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Protection of chlorophyllin against oxidative damage by inducing HO-1 and NQO1 expression mediated by PI3K/Akt and Nrf2

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Abstract
Green vegetables are thought to have a chemoprotective effect on the basis of epidemiologic evidence. This study investigated whether chlorophyllin (CHL) could induce antioxidant enzymes and confer protection against oxidative damage. The results showed that CHL could induce HO-1 and NQO1 expression in human umbilical vein endothelial cell (HUVEC) in a time- and dose-dependent manner and protect them against hydrogen peroxide caused oxidative damage. The induction of HO-1 and NQO1 by CHL was accompanied with the accumulation of transcription factor Nrf2 in nucleus and the activation of PI3K/Akt signalling pathway. Additionally, the specific inhibitor of PI3K/Akt could obviously decrease not only the induced expression of HO-1 and NQO1 but also the antioxidant effect of CHL. In conclusion, this study proved that CHL exerts antioxidant effect by inducing HO-1 and NQO1 expression mediated by PI3K/Akt and Nrf2. One thinks CHL may have promise to be prophylactic pharmaceuticals without adverse effects.

Keywords: *Free radicals, HO-1, NQO1, chlorophyllin, Nrf2, PI3K/Akt*

Abbreviations: *CO, carbon monoxide; CHL, chlorophyllin; DMSO, dimethyl sulphoxide; ESR, electron spin resonance; HO-1, heme oxygenase-1; HUVEC, human umbilical vein endothelial cell; NQO1, NAD(P)H: quinone oxidoreductase 1; β-NF, β-Naphthoflavone; Nrf2, NF-E2-related factor 2; PBS, phosphate buffered solution; PI3K, phosphoinositide 3-kinase; PI, propidium iodide; ROS, reactive oxygen species; rpm, rounds per minute; MTT, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ZnPPIX, zinc protoporphyrin IX.*

Introduction

Free radicals are continuously generated in aerobic cells during normal metabolism. Traditionally, reactive oxygen species (ROS) were viewed solely as non-regulated by-products of cellular respiration and metabolism. However, recent knowledge suggests that ROS are produced in a controlled fashion and have a critical signalling function [1–4]. However, excess generation of ROS in cells has great potential to damage cellular proteins, lipids, DNA, etc. Oxidative damage has been implicated in various diseases including cancer, diabetes, ageing, Alzheimer's and Parkinson's disease, arthritis, inflammatory skin diseases and vascular diseases such as atherosclerosis and restenosis [5].

Thus, it is valuable to identify some antioxidants that can antagonize the deleterious action of ROS on biomolecules. It is now well established that the human diet contains a diverse array of chemopreventive agents [6]. However, many phytochemicals elicit chemopreventive effect in experimental animals at doses far above the concentrations commonly

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encountered in the human diet. Fortunately, chlorophyll may be one promising exception. Both natural chlorophyll and commercial-grade derivatives such as sodium copper chlorophyllin (CHL) have been widely investigated for a range of beneficial biological activities including wound healing, anti-inflammatory properties, control of calcium oxalate crystals and internal deodourization [7–11]. When CHL is prepared from crude plant extracts, it contains two primary chemical species, copper chlorin e6 and copper chlorin e4. Notably, chlorophyll and CHL are both potent inducers of the detoxification enzymes. To some extent, it may account for the chemoprotective effect widely ascribed to green vegetables on the basis of epidemiologic evidence [12].

Induction of the phase II response is being progressively recognized as an effective and sufficient strategy for protecting cells against oxidants. NF-E2-related factor 2 (Nrf2) is the transcription factor that, upon activation by oxidative stress, binds to the antioxidant response element (ARE) and activates transcription of ARE-regulated genes [13,14]. ARE-regulated genes may contribute to the maintenance of redox homeostasis by serving as endogenous antioxidant systems through the action of proteins such as Heme oxygenase-1 (HO-1), ferritin, glutathione peroxidase (GPx), NAD(P)H: quinone oxidoreductase (NQO1), etc. [15]. Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme. Among the three HO isoforms reported, HO-1 is highly inducible by hemin and by a vast array of non-heme substances such as endotoxin, heavy metals, hydrogen peroxide, ultraviolet light or sulphydryl reagents [16,17], suggesting that HO-1 may play a significant role in protection tissues from oxidative injuries. NQO1 catalyses two-electron reduction and detoxification of quinones and its derivatives without formation of free radicals (semiquinones) and highly reactive oxygen species, so it can protect cells against adverse effect of quinones and their derivatives [18–20]. As a protective agent, NQO1 activity has been shown to prevent the formation of highly reactive quinone metabolites, detoxify benzo(a)pyrene quinone and reduce chromium (VI) toxicity [21]. Additionally, as many studies reported, the activation of the PI3K/Akt signalling was an important checkpoint in the induction of phase II enzymes [22–24].

In this study, we investigated whether CHL could induce antioxidant enzymes such as HO-1 and NQO1 through PI3K and Nrf2 and protect human umbilical vein endothelial cell (HUVEC) against oxidative damage.

Materials and methods

Chemicals and reagents

Chlorophyllin, hemin, β-Naphthoflavone (β-NF), zinc protoporphyrin IX (ZnPPIX), dicoumarol, propidium iodide (PI) and tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). LY294002 was obtained from Cell Signaling Technology (Beverly, MA). HUVEC was kindly provided by Professor Zong-yao Wen (Department of Biophysics, Peking University). All other reagents were of analytical grade.

Cell-survival assay by flow cytometry

After allowing the cells to grow to 70% confluence, $\sim 2 \times 10^6$ cells per sample were incubated in the presence or absence of CHL for 12 h at 37°C, then treated with H_2O_2 (1 mM). After 12 h, cells were harvested and stained with PI at a final concentration of 10 μg/ml at room temperature for 15 min. Flow-cytometric analysis was performed with a FACSCalibur flow cytometer and the CellQuest software (BD, Germany). Respectively, 488 nm excitation and 630 emission filters were selected. Total 10 000 cells were analysed. The percentage of PI-negative (surviving) cells was calculated by single-parameter analysis of the PI-related fluorescence [25].

Electron spin resonance (ESR) spectra of free radicals

Spin trap agent was dispensed as: PBN and DETAPAC dissolved in PBS buffer (pH 7.4) at a final concentration of 100 and 2 mM, respectively. To $\sim 2 \times 10^6$ cells, 1 ml of spin trap agent was added. HUVEC homogenates were centrifuged at 14 000 rounds per minute (rpm) for 10 min after adding 0.6 ml ethyl acetate. Finally, the ethyl acetate extracts were taken in a test tube and stored at 4°C in the dark for 2 h. The specimen was then transferred into a quartz tube for ESR measurement. An X-band ESR spectrometer (Varian, CA) was used to detect the levels of free radicals in HUVEC directly at room temperature (25°C) with measurement conditions as: X-band, central magnetic field (3445 G), scan width (200 G), microwave power (20 mW), frequency (100 kHz), modulation amplitude (2.5 G) and time constant (0.128 s) at 37°C. Before measurement, ESR spectrometer was standardized with diphenyl-picri-hydrazyl to make sure that the equipment was in the same condition every time the experiment was performed [26,27].

Analysis of mRNA levels by semi-quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (In-Vitrogen Lifetechnology, CA). Total RNA (1 μg) was reversely transcribed into cDNA with random hexamers. The primer pairs were as follows: HO-1 (size of PCR product, 273 bp): sense 5′-CGC TAC CTG GGT GAC CTG TCT-3′, antisense 5′-CTT GGT GTC ATG GGT CAG CAG-3′; NQO1 (size of PCR product, 264 bp): sense 5′-CTG CAT TTC TGT

GGC TTC CAA-3′, antisense: 5′-GAT GGA CTT GCC CAA GTG ATG-3′; β-actin (size of PCR product, 228 bp): sense 5'-AGC CAT GTA CGT AGC CAT CC-3', antisense 5'-CTC TCA GCT GTG GTG GTG AA-3'. Equal volume of RT-PCR products were separated on an agarose gel (1.5%, w/v) and visualized by ethidium bromide staining with a gel documentation system (Gene Genius Bio Imaging System, MA).

Preparation of cytoplasmic and nuclear proteins

At the indicated time points, HUVEC was washed with ice-cold PBS, then scraped in the presence of ice-cold hypotonic lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF) and placed on ice for 15 min. After they were homogenized gently, the nuclei were recovered by centrifugation at 3000 g for 15 min and the supernatant was kept as the cytoplasmic extract. The nuclei were washed once with nucleus wash buffer (10 mM Hepes, pH 7.9, 0.2 mM $MgCl_2$ and 10 mM KCl) and resuspended in ice-cold nucleus lysis buffer (20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT and 0.2 mM PMSF) for 30 min on ice. Insoluble material was removed by centrifugation at 21 000 g for 10 min. The supernatant was used as the nuclear extract. Protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma, MO).

Western blotting

Equal amounts of proteins were mixed with loading buffer and subjected to electrophoresis using 12% (w/v) SDS-polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membrane (Hybond-C Extra, Amersham) and the non-specific bindings were blocked with 5% (w/v) non-fat dried milk dissolved in TBS-T buffer. The membranes were then incubated for 1 h at room temperature with HO-1 (Chemicon, CA), NQO1 (Santa Cruz, CA), Nrf-2 (Abcam, Cambridge, UK) and β-actin (Santa Cruz, CA) antibodies diluted, respectively, at 1:1,000, 1:200, 1:500 and 1:1000. Subsequently, membranes were incubated with goat anti-rabbit (1:5000 dilution) horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized on X-ray film activated by chemiluminescence using the ECL Western blotting system (Amersham Pharmacia Biotech, Sweden). Intensities of each band signal were determined by densitometry and image densities of specific bands for HO-1, NQO1 and Nrf-2 at each time point were normalized with the density of respective β-actin band.

Immunofluorescence confocal laser microscopy

Cells were cultured on glass coverslips coated with poly-L-lysine. After they were fixed with formalin and permeabilized with cold acetone, immunofluorescence assay was performed using HO-1 or NQO1 primary antibody and FITC-labelled secondary antibody. The cells were stained with DAPI (Vector, CA) to visualize the nuclei and analysed under a confocal laser microscope (Leica, Germany). The fluorescent intensity and confocal laser microscopic images were held constant to allow for comparison of relative fluorescence intensities between control and treated cells.

Heme oxygenase activity assay

Heme oxygenase activity was using the method described by Ryter et al. [28] with some modifications. HUVEC was homogenized and centrifuged to obtain the microsomal fraction. The microsomal fraction was added to the reaction mixture containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 20 μM hemin, 100 mM potassium phosphate buffer, pH 7.4. After 2 mg of rat liver cytosol was added as a source of biliverdin reductase, the mixtures were incubated in the dark at 37°C for 1 h and placed on ice to terminate the reaction. Bilirubin formed was determined by calculating the difference in absorbance between 464 and 530 nm (extinction coefficient, 40 mm^{-1} cm^{-1} for bilirubin). HO activity was expressed as nanomoles of bilirubin formed per milligram of protein per hour.

NQO1 activity assay

The induction of NQO1 was determined by utilizing the 'Prochaska' bioassay with some modifications in both Hepa1c1c7 murine hepatoma cells and HUVEC [29,30]. β-NF was used as standard in all bioassays. When the cells grew to 70% confluence in 96-well micro-plates, the test compounds dissolved into dimethyl sulphoxide (DMSO) were diluted into the media for 24 h. After the cells were lysed by incubation with a solution containing 0.8% (w/v) digitonin and 2 mM EDTA, pH 7.8 at 37°C for 10 min, the complete reaction mixture was added to each well with the aid of a multiple pipetting device, which contained 25 mM Tris-Cl (PH7.4), 0.6 mg/ml bovine serum albumin, 5 μM FAD, 1 mM glucose 6-phosphate, 30 μM NADP, 2 U/ml glucose 6-phosphate dehydrogenase, 0.3 mg/ml MTT and 50 μM menadione. The reaction was arrested after 5 min by the addition of dicoumarol (50 μM). Then the plates were scanned at 610 nm. The potential of the inducers was expressed as the ratio of the treated and the control.

Statistical analysis

Statistical analyses were performed by SPSS 12.0 software and the results were presented as mean ± SD. Statistical significance of differences among groups was determined by analysis of variance, followed by the Student–Newman–Keuls test for between groups difference. A p-value < 0.05 was regarded as significant.

Results

CHL treatment induced expression of HO-1 and NQO1 in a dose- and time-dependent manner

The induction of phase II drug metabolizing and antioxidant enzymes represents a primary cellular defensive mechanism. However it is still unclear whether these enzymes are inducible in HUVEC by CHL treatments. As observed in Figure 1, CHL treatment caused a steady increase of HO-1 and NQO1 mRNA within the range of 10–200 μM, especially at the concentration of 50 μM. An obvious increase of HO-1 mRNA was detected at 1 h and reached a maximum of nearly 7-fold at 6 h, then declined to the basal level. While for NQO1 at the same concentration of CHL, an increase in mRNA level was detected at 2 h and reached a maximum of nearly 5-fold at 12 h, then decreased gradually.

For Western blotting, CHL treatment also significantly induced the protein expression of HO-1 and NQO1 in HUVEC within the range of 10–200 μM, especially at the concentration of 50 μM, as seen in Figure 2. When cells were treated with CHL (50 μM), an increase of HO-1 protein was detected at 2 h and reached a maximum of nearly 6-fold at 12 h and then declined to the basal level. While for NQO1 at the same concentration of CHL, an increase in protein level was detected at 6 h and reached a maximum of nearly 7-fold at 24 h and then declined gradually.

To further confirm the effect of CHL treatment on the expression of HO-1 and NQO1 in the view of morphology, immunofluorescence assay was used with confocal laser microscope. As shown in Figure 3A, HUVEC was pre-treated with CHL (50 μM) for 12 h and hemin (50 μM) was chosen as the positive control for the assay. CHL treatment induced significant expression of HO-1, although less than hemin. LY294002, the specific inhibitor of PI3K, could decrease HO-1 expression to some extent. In Figure 3B, CHL treatment induced significant expression of NQO1 after HUVEC was pre-treated with CHL (50 μM) for 24 h, but less than β-NF. β-NF (2 μm) was chosen as the positive control. LY294002 could also decrease NQO1 expression.

Increased enzymatic activity of HO-1 and NQO1 in CHL-treated HUVEC

To confirm whether increased expression of HO-1 and NQO1 resulted in the elevation of enzymatic activity, the enzymatic activity in CHL-treated HUVEC was measured. For HO-1, HUVEC was

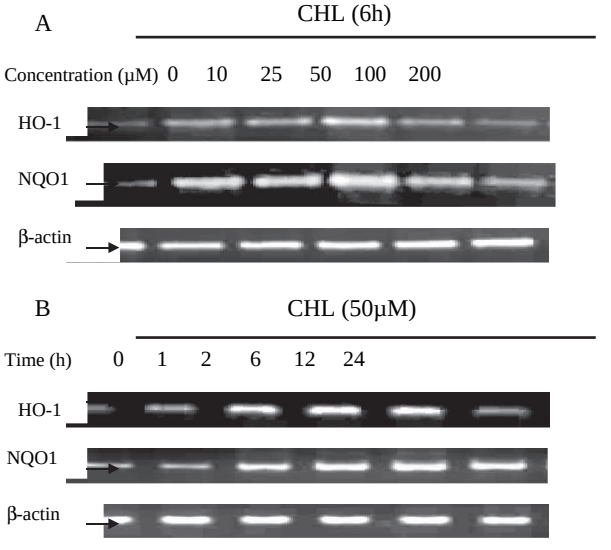

Figure 1. CHL treatment increased HO-1 and NQO1 mRNA in a dose- and time-dependent manner. (A) When the cells were exposed to various concentrations of CHL, an obvious increase of HO-1 and NQO1 mRNA was detected within the range of 10–200 μM, especially at the concentration of 50 μM. (B) When cells were treated with CHL (50 μM), the maximal transcriptional activation of HO-1 and NQO1 by CHL was observed at 6 h and 12 h, respectively, and then decreased gradually. The figures shown are representative of five independent experiments with similar results.

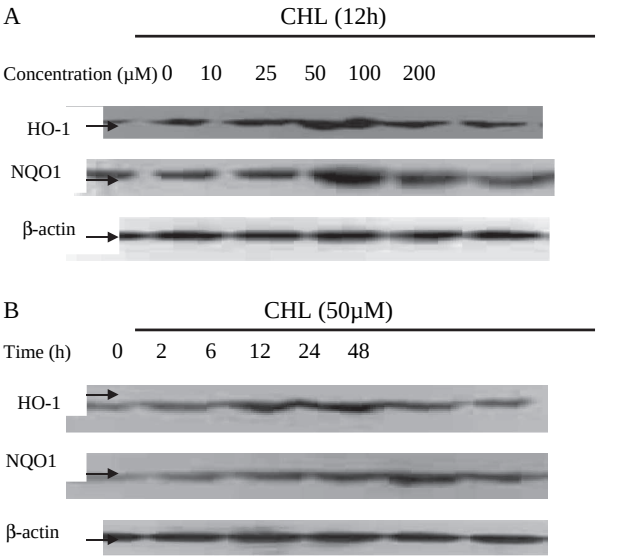

Figure 2. CHL treatment induced HO-1 and NQO1 protein in a dose- and time-dependent manner. (A) CHL treatment could increase the expression of HO-1 and NQO1 protein within the range of 10–200 μM and the most optimum concentration was at 50 μM. (B) The induction of HO-1 and NQO1 proteins by CHL was time dependent. The maximal induction of HO-1 and NQO1 was seen at 12 h and 24 h, respectively, after CHL treatment. The figures shown are representative of five independent experiments with similar results.

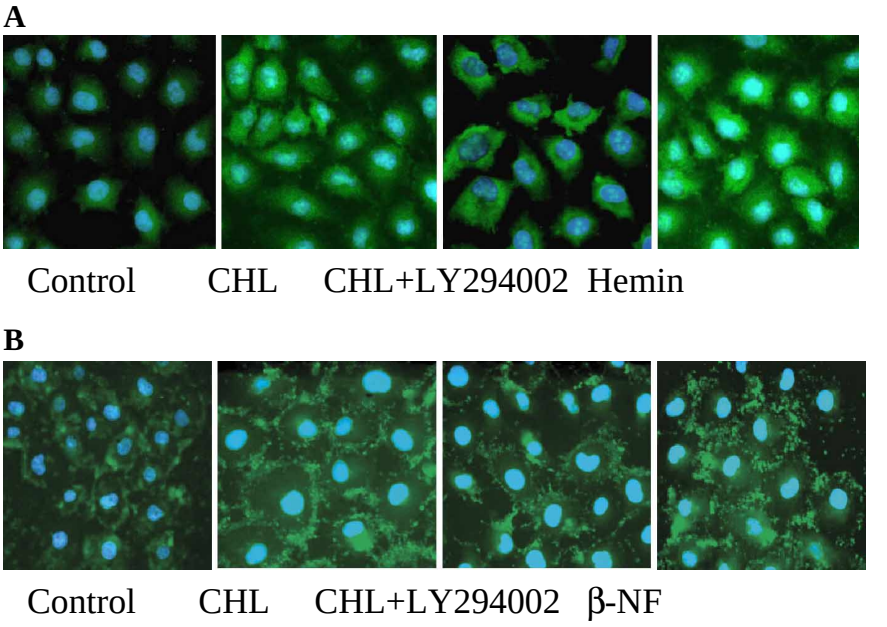

Figure 3. CHL treatment inducted HO-1 and NQO1 expression scanned by confocal laser microscope. (A) CHL induced significant expression of HO-1 after HUVEC was pre-treated with CHL (50 μM) for 12 h, although less than hemin. (B) CHL induced significant expression of NQO1 after HUVEC was pre-treated with CHL (50 μM) for 24 h, although less than *β*-NF. The induced expression of HO-1 and NQO1 could be decreased by LY294002, the specific inhibitor of PI3K. The figures shown are representative of three independent experiments with similar results. Original magnification ×400.

pre-treated with CHL (50 μM) for 12 h and harvested to prepare microsomal fractions. Hemin (50 μM) was chosen as the positive control for the assay. As shown in Figure 4A, CHL treatment induced a significant increase of HO-1 activity in HUVEC, although less than hemin. ZnPPIX, the specific inhibitor of HO-1, and LY294002 could both decrease HO-1 activity to some extent.

For NQO1, HUVEC was pre-treated with CHL (50 μM) for 24 h, then harvested and homogenated. NQO1 activity was determined by the Prochaska bioassay with some modifications as described in Materials and methods. *β*-NF (2 μM) was chosen as the positive control for the assay. As shown in Figure 4B, CHL treatment induced a significant increase of NQO1 activity in HUVEC, but less than *β*-NF. Dicoumarol, the specific inhibitor of NQO1, and LY294002 could both decrease NQO1 activity to some extent.

In our experiments, we also measured the NQO1 activity in Hepa1c1c7 murine hepatoma cells with the Prochaska bioassay. The effect of CHL treatment on NQO1 activity was similar with that in HUVEC (data shown).

Involvement of Nrf2 translocation during CHL induced HO-1 and NQO1 expression

It is well known that Nrf2 is sequestered in the cytoplasm by Keap1 protein under normal condition and the translocation of Nrf2 into the nucleus is essential for the transactivation of various target genes. The primary control of the Nrf2 function for phase II gene induction relies on subcellular distribution rather than induction of this transcription factor through *de novo* synthesis. To confirm whether CHL induces phase II genes through regulation of Nrf2, we compared subcellular localization of Nrf2 in CHL exposed cells. As shown in Figure 5, the Nrf2 protein level in the nucleus increased 0.5 h after the CHL treatment in parallel with lowering of the Nrf2 protein in the cytoplasm. The translocation of Nrf2 into the nucleus reached a maximum of nearly 5-fold at 1 h and then recovered to normal state gradually. The increase of Nrf2 protein in nucleus induced by CHL was shown to occur prior to the increase of HO-1 and NQO1 expression. Hence, CHL may trigger the translocation of Nrf2 into the nucleus and promote transcription of target genes.

Involvement of the PI3K/Akt pathway in CHL induced HO-1 and NQO1 expression

To identify the signalling cascades activated by CHL, we analysed the effects of CHL on the survival pathway, PI3K/Akt. The activation of the pathway was examined using specific antibodies which selectively recognized the phosphorylated and active form of Akt. As shown in Figure 6, an obvious activation of PI3K/Akt by CHL could be observed 10 min after stimulation and reached a maximum at 60 min and then declined gradually.

To confirm the effect of the inhibitor of PI3K on the activation of PI3K/Akt induced by CHL, HUVEC was

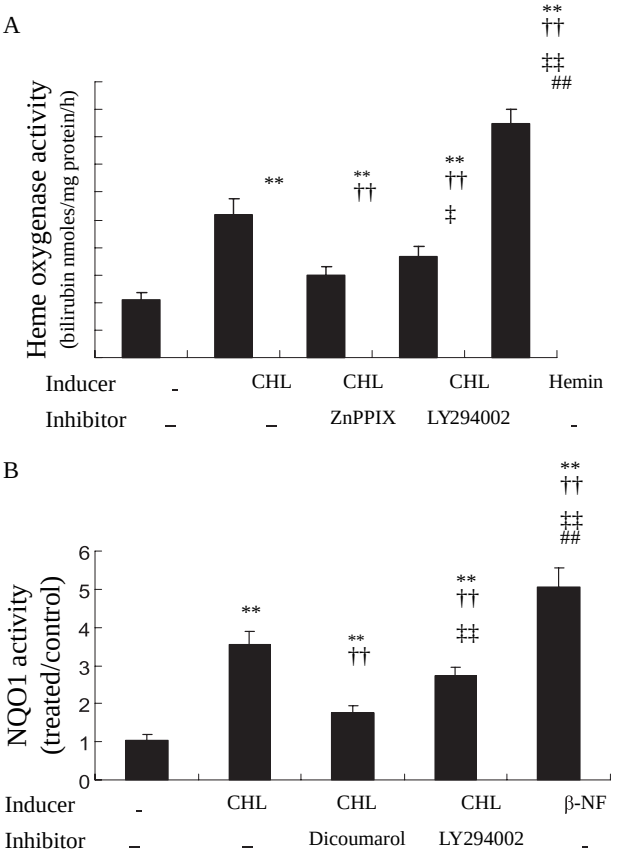

Figure 4. Effect of CHL treatment on HO-1 and NQO1 activity. (A) After pre-treatment with CHL (50 μM) for 12 h, CHL induced a significant increase of HO-1 activity, although less than hemin. ZnPPIX, the specific inhibitor of HO-1, and LY294002 could both decrease HO-1 activity to some extent. (B) After pre-treatment with CHL (50 μM) for 24 h, CHL treatment induced a significant increase of NQO1 activity, although less than β-NF. Dicoumarol, the specific inhibitor of NQO1, and LY294002 could both decrease NQO1 activity to some extent. Bar graph shows mean ± SD obtained from five independent experiments with similar results. $^{*}p<0.05$, $^{**}p<0.01$ compared with control group; $\dagger p<0.05$, $\dagger\dagger p<0.01$ compared with CHL group; $\ddagger p<0.05$, $\ddagger\ddagger p<0.01$ compared with ZnPPIX-CHL (HO-1) or Dicoumarol-CHL (NQO1) group; $\# p<0.05$, $\#\# p<0.01$ compared with LY294002-CHL group.

pre-incubated with LY294002 for 30 min and stimulated with CHL (50 μM) for 60 min. As shown in Figure 7A, the inhibitor markedly reduced the CHL

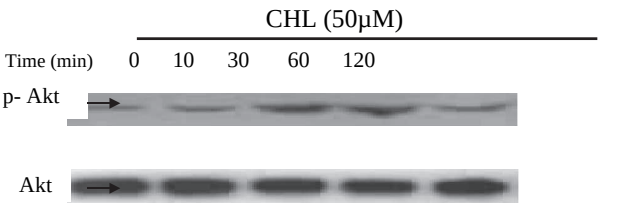

Figure 6. Roles of the PI3K/Akt pathway in CHL induced expression of HO-1 and NQO1. HUVEC was stimulated with CHL (50 μM) for indicated times. An obvious activation of PI3K/Akt could be observed 10 min after stimulation and reached a maximum at 60 min and then declined to the basal level. The figure shown is representative of five independent experiments with similar results.

induced activation of PI3K/Akt at the concentration of 10–50 μM. To further elucidate the role of PI3K/Akt signalling pathway, the effect of LY294002 on the up-regulation of HO-1 and NQO1 expression was examined. As shown in Figure 7B and C, the increase in mRNA and protein levels of HO-1 and NQO1 induced by CHL was significantly suppressed by LY294002. The effect of LY294002 was dose-dependent over the range of 5–50 μM. Additionally, the CHL induced translocation of Nrf2 to the nucleus was also suppressed in the presence of LY294002 (data not shown). It can be deduced that the PI3K/Akt signalling pathway is required in the CHL induced up-regulation of HO-1 and NQO1 expression.

CHL protected HUVEC against H_2O_2 caused oxidative damage

To examine the chemopreventive role CHL against oxidative damage, HUVEC was pre-treated with CHL and then exposed to H_2O_2. Cell-survival assay was analysed by flow cytometry as described in Materials and methods. As shown in Figure 8, CHL itself had nearly no effect on normal HUVEC. The cellular survival rate decreased sharply after treatment with H_2O_2 (1 mM), while pre-treatment with CHL (50 μM) for 12 h could protect HUVEC against oxidative damage. ZnPPIX, the specific inhibitor of HO-1, could attenuate the cytoprotective

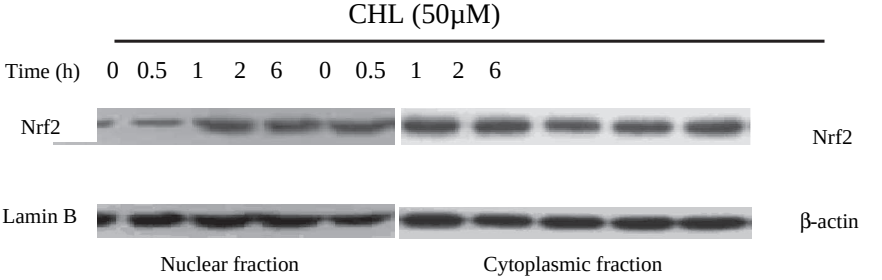

Figure 5. Effect of CHL treatment on Nrf2 translocation. After HUVEC was pre-treated with CHL (50 μM) for indicated times, cytoplasmic and nuclear proteins were prepared for Western blotting. Nrf2 protein level in the nucleus increased significantly 1 h after CHL treatment in parallel with lowering of the Nrf2 protein level in the cytoplasm. The figure shown is representative of five independent experiments with similar results.

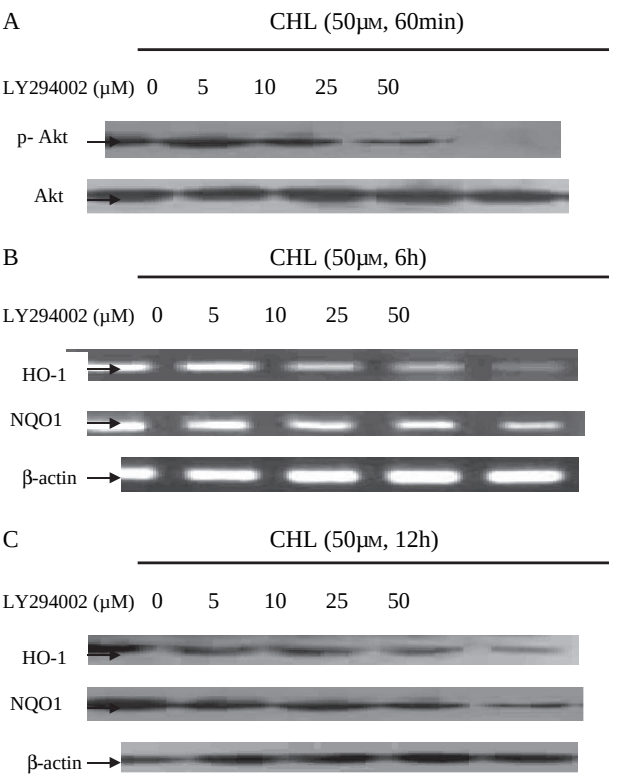

Figure 7. Effects of the inhibitor of PI3K on the activation of PI3K/Akt and on HO-1 and NQO1 expression induced by CHL. Cells were pre-incubated for 30 min with various concentrations of LY294002 and then treated with CHL. (A) LY294002 markedly decreased activation of Akt at the concentrations of 10–50 μM. (B and C) The CHL induced up-regulation of mRNA and protein of HO-1 and NQO1 was significantly suppressed by LY294002. The figures shown are representative of five independent experiments with similar results.

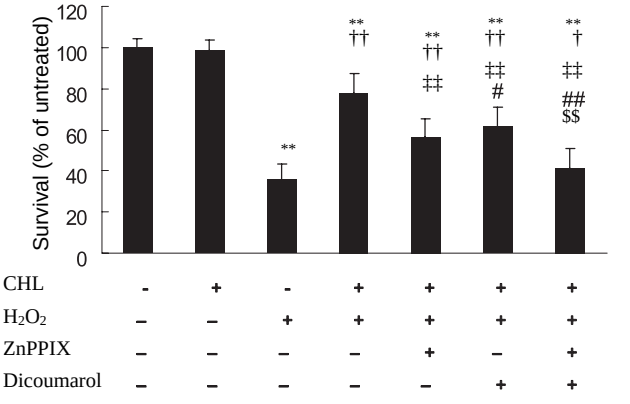

Figure 8. Protection of CHL against H_2O_2 caused oxidative damage. HUVEC was incubated in the presence or absence of CHL for 12 h and then treated with H_2O_2. Pre-treatment with CHL (50 μM) could protect HUVEC against oxidative damage. Either ZnPPIX (50 μM) or dicoumarol (50 μM) could attenuate the cytoprotective role by inhibiting respective enzymatic activity. The two inhibitors had synergetic effect. Bar graph shows mean ± SD, $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with H_2O_2 group; $\ddagger p < 0.05$, $\ddagger\ddagger p < 0.01$ compared with CHL-H_2O_2 group; $\#p < 0.05$, $\#\#p < 0.01$ compared with ZnPPIX -CHL-H_2O_2 group; $\$p < 0.05$, $\$\$p < 0.01$ compared with Dicoumarol-CHL-H_2O_2 group.

action of CHL by inhibiting HO-1 activity. Dicoumarol, the specific inhibitor of NQO1, could also attenuate the protective action. Additionally, the two inhibitors had synergetic effect when they were used together.

Effect of CHL on free radicals caused by H_2O_2

The ESR spectra of the spin trapping adduct of free radicals extracted from the HUVEC with PBN are shown in Figure 9. The peak height of spectrum represented relative concentration of free radicals (Gauss). As Figure 9 shows, nearly no signal of free radicals could be seen in the control and CHL groups. A significant increase of free radicals in the H_2O_2 group was detected 1 h after treatment with H_2O_2 (1 mM), while free radicals in CHL-H_2O_2 group were sharply lower than that in the H_2O_2 group. ZnPPIX could reduce the antioxidant effect of CHL. So did Dicoumarol, by inhibiting NQO1 activity. With the help of ESR technology, it can be shown directly that CHL can scavenge the free radicals in HUVEC caused by H_2O_2.

Collectively, the data above indicate that up-regulation of HO-1 and NQO1 expression plays a pivotal role in the protection of CHL against H_2O_2 caused oxidative damage, while the activation of PI3K/Akt signalling pathway and the translocation of transcriptional factor Nrf2 are required in the induction of the antioxidant enzymes such as HO-1 and NQO1.

Discussion

Free radicals have critical function in cellular physiological metabolism. However, excess generation of them in cells has a great deleterious effect through both damaging directly cellular components and disturbing cellular function. It is necessary to identify some compounds with chemopreventive action, which should not only antagonize the deleterious effect of ROS but also be non-toxic and non-stressful.

The induction of HO-1 and NQO1 may be protective against oxidative damage, but the classic chemicals, such as hemin and β-NF, can be regarded as another stress. Hemin can cause substantial generation of intracellular ROS [31,32]. *β*-NF can activate aryl hydrocarbon receptor (AhR) and the AhR-mediated responses may contribute to the carcinogenesis in various tissues [33]. As a result, the phase II gene induction by hemin or BNF failed to protect cells from H_2O_2-caused cellular damage, probably due to the side effects of themselves. While the use of plant-derived non-cytotoxic natural substances to trigger HO-1, NQO1 expression and other cellular defensive enzymes would offer a great advantage for protection against oxidative stress.

In this study we demonstrated that CHL could induce HO-1 and NQO1 expression without any

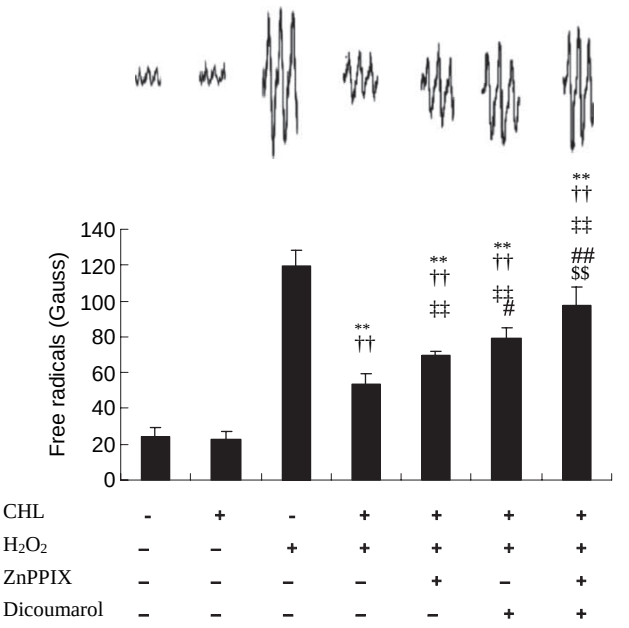

Figure 9. Effect of CHL on free radicals caused by H_2O_2. The peak height of ESR spectrum represented relative concentration of free radicals (Gauss). The signal of free radicals was very weak in the control and CHL treated groups. A significant increase of free radicals in the H_2O_2 group was detected 1 h after treatment with H_2O_2 (1 mM), while free radicals in CHL-H_2O_2 group were sharply lower than that in the H_2O_2 group. Bar graph shows mean ± SD, $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with H_2O_2 group; $\ddagger p < 0.05$, $\ddagger\ddagger p < 0.01$ compared with CHL-H_2O_2 group; $\#p < 0.05$, $\#\#p < 0.01$ compared with ZnPPIX -CHL-H_2O_2 group; $\$p < 0.05$, $\$\$p < 0.01$ compared with Dicoumarol-CHL-H_2O_2 group.

pro-oxidant potential and play an important role in blocking ROS-mediated endothelial cell damage.

As the results show, CHL could induce expression of HO-1 and NQO1 in HUVEC in a time- and dose-dependent manner. The maximal expression of HO-1 and NQO1 induced by CHL was observed at 12 h and 24 h, respectively, after treatment and then decreased gradually thereafter. The induction of HO-1 and NQO1 by CHL was also proved by immunofluorescence assay and enzymatic activity detection. Increased activity of HO-1 and NQO1 induced by CHL may alleviate the cellular damage by scavenging reactive free radicals and reducing reactive semiquinone, respectively.

The induction of HO-1 and NQO1 by CHL in cells is being regarded as an intracellular antioxidative mechanism. It is necessary to study the role of PI3K and Nrf2 in the induction so as to clarify the antioxidative mechanism of CHL.

It is known that the translocation of Nrf2 to the nucleus elicits up-regulation of target genes in various cell types. Nrf2 is sequestered by its cytoplasmic partner Keap1 in normal state [35]. CHL provoked a rapid translocation of Nrf2 into the nucleus, but the translocation was greatly abolished in the presence of a PI3K inhibitor, LY294002, suggesting that Nrf2 is a downstream effector of Akt. Hence, CHL treatment may activate PI3K/Akt pathway and the signals pass through the Keap1-Nrf2 complex, resulting in the dissociation of Keap1–Nrf2 followed by nuclear accumulation of Nrf2. The induction of HO-1 and NQO1 protein by CHL was accompanied with the accumulation of transcription factor Nrf2 in nucleus and the increase of the corresponding mRNA. It can be concluded that the induction of HO-1 and NQO1 expression may occur through transcriptional activation. This transcriptional modulation is presumably to keep the expression of antioxidant enzymes so as to maintain the cellular defenses active and to rapidly restore induced enzymes to normal levels.

Besides HO-1 and NQO1, we can not exclude the possibility that CHL also up-regulates other intracellular antioxidant systems along with HO-1 and NQO1 expression, which also contribute to the long-term antioxidant properties of this compound. The genes that are co-ordinately induced along with HO-1 and NQO1 may include glutathione S-transferase (GST), UDP-glucuronosyl transferase (UDP-GT), epoxide hydrolase (EH), g-glutamylcysteine synthetase (γ-GCS) and so on [36].

With CHL treatment, a battery of phase II enzymes can be induced. As the results (Figures 3 and 4) showed, compared with the classic chemicals such as hemin and β-NF, the inductive potential of CHL for HO-1 and NQO1, respectively, was less or moderate, but the comprehensive protective effect was far better than each of them [31–34]. Additionally, the time course of the enzymatic induction was sequential. As far as our study was concerned, the induction of HO-1 was earlier while NQO1 was later. We may speculate that this mode of induction of phase II enzymes by CHL can moderately protect cells from oxidative stress in a manner of co-ordinated action, which is in accordance with the normal physiological status of cells to some extent.

On the other hand, CHL may exert chemopreventive action through other mechanisms. CHL can form complexes with oxidants or mutagens to promote their excretion [37–39]. CHL may also directly scavenge ROS with its conjugated double bond, the details of which need to be further studied [40–42].

In conclusion, for the first time our study showed that CHL exerts an antioxidant effect by inducing HO-1 and NQO1 expression mediated by PI3K/Akt and Nrf2. It is possible that CHL may also up-regulate other intracellular antioxidant systems along with HO-1 and NQO1. The induction of phase II enzymes by CHL is pleiotropic and sequential, which may contribute to explaining the multiple chemopreventive actions of CHL such as antioxidant, anti-inflammatory and anti-carcinogenic. We think CHL may have promise to be prophylactic pharmaceuticals without side effects for people.

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