-LOX. (D) Shows the relative expression level of $cPLA_2$. Results are expressed as mean ± SEM. C denotes CUR, D denotes DHA and E denotes EPA. $^*p < 0.05$, compared with the LPS treated cells.

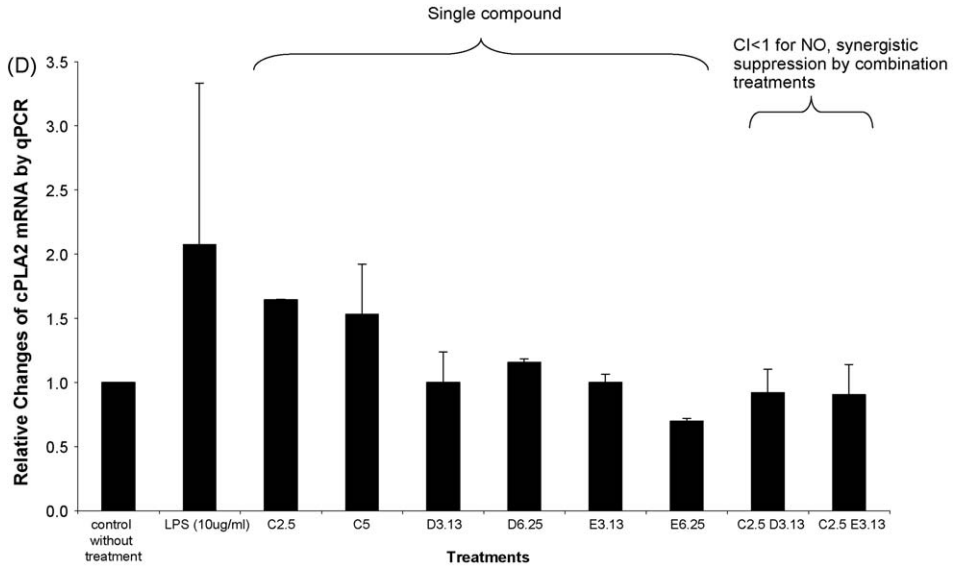

Fig. 4. (*Continued*).

subjects consuming fish 1–2 times a month had a detectable plasma DHA level of about 60 μg/mL and EPA of about 10 μg/mL, which is equivalent to 182 and 33 μM, respectively [23]. These concentrations are much higher than the concentrations tested in the present study. Therefore, the low doses of CUR in combination with DHA or EPA used in the present study provides strong evidence for translating the observed synergistic anti-inflammatory effects into human trials. The present study also shows that significant synergism exists between CUR and DHA or EPA, particularly in this anti-inflammatory model. To examine whether such synergism is specific to RAW 264.7 cells model, we had also performed similar combination treatments in a human colon cancer HT-29 cell line, significant synergistic suppression of NO production were also observed with CUR in combinations with DHA or EPA (unpublished observations). Ongoing experiments are being conducted to further study on the synergistic anti-inflammatory effects.

The synergistic anti-inflammatory effects of low doses of CUR in combination with DHA or EPA not only inhibited NO production in the LPS-stimulated RAW 264.7 cells, but also clearly inhibited in the non-LPS treated cells (Fig. 2B). Over the past 1–2 decades research on NO has shown its importance in maintaining normal physiological functions in the *in vivo* settings. The suppression of the endogenous NO may be unfavorable in certain normal physiological activities such as smooth muscle relaxation, however, in the context of the present study which would be under pathological conditions as with RAW 264.7 cell line, since it is a leukemic macrophage cell line that has been associated with tumor progression [24]. Macrophage generally is an important component in the human immune defense mechanism and often time enhanced immunity has been considered to be beneficial in the prognosis and survival of cancer patients [25,26] Recent report suggests that tumor-associated macrophages are implicated in the pathophysiology of cancer formation and progression, and they could play a role in the microenvironments of numerous malignancies [27].

In the present study, low doses of CUR, DHA and EPA alone caused an increase of iNOS and COX-2 proteins and in the presence of inflammatory agent such as LPS, low doses of CUR, DHA and EPA alone were unable to suppress the protein expression of iNOS and COX-2 (Fig. 3). However, with these similar low concentrations, the anti-inflammatory synergistic effects for CUR with DHA or EPA were evident. The reasons for these observations are unclear, but could be related to differential signaling at these low doses. Certainly further study into the potential mechanism would be needed to explain such observations.

Previously, our laboratory had identified more than 1000 Nrf2-dependent genes regulated by CUR [8] and the oxidative stress response gene, HO-1 was one of them. In the current study, the induction of HO-1 by CUR (Fig. 3) is also observed which is consistent with previous studies [7,8]. The present study also identifies the synergistic induction of HO-1 by CUR with DHA or EPA (Fig. 3), which possibly mediated by the Nrf2/ARE signaling pathway. In addition, the suppression of inflammatory mediators such as NO, PGE_2, 5-LOX and $cPLA_2$ also shows synergism with CUR and DHA or EPA.

The development of inflammatory diseases, including cancer, is commonly accompanied by increased production of leukotrienes and prostaglandins via the arachidonic acid (AA) pathway [28]. Typically the cytosolic phospholipase A_2 ($cPLA_2$) cuts the AA out of the cellular phospholipids and when free AA are exposed to the enzymes such as 5-LOX and COX-1/2, leukotrienes and PGE_2 are produced, respectively. Clinically, inhibition of COX by nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors reduces the levels of prostaglandins, resulting in a reduction of pain and inflammation. However, this inhibition may also cause alternative processing of AA via the 5-LOX pathway, resulting in an increase of production of pro-inflammatory and gastrotoxic leukotrienes. Theoretically, the dual inhibitors of COX and 5-LOX could decrease the production of both leukotrienes and prostaglandins, and they could display enhanced anti-inflammatory effects [29]. A recent study proposes a potential role of $cPLA_2$ in LPS-induced p38 MAPK pathways, and that the p38 MAPK/$cPLA_2$/COX-2 pathway was implicated in the production of LPS-induced interleukin-1-beta and interleukin-6 in differentiated U937 cells [30]. Previously Hong et al. [31] showed that CUR at 20 μM potently inhibited 5-LOX, however, this 5-LOX inhibitory activity was not observed in our current study, although we used much lower concentrations of 2.5 and 5 μM CUR. Our present study shows that the $cPLA_2$ was decreased by all the low doses of CUR, DHA and EPA when they were used alone (Fig. 4D). The synergistic suppression of 5-LOX and $cPLA_2$ were clearly demonstrated with 2.5 μM CUR when combined with 3.13 μM of DHA or EPA.

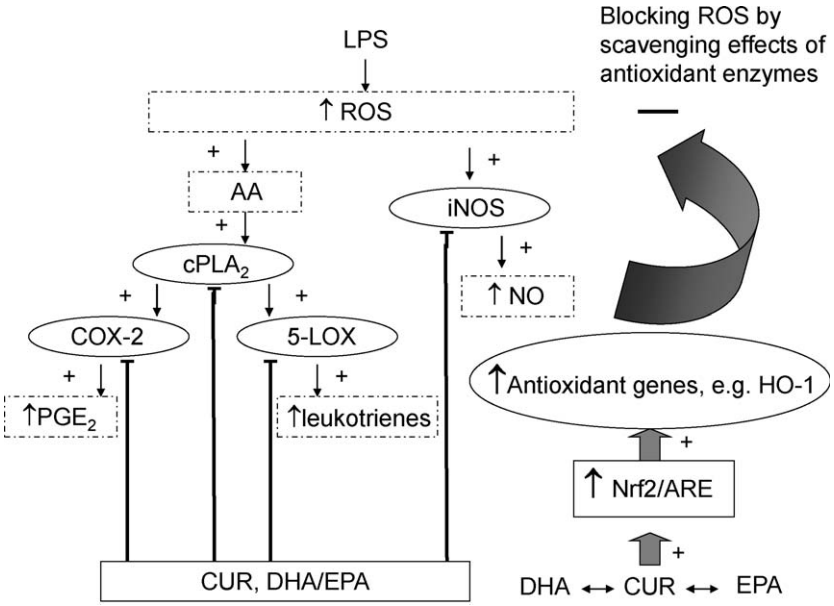

Fig. 5. Schematic diagram of the proposed targets for the synergistic chemopreventive anti-inflammatory effects of CUR with DHA or EPA in the RAW 264.7 cells stimulated by LPS, potentially leading to the inhibition of the pro-inflammatory cytokines and related mediators. (+) Denotes stimulation, (−) denotes inhibition, (↑) denotes increase and (↔) denotes interaction of CUR with DHA or EPA.

One should be cautious regarding the possible effects of CUR, DHA and EPA might have on the normal physiological processes. Some studies found that CUR did not affect the expression levels of COX-1 [32,33] and CUR or its analogues had increased binding of COX-2 [34]. CUR had also been shown to decrease [35,36] or not affecting [37] the activity of endothelial NOS. The effects of DHA or EPA on endothelial NOS were also not consistent. It was reported that both endothelial and neuronal NOS were increased with DHA treatment [38] but DHA did not protect against oxidative stress whereas EPA did [39]. Further in depth studies on these interplays and the significance of these signaling pathways modulated by these compounds would be needed.

In summary, a proposed mechanism is presented in Fig. 5, to illustrate the possible interactions between CUR, DHA and EPA, with the various genes/enzymes investigated in this study. CUR, DHA/EPA could directly inhibit the enzymatic activities and/or transcription or translation of iNOS, $cPLA_2$, COX-2 and 5-LOX. These inhibitions could also be caused indirectly via the induction of antioxidant enzymes such as HO-1. The induction of antioxidant genes would attenuate the production of LPS-mediated ROS, which would lead to a decrease in the production of iNOS and NO, as well as decrease levels of $cPLA_2$, COX-2 (PGE_2) and 5-LOX (Fig. 5). The current study suggests that the anti-inflammatory and the antioxidant activities of CUR can be enhanced in a synergistic fashion by combining with low doses of PUFA, DHA or EPA. Future *in vivo* animal studies would be needed to help translate these results to the clinical settings.

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