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Induction of NRF2-mediated gene expression by dietary phytochemical flavones apigenin and luteolin

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ABSTRACT: Apigenin (API) and luteolin (LUT) have been used as therapeutic agents in folk medicine for thousands of years. These compounds exert a variety of biological activities, including anticancer, antioxidant and antiinflammatory activities. This study investigated whether API and LUT could activate Nrf2-antioxidant response element (ARE)-mediated gene expression and induce antiinflammatory activities in human hepatoma HepG2 cells. The compounds did not exhibit any substantial toxicity at low doses (1.56–6.25 μM). The induction of ARE activity was assessed in HepG2-C8 cells after treatment with low doses of API and LUT for 6 and 12 h. It was found that the induction of ARE activity by these compounds at the higher doses was comparable to the effects of the positive control, SFN at a dose of 6.25 μM. Exposure to the PI3K inhibitor LY294002 abolished ARE activation by both API and LUT, whereas the ERK-1/2 inhibitor PD98059 only decreased ARE activity induced by API. Both compounds significantly increased the endogenous mRNA and protein levels of Nrf2 and Nrf2 target genes with important effects on heme oxygenase-1 (HO-1) expression. API and LUT significantly and dose-dependently decreased the production of nitric oxide (NO), nitric oxide synthase (iNOS) and cytosolic phospholipase A2 (cPLA2), which were induced by the treatment of HepG2 cells with 1 μg/ml of lipopolysaccharide (LPS) for 24 h. The results indicate that API and LUT significantly activate the PI3K/Nrf2/ARE system, and this activation may be responsible for their antiinflammatory effects, as demonstrated by the suppression of LPS-induced NO, iNOS and cPLA2. Copyright © 2015 John Wiley & Sons, Ltd.

Key words: apigenin; luteolin; Nrf2; HepG2; LPS

Introduction

Increased consumption of fruits and vegetables is associated with a decreased risk of cancer, and this correlation is thought to be driven by the high levels of phytochemicals with anticancer properties that are contained in fruits and vegetables [1]. Apigenin (4′,5,7,-trihydroxyflavone; API) and luteolin (3′,4,5,7-tetrahydroxyflavone; LUT) are low molecular weight polyphenolic compounds and two of the most common dietary flavonoids in the human diet (Figure 1A, B, respectively) [2]. API and LUT are abundant in a wide variety of common herbs and vegetables, such as parsley, chamomile, celery and citrus fruits [2]. Although API and LUT are usually found in small quantities with a low oral

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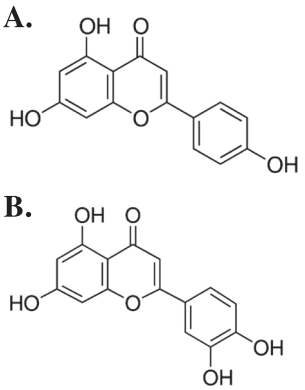

Figure 1. Chemical structures of API (A) and LUT (B)

bioavailability (similar to other flavonoids), they display relatively rapid absorption, complex biotransformation/metabolism processes and a slow elimination phase that seems to account for their accumulation *in vivo* and biological effects [3]. For instance, rats subjected to a single oral dose of *Chrysanthemum morifolium* extract (200 mg/kg), containing 5.2% of API and 7.6% of LUT, showed that both flavones are absorbed in less than 4 h after administration (API 3.9 h and LUT 1.1 h, respectively) with the highest area under the curve for API (237.6 μg·h/ml) in comparison with LUT (23.03 μg·h/ml) [3]. API displayed a lower apparent distribution volume, lower renal clearance and higher elimination half-life (13.781/kg, 0.85691/kg/h and 3.4 h, respectively) in comparison with LUT (65.121/kg, 8.4731/kg/h and 2.7 h, respectively), with a total recovery in feces and urine lower than 50% for both flavones at 72 h mainly as phase II metabolites [3]. API and LUT intake has been associated with a decreased risk of several types of cancer [4,5]. In this context, various studies suggest that these flavonoids inhibit critical events associated with carcinogenesis, including cell transformation, invasion, metastasis and angiogenesis, by modulating kinases, inhibiting transcription factors, regulating the cell cycle and inducing apoptosis [6,7]. Subsequently, several molecular targets have been identified to contribute to these effects, including the activation of phosphatidylinositol 3′-kinase (PI3K)/Akt, nuclear factor kappa B (NF-kB), mitogen-activated protein kinase (MAPK), p53, and many others; however, the mechanisms underlying their chemopreventive effects are not completely understood [7,8].

It has been increasingly reported that the biological activities of LUT and API are related to their ability to modulate reactive oxygen species (ROS) [9,10]. ROS are highly reactive chemical species that play important roles in cell signaling and homeostasis when they are present at low levels but can lead to carcinogenesis at high levels by promoting DNA damage, genomic instability and neoplastic transformation [11,12]. The induction of cytoprotective mechanisms, such as the expression of phase II detoxification/antioxidant enzyme orchestrated by nuclear factor (erythroid-derived 2)-like 2 (Nrf2), is believed to have an important role in preventing carcinogenesis [13,14]. After translocation into the nucleus, Nrf2 induces the transcriptional activity of a *cis*-acting DNA element known as the antioxidant response element (ARE)/electrophile response element (EpRE), which leads to the expression of protective genes, such as phase II drug metabolizing enzymes UDP-glucuronosyltransferases (UGT), glutathione *S*-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO-1) and antioxidative stress enzymes heme oxygenase-1 (HO-1) [13].

API and LUT have been reported to protect against oxidative stress through the upregulation of glutamate cysteine ligase, the glutamate cysteine ligase catalytic subunit and HO-1 in primary rat hepatocytes via the Nrf2 signaling pathway [15]. In addition, it was demonstrated previously that API targets key CpG sites in the Nrf2 promoter region of epithelial JB6 P+ cells through epigenetic mechanisms, and this leads to the reactivation of Nrf2-dependent antioxidant/detoxifying enzymes [16]. Interestingly, increasing evidence has indicated that important cross talk occurs between Nrf2 and NF-κB under conditions of oxidative stress, suggesting that Nrf2 plays an important role in the regulation of inflammation [17–20]. For example, HepG2 cells challenged with lipopolysaccharide (LPS) show significantly enhanced NF-κB transcriptional activity when Nrf2 is silenced [21]. Additionally, it was reported that sulforaphane (SFN) treatment of Nrf2 (+/+), but not Nrf2 (−/−), mice restored the number of healthy cells back to basal levels by 8 days after UVB irradiation, demonstrating a decreased activity of inflammatory biomarkers in SFN-treated Nrf2 (+/+) mice compared with KO mice and revealing a protective role of Nrf2 against

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UVB-induced skin inflammation [22]. Subsequent studies in our laboratory demonstrated that the protective effects of Nrf2 in response to UVB irradiation are mediated in part by increased HO-1 protein expression [23].

API and LUT have been reported extensively to possess strong antiinflammatory activities by repressing NF-kB and inhibiting proinflammatory mediators *in vitro* and *in vivo* [7,8,24,25]. Therefore, the Nrf2-ARE activation mediated by API and LUT and the antiinflammatory activity of both compounds was examined in LPS-stimulated HepG2 cells. This research suggests that API and LUT activate the antioxidative Nrf2-ARE pathway and that this activation may be involved in their attenuation of LPS-induced inflammation.

Materials and Methods

Reagents and cell culture

Dimethyl sulfoxide (DMSO), API (Figure 1A; ≥ 97% purity, from parsley) and LUT (Figure 1B; ≥ 98% purity) were purchased from Sigma (St Louis, MO). Sulforaphane (SFN) was purchased from LKT Laboratories (St Paul, MN). The inhibitors of phosphatidylinositol 3-kinase (PI3K) (LY294002), p38 MAPK (SB203580), JNK-1/2 (SP600125) and ERK-1/2 (PD98059) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Human hepatoma HepG2 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The HepG2-C8 cell line was established in Dr Ah-Ng Tony Kong's laboratory by transfecting HepG2 cells with a pARE-T1-luciferase construct (kindly provided by Dr William Fahl, University of Wisconsin) using the FuGENE 6 method as described previously [12]. The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1.17 g/l sodium bicarbonate, 100 units/ml penicillin and 100 μg/ml streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were grown to 80% confluence, split and then sub-cultured in fresh medium three times per week after washing with Versene and detaching with trypsin (Gibco, Carlsbad, CA, USA).

Cell viability assay

The HepG2 cells were seeded in 96-well plates in medium containing 1% FBS per well (1×10^4 cells/well) at an initial density of 1×10^5 cells/ml in a volume of 100 μl. After overnight incubation, the cells were treated with DMEM/1% FBS and various concentrations of API and LUT (1.56–100 μM) using 0.1% DMSO as a control for 24 h. The cytotoxicity of the API and LUT was tested by using the CellTiter 96® aqueous non-radioactive cell proliferation MTS assay kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison, WI). Absorbance of the formazan product was measured at 490 nm using a μQuant Biomolecular spectrophotometer from Bio-Tek Instruments Inc. (Winooski, VT). The cell viability was calculated by comparing the optical density of the treated samples with the optical density of DMSO, which was used as the negative control.

Evaluation of ARE reporter gene activity by luciferase assay

The HepG2-C8 cells were seeded in 12-well tissue culture plates at a density of 1×10^5 cells/ml in 1 ml of DMEM/1% FBS per well. The cells were treated with various concentrations of API and LUT, and the negative control group was treated with 0.1% DMSO. The positive control group was treated with 6.25 μM SFN, a well-known inducer of ARE activity [12,14]. After 6 h and 12 h of incubation, the luciferase activities in the cell extracts were measured with a Promega luciferase kit (Madison, USA) according to the manufacturer's protocol. The inhibitors LY294002 (50 μM), SB203580 (10 μM), SP600125 (50 μM) or PD98059 (50 μM) were added to the cultures 1 h prior to treatment and incubated with the cells for 12 h during stimulation according to the manufacturers' instructions. Briefly, after the treatment, the cells were washed with ice-cold phosphate buffered-saline (1× PBS, pH 7.4) and immediately harvested in 1x Promega luciferase cell culture lysis buffer (Madison, USA). The homogenates were centrifuged at 12000 rpm at 4 °C for 5 min. An aliquot of 10 μl of supernatant was assayed for luciferase activity using a Sirius luminometer from Berthold Detection Systems GmbH (Pforzheim, Germany). The luciferase

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activity was normalized relative to the protein concentration, which was determined using the BCA protein assay from Pierce (Rockford, USA). The results are expressed as the fold induction over the luciferase activity of the control DMSO-treated cells.

Evaluation of nitric oxide (NO) increase

The concentrations of NO in the supernatants of HepG2 cells were detected using a fluorometric assay, as reported previously [26]. Briefly, HepG2 cells were seeded at a density of 1.0×10^5 cells/ml in 96-well plates overnight. Then, the medium was discarded, and the cells were pretreated with API or LUT for 12 h. Lipopolysaccharide (LPS) from Sigma, Inc. (St Louis, MO, USA) was then added to a final concentration of 1 μg/ml and incubated for 24 h as described by Kang *et al*. [27]. The MTS assay was used to evaluate any potential toxicity as described above. The controls used were 0.1% DMSO with and without LPS. After 24 h of treatment, 50 μl of supernatant from each well was mixed with 10 μl of 2,3-diaminonaphthalene (0.05 mg/ml in 0.62 M HCl) from Sigma, Inc. (St Louis, MO, USA), and the NO concentration was determined by comparison with a calibration curve generated using NO standards in deionized water. After 10 min of incubation at room temperature in the dark, the reaction was terminated with 5 μl of 2.8 M NaOH. The formation of 2,3-diaminonaphthotriazole in black opaque 96-well plates was measured with a FLx-800 microplate fluorescence reader from Bio-Tek Instruments Inc. (Winooski, VT) at 360 nm excitation and 460 nm emission using a gain setting of 75%. The results are expressed as the fold of NO increase as follows: NO increase (fold) = [(nitric oxide content in LPS or sample treatments (μM)]/(nitric oxide content in DMSO control (μM)].

RNA extraction and quantitative real-time PCR

The total RNA was extracted from treated HepG2 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. The cDNA was used as the template for quantitative real-time PCR (qPCR) with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) in an ABI7900HT system. The following primers were used: Nrf2, 5′-GGCAGAGACATTCCCATTTG TAG-3′ (sense) and 5′-TCGCCAAAATCTGTGT TTAAGGT-3′ (antisense); NQO1, 5′-CAGAAAT GACATCACAGGTGAGC-3′ (sense) and 5′-CTAAGACCTGGAAGCCACAGAAA-3′ (antisense); HO-1, 5′- GCTCGAATGAACACTCTG GAGAT-3′ (sense) and 5′- TCCAGAGAGAAA GGAAACACAGG-3′ (antisense). β-Actin was used as an internal control with 5′-CGTTCA ATACCCCAGCCATG-3′ (sense) and 5′-GACC CCGTCACCAGAGTCC-3′ (antisense) primers.

Protein lysate preparation and western blotting

The HepG2 cells were treated with API and LUT according to the procedures described previously. DMSO and SFN were used as negative and positive controls, respectively. After 12 h of treatment, the cells were harvested using RIPA buffer supplemented with a protein inhibitor cocktail from Sigma (St Louis, MO). The protein concentrations of the cleared lysates were determined using the bicinchoninic acid (BCA) method according to the protocol provided by Pierce (Rockford, IL). Then, 20 μg of total protein from each sample was mixed with 5 μl Laemmli SDS sample buffer from Boston Bioproducts (Ashland, MA) and denatured for 5 min at 95 °C. The proteins were separated on a Bio-Rad 4-15% SDS-polyacrylamide gel (Hercules, CA) and transferred to a Millipore polyvinylidene difluoride (PVDF) membrane (Bedford, MA), and the membranes were blocked with 5% BSA in Tris-buffered saline–0.1% Tween 20 (TBST) buffer. Then, the membrane was sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. The bands were visualized using the SuperSignal enhanced chemiluminescence (ECL) detection system and recorded using a Bio-Rad Gel Documentation 2000 system (Hercules, CA). The primary antibodies were purchased from the following sources: anti-Nrf2 from Abcam (Cambridge, MA), anti-HO-1 from Cell Signaling (Boston, MA), and anti-cPLA2, anti-iNOS, anti-NQO-1 and anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA).

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Statistical analysis

All experiments were performed at least three times with similar results. Statistical tests were performed using Student's *t*-test. All *p* values were two-sided, and a *p* value of < 0.05 was considered statistically significant.

Results

API and LUT display differential profiles in inducing ARE-luciferase reporter activity

The MTS assay was used to determine the cytotoxicity of API and LUT in HepG2 cells after 24 h of treatment. Neither compound presented significant toxicity at concentrations up to 12.5 μM (Figure 2A, B); therefore, these concentrations were used for the subsequent studies. To evaluate the transcriptional activation of ARE-luciferase by API and LUT, HepG2-C8 cells were incubated with different concentrations of these compounds (1.56–6.25 μM) for 6 and 12 h. The results demonstrated that API and LUT both induced ARE-luciferase after as little as 6 h of exposure at all the concentrations evaluated, exhibiting significant differences at 6 h for LUT and 12 h for API that were comparable with the effects of the positive control, SFN (6.25 μM) ($p < 0.05$; Figure 3A, B).

API and LUT increase ARE-luciferase reporter activity in part through the PI3K and MAPK signaling pathways

Then it was decided to examine the potential pathways involved in the underlying effect of the ARE activation by API and LUT. It has been suggested that these compounds activate Nrf2 and ARE-related genes through the extracellular signal-regulated protein kinase 2 (ERK2) [15] and PI3K/Akt pathways [28]. Therefore, HepG2-C8 cells were treated with LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K; an upstream kinase of Akt), or with PD98059, an inhibitor of the ERK-1/2 mitogen-activated protein kinase (MAPK) pathway, prior to exposure to API or LUT. The luciferase activity of HepG2-C8 cells with the inhibitors alone was examined; however, no luciferase activity was registered for these treatments (data not shown), whereas the luciferase activity induced by both compounds was nearly abolished in the presence of the PI3K inhibitor (Figure 4A, B). Interestingly, the luciferase activity was significantly decreased ($p < 0.05$) in the presence of the ERK-1/2 inhibitor PD98059 in cells treated with API (Figure 4A), but not in cells treated with LUT (Figure 4B). In addition, no changes were observed in the ARE induction in cells treated with the inhibitors of p38 MAPK (SB203580) or JNK-1/2 (SP600125) (data not shown).

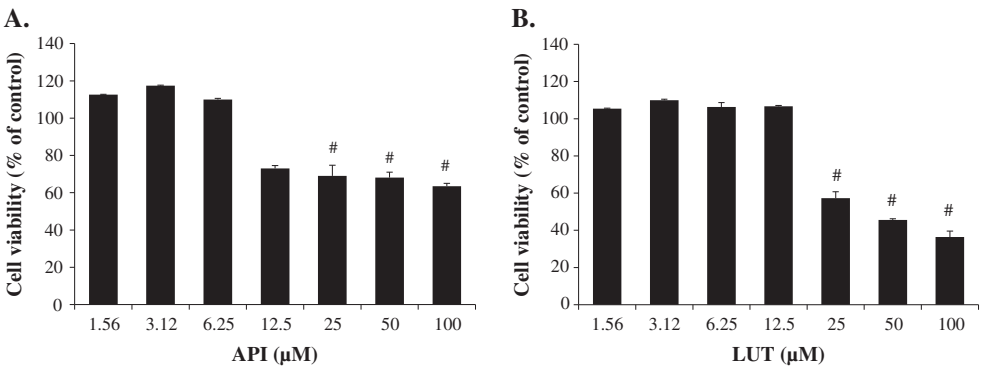

Figure 2. Viability of HepG2 cells after treatment with API (A) or LUT (B) for 24 h. The cells were seeded in 96-well plates in DMEM/1% FBS for 24 h. The cells were then incubated in fresh medium with different concentrations (1.56–100 μM) of API or LUT for 24 h, as described in the Materials and Methods section. Cell viability was determined and calculated using the MTS assay. The data are expressed as the mean ± SD ($n = 3$). Number signs indicate significant differences ($p < 0.05$) in cell viability compared with the control

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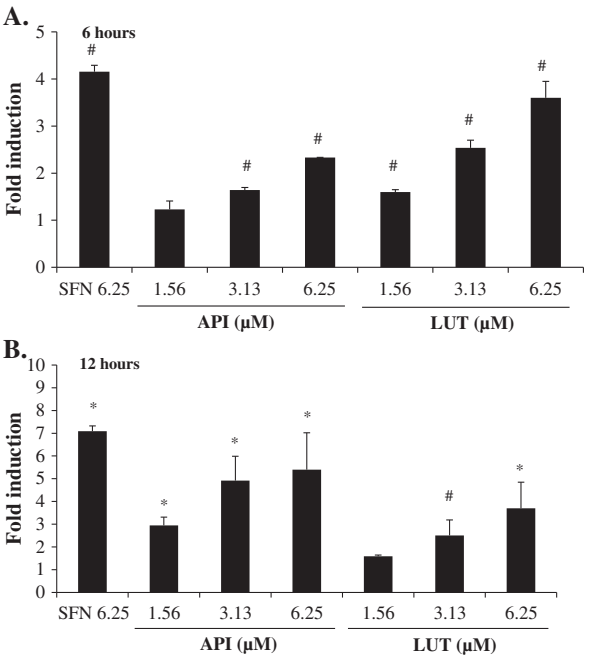

Figure 3. Induction of ARE-luciferase activity by API and LUT at concentrations from 1.56 to 6.25 μM in HepG2-C8 cells after 6 h (A) and 12 h (B). The normalization of the luciferase activity was performed based on the protein concentrations, which were determined using a BCA protein assay, as described in the Materials and Methods section. The data were obtained from three independent experiments and expressed as the inducible fold change compared with the vehicle control. Asterisks and number signs indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between the treatment and the control group

API and LUT increase the mRNA and protein levels of Nrf2 and Nrf2-regulated genes

Because API and LUT showed the strongest induction of ARE-luciferase activity at 12 h (Figure 3A, B), it was decided to evaluate the expression of Nrf2 and its target genes NQO-1 and HO-1 at the mRNA and protein levels in HepG2 cells by quantitative real-time polymerase chain reaction and western blotting, respectively. The results revealed a moderate increase in NQO-1 expression and a significant increase in the mRNA levels of Nrf2 and HO-1 ($p < 0.05$) after treatment with API and LUT compared with the control; this induction was comparable to that observed in the positive control, SFN (6.25 μM) (Figure 5A). At the protein level, a moderate elevation of NQO-1 was observed with all compounds after 12 h of treatment, whereas an increase of Nrf2 and HO-1 expression was observed with API and LUT for all doses evaluated ($p < 0.05$; Figure 5B). Interestingly, when the cells were treated with LUT, the Nrf2 protein level was increased by approximately 5-fold, which is greater than that induced by SFN (6.25 μM).

API and LUT inhibit NO production and reduce iNOS and cPLA2 protein expression in LPS-induced HepG2 cells

Nrf2 and its downstream gene HO-1 have been reported to be key antiinflammatory mediators that suppress the nuclear factor (NF) kappa B (NF-κB) signaling pathway in LPS induction experiments [29,30]. Furthermore, API and LUT have been reported to block this pathway in both *in vitro* and *in vivo* studies [24,25,31]. Therefore, it was decided to investigate whether the activation of the Nrf2 signaling pathway by API and LUT is related to their potential antiinflammatory effects by evaluating NO production in LPS-induced HepG2 cells. The HepG2 cells were pretreated with API or LUT for 12 h before being challenged with LPS for 24 h. No toxicity was observed in the cells treated with LPS in combination with API or LUT, as demonstrated by the MTS assay (Figure 6A). Next, the nitric oxide level in LPS-treated cells was evaluated with and without pretreatment with API and LUT. It was observed that API and LUT significantly reduced LPS-stimulated NO production in a dose-dependent manner ($p < 0.05$; Figure 6B). LUT exhibited a stronger inhibitory activity than API at all concentrations. Because cPLA2 and iNOS are important inflammatory mediators [32], it was decided to further examine whether their protein levels were affected by API or LUT relative to the control LPS-treated cells. Thus, it was found that LPS stimulated the expression of iNOS and cPLA2 (Figure 6C) and that API and LUT markedly blocked the induction of these proteins in a dose-dependent manner ($p < 0.05$).

Discussion

Extensive reports have shown that the plant flavones API and LUT exert a variety of biological

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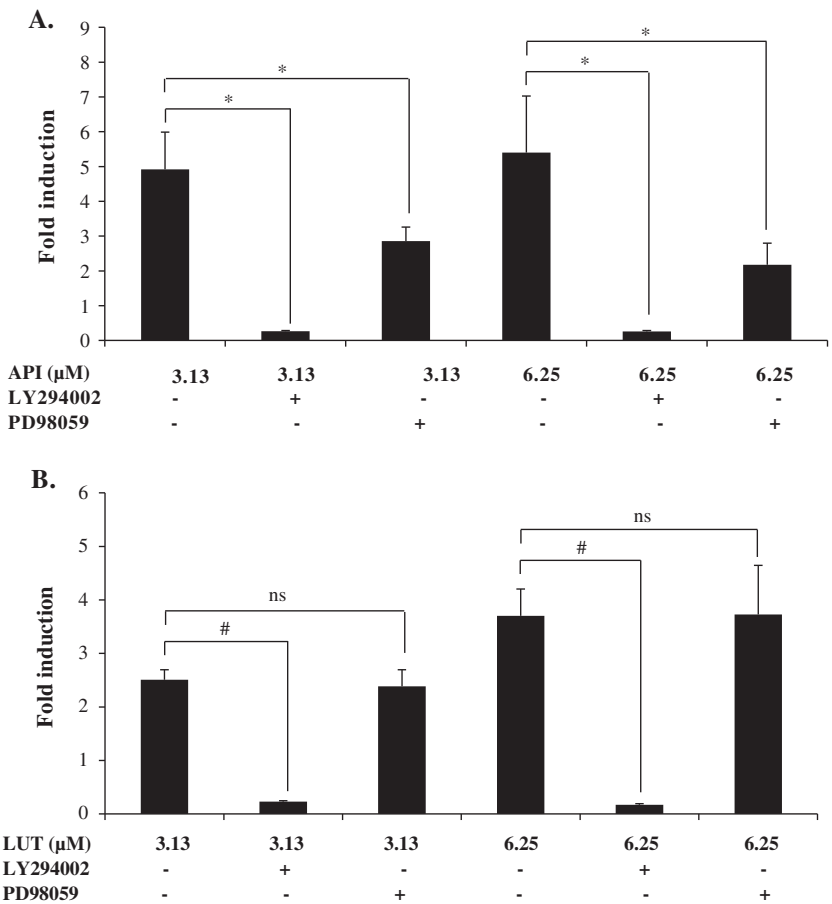

Figure 4. Induction of ARE-luciferase activity by the treatment of HepG2-C8 cells with API (A) and LUT (B) in combination with the phosphoinositide 3-kinase inhibitor, LY294002, or the ERK-1/2 inhibitor, PD98059, during a 12 h treatment, as described in the Materials and Methods section. The data were obtained from three independent experiments and expressed as the inducible fold change compared with the vehicle control. Asterisks and number signs indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between the treatment and control groups

activities, including the modulation of inflammation and carcinogenesis, and thus constitute promising molecules for cancer prevention [4,33]. Recent data indicate that API and LUT protect against oxidative stress through the upregulation of HO-1 mediated via Nrf2 [15,16]. In agreement with the above findings, our data show that API and LUT significantly induced ARE-luciferase activity in HepG2-C8 cells in a dose-dependent manner at 6 h (Figure 3A) and that this activity was maintained or enhanced after 12 h of treatment (Figure 3B). It was also observed that API and LUT significantly induced the mRNA and protein levels of Nrf2 and its target gene HO-1 after 12 h of treatment (Figure 5A, B). The Nrf2-ARE system is regulated by several kinases, such as extracellular signal-regulated kinase (ERK), c-JUN NH_2-terminal protein kinase (JNK), p38, and phosphoinositide 3-kinase (PI3K), and API and LUT exert important biological activities through these pathways [7,8,13]. Therefore, the role of MAPKs in the ARE activation mediated by the flavones was evaluated by using the inhibitors PD98059, SB203580 and SP600125 to perturb signaling through ERK-1/2, p38 MAPK and JNK, respectively. In the presence of the ERK-1/2 inhibitor, the induction of ARE activity by API was significantly decreased, but the other MAPK inhibitors had no effect, indicating a potential contribution of ERK-1/2 to ARE modulation by API

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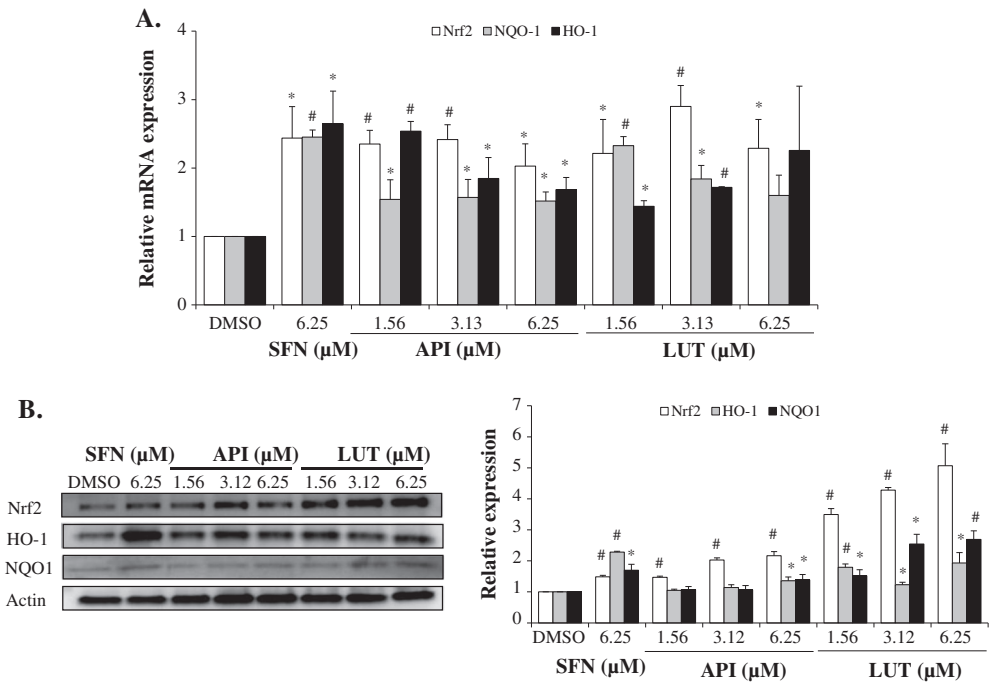

Figure 5. Effect of API and LUT on the relative fold changes of the mRNA (A) and protein (B) levels of Nrf2 and the Nrf2 target genes NQO-1 and HO-1 in HepG2 cells. Cells were incubated with different concentrations of API and LUT (1.56–6.25 μM) for 12 h, as described in the Materials and Methods section. Normalization of the mRNA expression data was performed using β-actin as an internal control. The protein expression level was normalized to β-actin (a complete description of the procedure and antibodies used is provided in the Materials and Methods section). The images were analysed using ImageJ software (NIH, http://rsbweb.nih.gov/ij/). The data are expressed as the mean ± SD of three independent experiments. Asterisks and number signs indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in the relative mRNA and protein expression levels compared with DMSO, which was used as a negative control

(Figure 4A). In contrast, the ARE activity induced by LUT was not affected by the three MAPK inhibitors evaluated (Figure 4B).

These contrasting results may be explained by the nature of the stimuli, the cell type, the sequence of the ARE or the chemical characteristics of these flavones [34]. Although the Nrf2-ARE system has been reported to be modulated by different MAPKs, this topic is poorly studied and highly controversial because modulation may occur through indirect mechanisms with limited effects [35]. Accordingly, after 12 h of treatment, no substantial differences in the activation of the Nrf2 signaling pathway were observed based on the ARE-luciferase activity or in the mRNA levels of the flavones. However, at the protein level, LUT had a moderately stronger effect on Nrf2 expression, but no significant differences between the flavones were observed with regard to HO-1 expression (Figure 5B). In contrast, it was observed that the PI3K inhibitor strongly abrogated ARE activation by API and LUT (Figure 4A, B), which is consistent with a previous report by Lim *et al*. regarding the stimulation of the Nrf2 signaling pathway through PI3K/Akt by the related compound luteolin 6-C-beta-D-glucoside in HepG2 cells [28].

Increasing evidence suggests that Nrf2 is involved in the activation of antioxidant/phase 2 gene transcription machinery and in the regulation of inflammation by modulating the NF-κB pathway through a regulatory feedback loop [20,36,37]. For example, Nrf2-deficient mice challenged with LPS or dextran sulfate sodium (DSS) show a dramatic increase in the activity of the NF-κB inflammatory signaling cascade, and reports indicate that Nrf2 affects the redox status of the cells and modulates NF-κB activation [30,38]. Similarly, Nrf2 knockdown by siRNA in LPS-stimulated HepG2 cells significantly increased the NF-κB transcriptional activity and

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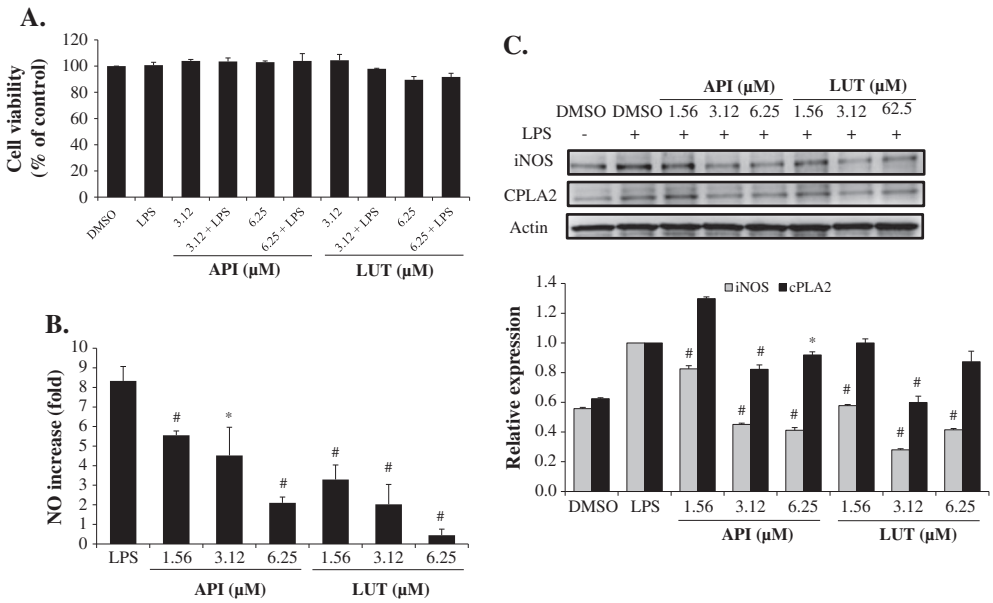

Figure 6. Effect of API and LUT on cell viability (A), NO increase (B) and iNOS and cPLA2 protein expression (C) in LPS-stimulated HepG2 cells. HepG2 cells were pretreated for 12 h before being challenged with 1 μg/ml LPS for 24 h, as described in the Materials and Methods section. The protein expression level was normalized to β-actin, and the images were analysed using ImageJ software (NIH, http://rsbweb.nih.gov/ij/). The data are expressed as the mean ± SD of three independent experiments. Asterisks in (A) indicate significant differences ($p < 0.05$) in cell viability compared with DMSO, which was used as a negative control. Asterisks and number signs in (B) and (C) indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in NO production and relative protein levels compared with LPS treatment

the levels of NF-κB-dependent transcription factors, such as interleukin 6, B-cell lymphoma 2, tumor necrosis factor alpha and cellular inhibitor of apoptosis 2, whereas Nrf2 overexpression reduced NF-κB transcriptional activity, perhaps by regulating the activity MafK-dependent NF-κB [21]. It was observed previously that LPS-induced NF-κB activation can be attenuated by Nrf2 inducers such as phenethyl isothiocyanate, sulforaphane and curcumin [39]. Although the mechanism by which Nrf2 modulates inflammatory signals is not fully understood, some studies suggest that the Nrf2 target gene HO-1 may be involved in this activity because its upregulation has been correlated with cytoprotective effects in various inflammatory diseases [40].

API and LUT both significantly increased HO-1 expression at the mRNA and protein levels (Figure 5A, B); therefore, it was hypothesized that API and LUT would demonstrate antiinflammatory activity in HepG2 cells challenged with LPS because NF-κB plays a pivotal role in the response to LPS via Toll-like receptor 4 (TLR4) [41,42]. Activated TLR4 induces the expression of a broad spectrum of mediators, including inducible nitric oxide synthase (iNOS), which has been shown to depend on NF-κB activation [43]. iNOS catalyses the oxidative deamination of L-arginine to produce NO, which is a potent proinflammatory and tumorigenesis mediator [43–45]. In our study, it was observed that the treatment of LPS-induced HepG2 cells with API or LUT reduced the production of NO in a dose-dependent manner (Figure 6B); this result correlates with the suppression of iNOS protein expression by these flavones (Figure 6C) and is consistent with the results of other studies regarding their antiinflammatory activities [46,47].

In addition, it was reported that Nrf2 plays an important role in the regulation of proinflammatory mediators, such as cyclooxygenase (COX2), arachidonate 5-lipoxygenase (5-LOX), prostaglandin E2, leukotriene B4, iNOS and cytosolic phospholipase A2 (cPLA2), by using Nrf2-knockout mice [20,38,48]. cPLA2 catalyses the release of arachidonic acid, which is the limiting substrate

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for enzymes such as 5-LOX and COX, and it was observed that cPLA2 is a consistent pro-inflammatory pathway affected by Nrf2 deletion [20,38,48]. cPLA2 is of interest because its overexpression has been associated with the pathogenesis of several types of cancer by stimulating proliferation and angiogenesis, which affect tumor progression and resistance to therapies [49,50]. In our study, it was observed that API and LUT significantly suppressed the induction of cPLA2 protein in LPS-treated HepG2 cells in a dose-dependent manner (Figure 6C).

Conclusion

The results of this study demonstrate that the natural flavones API and LUT both activate Nrf2 and downstream genes, particularly HO-1, in HepG2 cells at low doses, and this activation of Nrf2 seems to be related to their potent antiinflammatory activities seen in LPS-stimulated HepG2 cells. Therefore, API and LUT hold great promise as chemopreventive agents that promote antioxidative/antiinflammatory activities.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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