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Role of nuclear factor-κB and heme oxygenase-1 in the mechanism of action of an anti-inflammatory chalcone derivative in RAW 264.7 cells

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1 The synthetic chalcone 3′,4′,5′,3,4,5-hexamethoxy-chalcone (CH) is an anti-inflammatory compound able to reduce nitric oxide (NO) production by inhibition of inducible NO synthase protein synthesis. In this work, we have studied the mechanisms of action of this compound.

2 CH (10–30 μM) prevents the overproduction of NO in RAW 264.7 macrophages stimulated with lipopolysaccharide (1 μg ml^{-1}) due to the inhibition of nuclear factor κB (NF-κB) activation.

3 We have shown that treatment of cells with CH results in diminished degradation of the NF-κB–IκB complex leading to inhibition of NF-κB translocation into the nucleus, DNA binding and transcriptional activity.

4 We also demonstrate the ability of this compound to activate NfE2-related factor (Nrf2) and induce heme oxygenase-1 (HO-1).

5 Our results indicate that CH determines a rapid but nontoxic increase of intracellular oxidative species, which could be responsible for Nrf2 activation and HO-1 induction by this chalcone derivative.

6 This novel anti-inflammatory agent simultaneously induces a cytoprotective response (HO-1) and downregulates an inflammatory pathway (NF-κB) with a mechanism of action different from antioxidant chalcones.

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Keywords: 3′,4′,5′,3,4,5-Hexamethoxy-chalcone; nuclear factor-κB; heme oxygenase; NfE2-related factor; nitric oxide

Abbreviations: BSO, buthionine sulfoximine; CH, 3′,4′,5′,3,4,5-hexamethoxy-chalcone; CO, carbon monoxide; DHR, dihydrorhodamine 123; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GSH, glutathione; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; IκB-α, NF-κB inhibitory protein-α; IKK, IκB kinase; LSC, laser scanning cytometry; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; mET, 2-mercaptoethanol; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κB; Nrf2, NfE2-related factor; iNOS, inducible nitric oxide synthase; NO, nitric oxide; Rho, rhodamine; ROS, reactive oxygen species; StRE/ARE, stress- or antioxidant-response element

Introduction

Chalcones are a group of plant metabolites able to modify cell functions involved in inflammatory responses. A number of chalcone derivatives exhibit antioxidant and anti-inflammatory activities (Herencia *et al*., 2001), although the mechanisms of action of this class of compounds are not yet fully understood.

Nitric oxide (NO) mediates the inflammatory and cytotoxic actions of macrophages, which produce high levels of this endogenous free radical upon induction of nitric oxide synthase (iNOS) by endotoxins, cytokines and other stimuli (Knowles & Moncada, 1994). Simultaneous production of NO and superoxide by macrophages leads to formation of peroxynitrite, a reactive species mediating tissue injury (Ischiropoulos *et al*., 1992). The transcription of iNOS in macrophages has been shown to depend on nuclear factor κB (NF-κB) activation (Xie *et al*., 1994), a key factor regulating the expression of crucial mediators involved in chronic inflammatory diseases and cancer, including inflammatory cytokines, inducible enzymes and adhesion molecules (Tak & Firestein, 2001). Consequently, agents that inhibit the activation of this transcription factor have the potential for therapeutic interventions. It is interesting to note that some chalcones with antioxidant properties have been shown to inhibit NO production and iNOS expression in activated macrophages by interfering with NF-κB activation (Cheng *et al*., 2001).

Heme oxygenase (HO) activity catalyzes heme degradation leading to iron release and production of carbon monoxide (CO) and biliverdin that is reduced to bilirubin. CO activates soluble guanylyl cyclase and may exert effects similar to those of NO, including regulation of vascular tone, neuronal signaling or modulation of apoptosis (Maines, 1997; Petrache *et al*., 2000; Baranano & Snyder, 2001), whereas biliverdin and bilirubin are antioxidant molecules. HO-1 can be induced by a

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variety of agents such as heme, cytokines, endotoxin, mitogens, metals, oxidative stress, heat shock, UV radiations and NO (Otterbein & Choi, 2000), in contrast to the constitutive isoenzymes HO-2 and HO-3 (Maines *et al*., 1986; McCoubrey *et al*., 1997).

Reactive oxygen species (ROS) have been implicated as signaling intermediates in different cell types with the ability to upregulate redox-sensitive nuclear factors. Induction of HO-1 can be a general response to oxidative stress as a cellular defense mechanism. It is known that genes encoding enzymes with an antioxidant role can be regulated by Nrf2 (NfE2-related factor) *via* the stress- or antioxidant-response elements (StRE/ARE). This transcription factor is bound to different proteins in the cytoplasm and redox-dependent stimuli would antagonize repression by proteins such as Keap1, leading to Nrf2 nuclear translocation and gene transcription (Itoh *et al*., 1999). Thus, electrophilic compounds stimulate Nrf2-mediated transcription of HO-1 (Balogun *et al*., 2003).

Several lines of evidence indicate that expression of HO-1 induced by either pharmacological treatment or gene transfer results in the attenuation of cellular damage and inflammation. For example, an anti-inflammatory role has been suggested for HO-1 (Willis *et al*., 1996; Inoue *et al*., 2001), as we have recently observed in the zymosan-injected mouse air pouch (Vicente *et al*., 2003) and chondrocytes from osteoarthritic patients (Fernandez *et al*., 2003).

We have previously reported the anti-inflammatory properties of a synthetic chalcone derivative (3′,4′,5′,3,4,5-hexamethoxy-chalcone, CH) *in vitro* and *in vivo* (Herencia *et al*., 1999). This compound reduced NO production in mouse macrophages stimulated with lipopolysaccharide (LPS) by inhibition of iNOS *de novo* protein synthesis without affecting iNOS activity. The expression of iNOS was also inhibited by CH in the zymosan-injected mouse air pouch. In the present work, we have investigated the mechanisms responsible for the observed inhibition of NO production as well as the influence of CH on HO-1. This study provides new insights into the anti-inflammatory mechanisms of chalcone derivatives.

Methods

Cell culture

The mouse macrophage RAW 264.7 cell line (American Type Culture Collection, Manassas, VA, U.S.A.) was cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan. After appropriate stimulation times, cells were incubated with MTT (200 μg ml^{-1}) for 60 min. The medium was then removed and the cells solubilized in dimethyl sulfoxide (100 μl) to quantitate formazan at 550 nm (Gross & Levi, 1992). CH was dissolved in methanol and then diluted in the culture medium (0.1% v v^{-1}). This vehicle was included in control groups.

Northern blot analysis

RAW 264.7 cells (20–40 $\times$ 10^6) were incubated in 10-cm Petri dishes. Total RNA was extracted using the Trizol reagent (Life Technologies Inc., Barcelona, Spain), separated by electrophoresis (12 μg lane^{-1}) and transferred to a nylon membrane. The blots were hybridized at 42°C using α-^{32}P-labeled mouse HO-1 cDNA (Alcaraz *et al*., 2001) or iNOS cDNA (Cayman Chemical, Ann Arbor, MI, U.S.A.), washed and rehybridized with human GAPDH cDNA (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.).

NF-κB promoter activity

Cells (10^6 well^{-1}) were seeded in six-well plates and after 24 h incubation, macrophages were transiently transfected overnight with the pNF-κB-Luc plasmid (Stratagene, La Jolla, CA, U.S.A.) using Fugene 6 (Roche Applied Science, Indianapolis, IN, U.S.A.) according to the supplier's recommendations. Cells were then stimulated with LPS (1 μg ml^{-1}) for 8 h in the presence or absence of CH at different concentrations. After lysis and centrifugation, 100 μl aliquots of supernatants were used to assay luciferase activity using the kit from Promega Corp. (Madison, WI, U.S.A.). Luminescence was measured in a Microbeta counter (Wallac, Turku, Finland).

Electrophoretic mobility shift assay (EMSA)

Macrophages (3 $\times$ 10^6) in 5-cm Petri dishes were preincubated with different concentrations of CH for 30 min and then stimulated with LPS (1 μg ml^{-1}) for 1 h. Nuclear extracts were prepared as described (López-Collazo *et al*., 1998). Cells were washed twice with ice-cold phosphate-buffered saline, and then treated with 0.2 ml of buffer A (20 mM Tris-HCl, pH 7.8, 10 mM KCl, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethyl sulfonyl fluoride and 10 μM leupeptin) for 15 min followed by addition of Nonidet P-40 (0.5% w v^{-1}). The tubes were vortexed for 15 s, and nuclei were sedimented by centrifugation at 8000 $\times$ *g* for 30 s. Aliquots of the supernatant were stored at −80°C (cytoplasmic extract), and the nuclei pellet was resuspended in 50 μl of buffer A supplemented with 0.4 M KCl. After centrifugation at 13,000 $\times$ *g* for 15 min, aliquots of the supernatant (nuclear extract) were stored at −80°C. Protein was determined by the DC Bio-Rad protein reagent (Richmond, CA, U.S.A.). Double-stranded oligonucleotides containing either the consensus NF-κB (Promega Corp., Madison, WI, U.S.A.) or NRE2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) sequences were end-labeled using T4 polynucleotide kinase (Amersham Biosciences, Barcelona, Spain) and [γ-^{32}P]ATP, followed by purification using G-25 microcolumns (Amersham Biosciences, Barcelona, Spain). Incubations were performed on ice with 6 μg of nuclear extract, 100,000 c.p.m. of labeled probe, 2 μg poly(dI-dC), 5% v v^{-1} glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl and 10 mM Tris-HCl buffer (pH 8.0) for 15 min. Complexes were analyzed by nondenaturing 6% polyacrylamide gel electrophoresis in 45 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA followed by autoradiography of the dried gel. To assess a direct interaction of CH with nuclear proteins, in another series of experiments CH was incubated for 30 min with nuclear extracts from LPS-stimulated cells prior to the addition of the oligonucleotide probe. In some experiments, for cell-free activation of latent NF-κB, aliquots (10 μg protein) of cytoplasmic extracts from nonstimulated cells were incubated on ice with Nonidet P-40 (1.1% w v^{-1}) and sodium

deoxycholate (0.8% w v^{-1}) for 10 min before adding the oligonucleotide (Brennan & O'Neill, 1998). In other experiments, antibody against either p65 or p50 (1 μg) was incubated with the released NF-κB to assess the specificity of bands in the EMSA assay.

Western blot analysis

For Western blotting of the proteins of NF-κB and Nrf2 pathways, cytoplasmic or nuclear extracts from RAW 264.7 cells were obtained as indicated in EMSA, and membranes were incubated with polyclonal antibodies against p65 (1/500), NF-κB inhibitory protein (IκB-α, 1/500) or Nrf2 (1/200). Anti-phospho-(Ser^{32}) IκB-α antibody (1/750) was used according to the manufacturer's instructions. Blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1/20,000). The immunoreactive bands were visualized by an enhanced chemiluminescence system (Amersham Biosciences, Barcelona, Spain). For HO-1 protein expression, RAW 264.7 macrophages (10^6 $well^{-1}$) were incubated with CH (10–30 μM) for different times. The supernatants were used for bilirubin determination and cell pellets were lysed in 100 μl of buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl and 25 mM Tris, pH 7.4) and centrifuged at 4°C for 5 min at $10{,}000 \times g$. The protein content was determined by the Bradford method using bovine serum albumin as standard. Cell lysate (40 μg of protein) was mixed with Laemmli sample buffer under reducing conditions. Samples were size-separated by 12.5% SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Barcelona, Spain), which were blocked in phosphate-buffered saline (0.02 M, pH 7.0)–Tween 20 (0.1%) containing 3% fat-free dry milk. Membranes were incubated with specific polyclonal anti-HO-1 antibody (1/1000; Alcaraz *et al*., 2000) or anti-β-actin (1/1000, Sigma Chemical Co., St Louis, MO, U.S.A.). Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/20,000) and the immunoreactive bands were visualized as above.

IκB-α kinase (IKK) activity

Cytoplasmic extracts (100 μg) in buffer A (EMSA) were immunoprecipitated with 1 mg of anti-IKKα/β Ab and then treated with protein A–sepharose. After extensive washing with buffer A, the pellet was resuspended in kinase buffer to assay the activity of the IKK complex using 1 mM MgATP, 5 mM $MgCl_2$ and 100 ng of GST-IκB-α(1–54) as substrate. Proteins were separated by 12.5% SDS–polyacrylamide gel electrophoresis as indicated above, transferred to polyvinylidene difluoride membranes and detected by using a specific anti-phospho-(Ser^{32}) IκB-α antibody. In another set of experiments, [γ^{32}P]ATP (1 μCi) was used instead of cold ATP, and the gels were dried and subjected to autoradiography.

Determination of bilirubin

Bilirubin was quantified spectrophotometrically according to Turcanu *et al.* (1998). Briefly, $BaCl_2 \cdot 2H_2O$ (250 mg) was added to 0.5 ml of culture supernatant. After vortexing, 0.75 ml of benzene was added and it was vortexed again vigorously. Samples were then centrifuged at $13{,}000 \times g$ for 30 min. The upper benzene layer was collected and the absorbance at 450 nm (reference wavelength: 600 nm) was measured. Bilirubin was calculated using the molecular extinction coefficient $\varepsilon^{450} = 27.3$ mM^{-1} cm^{-1}.

Laser scanning cytometry (LSC)

Formation of intracellular ROS was detected using LSC Bx50 CompuCyte Olympus, (Cambridge, MA, U.S.A.) using dihydrorhodamine 123 (DHR), which is oxidized to fluorescent rhodamine (excitation at 485 nm and emission at 534 nm). RAW 264.7 cells (200,000 $well^{-1}$) were incubated with DMEM without phenol red and DHR (5 μM) for 10 min at 37°C. After washing, fresh medium was added and cells were incubated with or without CH (10–30 μM) for 20 min. Cultures were washed twice with Hank's balanced salt solution. Toxicity was determined by LSC by differential analysis of apoptotic and necrotic cells with the Vybrant Apoptosis Assay Kit (Molecular Probes, Leiden, the Netherlands), according to the manufacturer's protocol.

Determination of glutathione

Cells (10^6 $well^{-1}$) were incubated with CH (20 or 30 μM) for different times. After incubation, cells were kept on ice and sonicated in 5% trichloroacetic acid/2 mM EDTA. After centrifugation at $10{,}000 \times g$ for 10 min at 4°C, fluorescence was measured in the supernatants by the Hissin & Hilf method (Hissin & Hilf, 1976) in a Victor2 microplate reader (Wallac, Turku, Finland). Reduced glutathione (GSH) concentrations were estimated from a standard curve.

Materials

CH was synthesized as previously described (Herencia *et al.*, 1998). Z-Leu-Leu-Leu-CHO (MG-132) was purchased from Biomol Res. Labs. Inc. (Plymouth Meeting, PA, U.S.A.). Culture reagents came from Life Technologies Inc. (Barcelona, Spain) and radioactive compounds from NEN Life Science Products Inc. (Boston, MA, U.S.A.). Tin protoporphyrin was purchased from Frontier Scientific Europe Ltd, Carnforth (U.K.). Polyclonal antibodies against Nrf2, p65, p50, IκB-α and IKKα/β, and GST-IκB-α (1–317) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.), and anti-phospho-(Ser^{32}) IκB-α antibody from New England Biolabs (Beverly, MA, U.S.A.). The peroxidase-conjugated IgGs were purchased from Dako (Copenhagen, Denmark), and the rest of the reagents were from Sigma Aldrich (St Louis, MO, U.S.A.).

Data analysis

Results are presented as mean $\pm$ s.e.m. of n separate experiments. Statistical analyses were performed using one-way ANOVA followed by Dunnett's t-test for multiple comparisons.

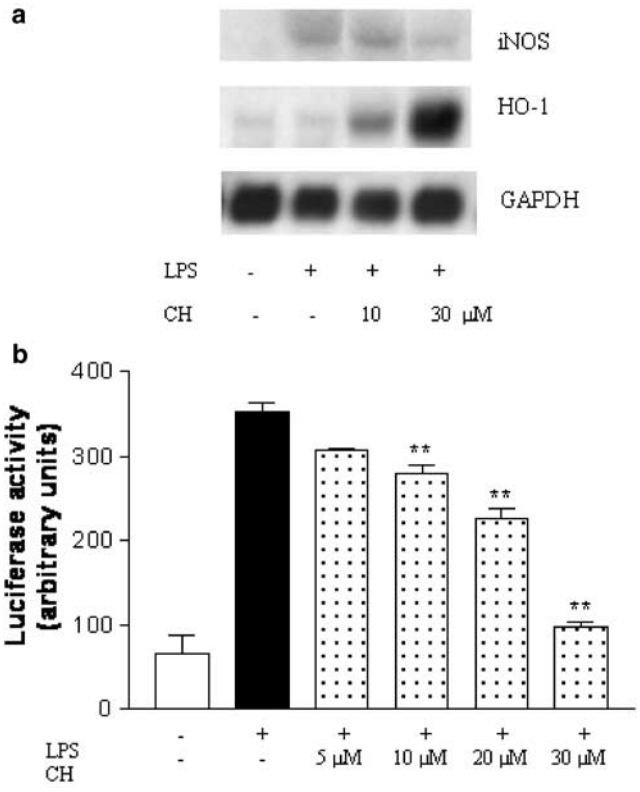

Figure 1 (a) Effect of CH on iNOS and HO-1 mRNA expression in LPS-stimulated cells. Cells were treated with CH for 30 min and stimulated with LPS ($1\,\mu g\,ml^{-1}$) for 4 h. Northern blot was performed as indicated in Methods. Results are representative of three separate experiments. (b) Inhibition of NF-κB reporter activity. RAW 264.7 cells grown in six-well plates were transfected with the NF-κB reported plasmid and assayed for luciferase activity. Data are expressed as mean $\pm$ s.e.m. ($n=3$). $^{**}P<0.01$ compared with LPS-treated cells.

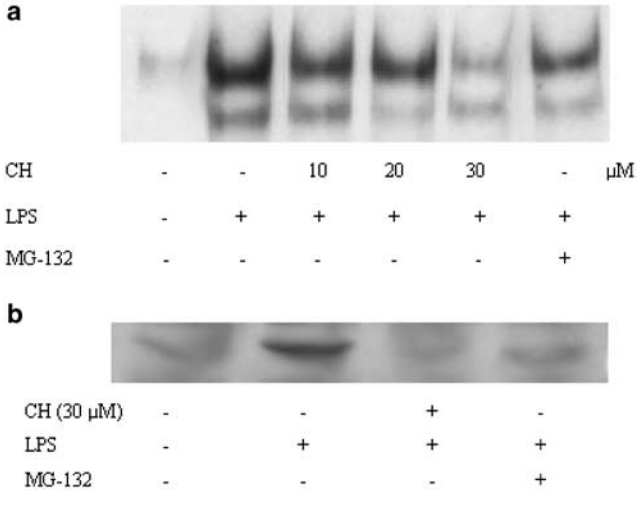

Figure 2 Effect of CH on NF-κB–DNA binding activity (a) and p65 translocation (b). Cells were preincubated with different concentrations of CH or MG-132 ($10\,\mu M$) for 30 min and then stimulated with LPS ($1\,\mu g\,ml^{-1}$) for 1 h. Nuclear fractions were then obtained for the analyses. Results are representative of three separate experiments.

Results

Effect of CH on iNOS mRNA expression

First of all we excluded the possibility that CH could modify cell viability at the range of concentrations and incubation times used in this paper, either in the presence or absence of LPS, by using the MTT assay. The possible effects of CH in this study would not be associated with macrophage cell death since this compound did not inhibit the oxidation of MTT (after 24 h incubation with $30\,\mu M$ CH $+ 1\,\mu g\,ml^{-1}$ LPS, viability was $96.6 \pm 1.5\%$ of control; $n=6$).

Since CH inhibits the production of NO and the synthesis of iNOS protein in macrophages stimulated with LPS, we assessed whether iNOS mRNA expression was inhibited by this compound. As shown in Figure 1a, when RAW 264.7 macrophages were treated with CH, a reduction in the iNOS mRNA levels induced by LPS was observed. Under the same conditions, CH caused a strong enhancement in HO-1 mRNA.

Effect of CH on NF-κB activation

Because NF-κB activity plays a key role in the induction of iNOS by LPS, we examined the effect of CH on this transcription factor. Previously, we observed that the incubation of cells with CH at concentrations between 5 and $30\,\mu M$ in the absence of LPS did not result in NF-κB activation (data not shown). Transfection experiments using an NF-κB binding site luciferase reporter indicated that CH inhibits the induction of luciferase expression by LPS (Figure 1b). In another series of experiments, nuclear extracts of RAW 264.7 cells stimulated with LPS in the absence or presence of CH were analyzed by EMSA to determine the influence of this compound on NF-κB–DNA binding. At 1 h after stimulation with LPS, CH was found to inhibit NF-κB–DNA binding in a concentration-dependent manner (Figure 2a). We used as reference MG-132 since this proteasome inhibitor is capable of suppressing LPS-induced NF-κB activation in RAW 264.7 cells (Chen *et al.*, 1997). LPS induces the accumulation of NF-κB in the nucleus. Figure 2b shows that this translocation process is inhibited in cells treated with either CH or MG-132, as revealed by immunoblotting the p65 protein in nuclear extracts.

Since NF-κB activation is dependent on the phosphorylation and degradation of IκB proteins, we also determined the effect of CH on both processes. Pretreatment of RAW 264.7 macrophages with either CH or MG-132 resulted in increased IκB-α protein expression in extracts from cells stimulated with LPS (Figure 3a). The effect of CH or MG-132 on IκB-α phosphorylation was studied by Western blotting using a specific anti-phospho-(Ser^{32}) IκB-α antibody. We observed that CH did not modify the expression of phosphorylated IκB-α, whereas the proteasome inhibitor increased it. The possible influence of CH on IκB-α phosphorylation was also assessed by performing the IKK activity assay. Macrophages were treated with CH followed by stimulation with LPS, and the IKK complex was immunoprecipitated and assayed for kinase activity using GST-IκB-α as substrate. The phosphorylated derivative was detected either by Western blotting using a specific anti-phospho-(Ser^{32}) IκB-α antibody (Figure 3b) or by autoradiography (Figure 3c). In both cases, IKK activity in CH-treated cells was comparable to that of LPS controls, confirming that IκB-α phosphorylation is not a target for CH.

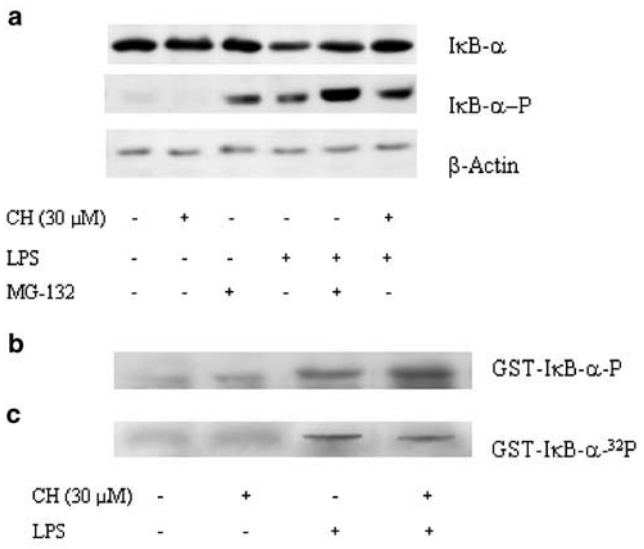

Figure 3 Effect of CH on IκB-α expression and phosphorylation. (a) IκB-α and IκB-α-P protein expression. Cells were preincubated with CH (30 μM) and/or MG-132 (10 μM) for 30 min and then stimulated with LPS (1 μg ml^{-1}) for 30 min. (b, c) IKK activity. Cells were stimulated as above and IKK protein was immunoprecipitated as indicated in Methods. The formation of GST-IκB-α-P was assessed by Western blot and that of GST-IκB-α-^{32}P by autoradiography. Results are representative of four separate experiments.

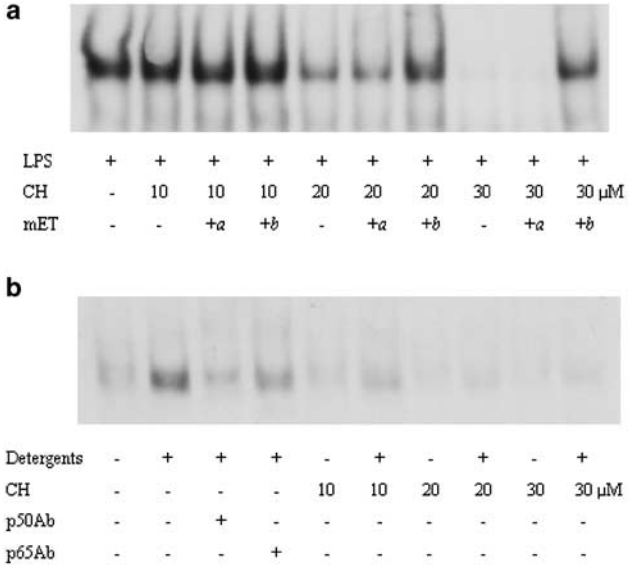

Figure 4 *In vitro* interaction with DNA and inhibition of NF-κB release. (a) *In vitro* interaction of CH with DNA binding. Cells were stimulated with LPS (1 μg ml^{-1}) for 1 h and then nuclear fractions were obtained. CH was incubated with nuclear fractions for 30 min and EMSA was carried out. In some experiments, 2-mercaptoethanol (mET, 140 mM) was added to the reaction tube and incubated for 30 min: *a* (mET was added after the 30 min incubation of nuclear fractions with CH) or *b* (mET was added before the 30 min incubation of nuclear fractions with CH). Results are representative of three separate experiments. (b) Release of NF-κB from the IκB complex. Unstimulated cells were treated with CH and then cytoplasmic extracts were obtained. Aliquots of these extracts were treated with detergents as indicated in Methods to release NF-κB, and then EMSA was performed. In some experiments, antibody against either p65 or p50 was incubated with the released NF-κB before performing EMSA.

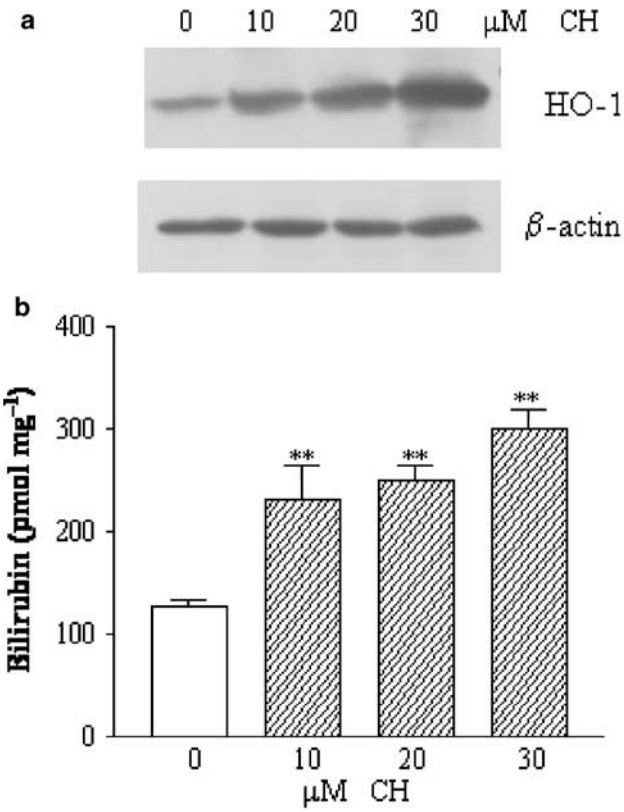

Figure 5 Concentration-dependent induction of HO-1 by CH. (a) Protein expression. (b) Bilirubin accumulation in the culture medium as an index of enzyme activity. RAW 264.7 macrophages were incubated with different concentrations of CH for 8 h. After centrifugation, cell pellets were used for Western blot analysis and bilirubin was measured in the supernatants as indicated in Methods. (a) Results are representative of three separate experiments. (b) Data are the mean ± s.e.m. of six determinations. $^{**}P<0.01$.

We also examined the ability of CH to inhibit directly NF-κB–DNA binding. For this, CH was incubated *in vitro* with nuclear extracts from LPS-activated cells, which resulted in strong inhibition (Figure 4a). This effect was prevented by 2-mercaptoethanol (140 mM), but this agent was ineffective if added after CH incubation with nuclear extracts, suggesting that CH can interact with NF-κB proteins in an irreversible manner.

In another series of experiments, cells were treated with CH in the absence of LPS stimulation, and then cytoplasmic extracts were prepared. In nonstimulated cells, NF-κB proteins are retained in the cytoplasm bound to IκB in an inactive state, but these proteins can be released after detergent treatment to bind to DNA specific sequences. EMSA analyses of these cytoplasmic extracts incubated with an antibody against either p65 or p50 indicated the presence in these extracts of both fractions of the NF-κB complex (Figure 4b). Our results also showed that CH treatment of cells limited the release of NF-κB proteins. Experiments were also carried out to assess the possible influence of HO-1 on NF-κB inhibition by CH. For this purpose, EMSA analyses were performed with nuclear extracts from cells stimulated with LPS for different times (1–8 h) in the absence or presence of CH (10–30 μM) and either tin protoporphyrin or zinc protoporphyrin at concentrations between 1 and 10 μM. In these assays, none of these HO inhibitors modified the effect of CH on NF-κB–DNA binding

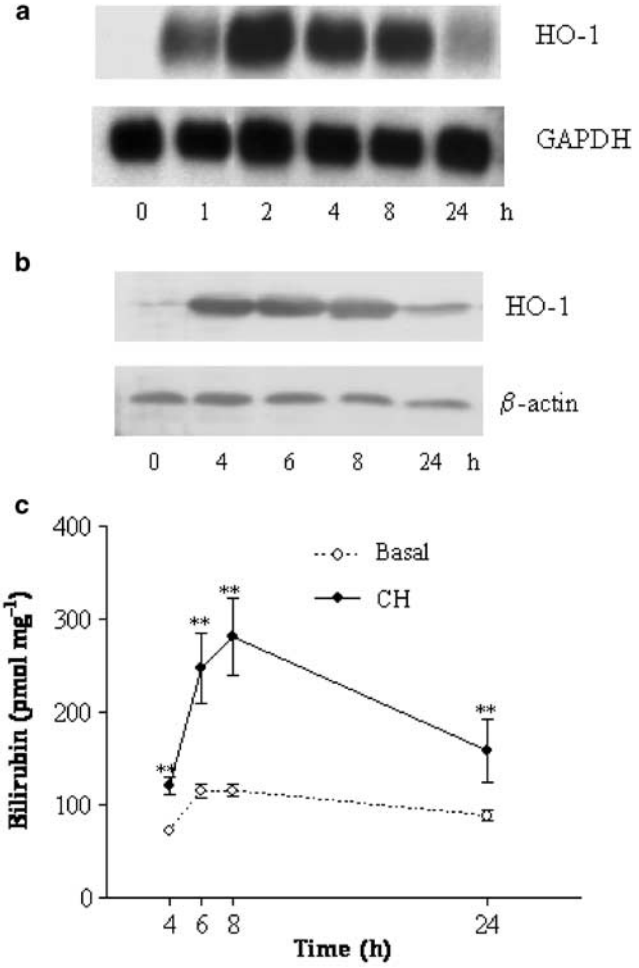

Figure 6 Time course of HO-1 induction by CH. (a) mRNA expression. (b) Protein expression. (c) Bilirubin accumulation in the culture medium. RAW 264.7 macrophages were incubated with 30 μM CH for different times. After centrifugation, cell pellets were used for Western blot analysis and bilirubin was measured in the supernatants as indicated in Methods. In another series of experiments, mRNA was extracted from cell pellets as indicated in Methods. (a, b) Results are representative of three separate experiments. (c) Data are the mean$\pm$s.e.m. of six determinations. $^{**}P<0.01$.

in intact cells, and neither did they have any effect on NF-κB activation by LPS (data not shown).

Induction of HO-1 by CH

Our results showing that CH was able to induce strongly mRNA HO-1 at concentrations inhibiting iNOS expression led us to further characterize the effect of this chalcone on HO-1. In RAW 264.7 macrophages incubated with CH in the absence of LPS, HO-1 induction was concentration-dependent (Figure 5a) and paralleled by HO-1 activity, as reflected by the accumulation of bilirubin in the culture medium (Figure 5b). The time course of HO-1 induction revealed that mRNA was already detected 1 h after treatment and showed a maximum at 2 h (Figure 6a). In addition, cell extracts prepared at different times after treatment with 30 μM CH were subjected to Western blot analysis. As shown in Figure 6b, maximal levels in HO-1 protein were observed between 4 and 8 h and decreased at 24 h, whereas HO-1 activity peaked at 8 h (Figure 6c).

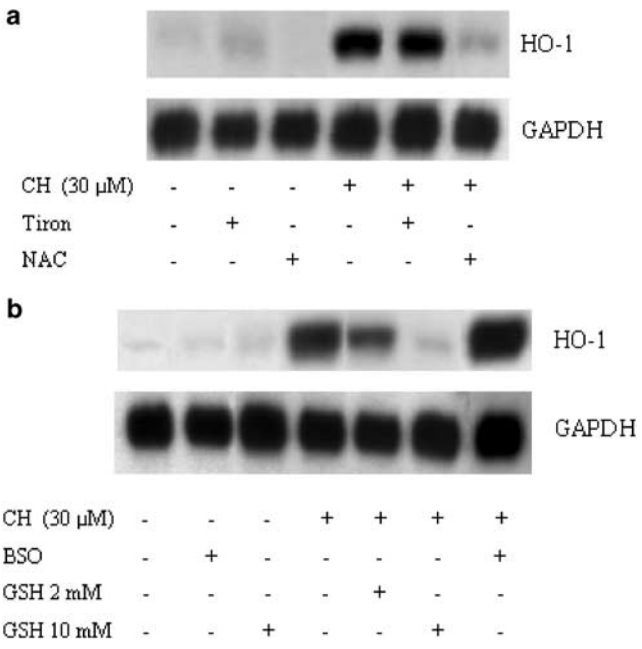

Figure 7 (a, b) Effect of agents modulating oxidative stress and GSH on HO-1 mRNA expression. Cells were pretreated with Tiron (500 μM), NAC (10 mM) or GSH (2 or 10 mM) for 30 min and then incubated with 30 μM CH for 2 h. In the case of BSO (200 μM), cells were pretreated for 6 h to deplete GSH and then incubated with CH as above. Northern blot analysis was performed as indicated in Methods. Results are representative of three separate experiments.

Influence of agents modulating oxidative stress and GSH

In order to determine the mechanisms involved in HO-1 induction by CH, we analyzed mRNA expression in the presence of different agents. Figure 7a shows that pretreatment with 10 mM *N*-acetylcysteine (NAC) strongly reduced HO-1 mRNA levels induced by CH. In contrast, the cell-permeable superoxide scavenger Tiron (500 μM) failed to modify HO-1 mRNA significantly. Interestingly, GSH depletion by buthionine sulfoximine (BSO) (200 μM) did not induce HO-1, but increased the effect of CH (Figure 7b). In addition, exogenous GSH (2 or 10 mM) inhibited HO-1 mRNA induction by this chalcone derivative.

Effect of CH on oxidative stress and intracellular GSH levels

Because oxidative stress is a well-recognized signal for HO-1 induction, we measured the production of ROS after CH treatment by laser cytometry. As shown in Figure 8a and c, it was found that CH at the concentrations able to induce HO-1 significantly augmented intracellular oxidative stress after 20 min treatment. This effect did not result in either apoptosis or necrosis of cells, as determined by laser cytometry using the annexin V–propidium iodide assay system (Figure 8b). Similar results were obtained at later times (6 and 24 h) either in the presence or absence of 1 μg ml^{-1} LPS (data not shown). We also examined the influence of this chalcone on GSH levels. As shown in Figure 9a, incubation of RAW 264.7 cells with CH

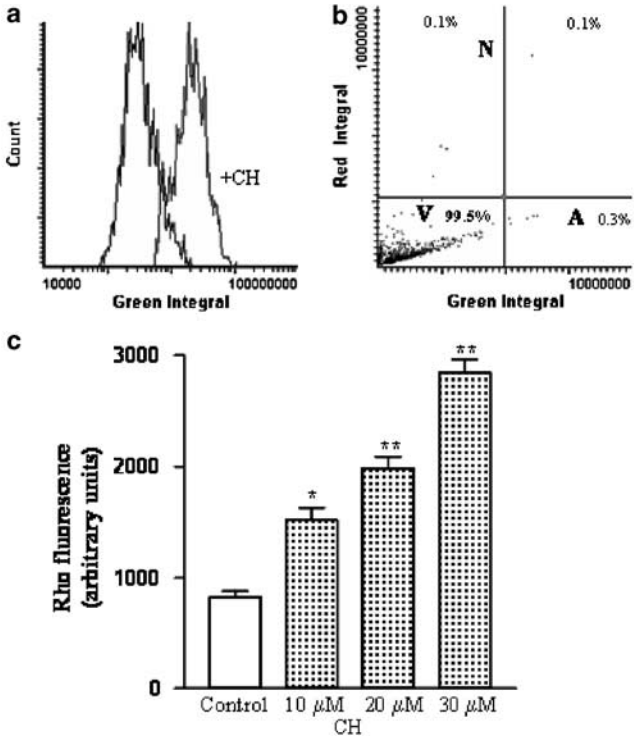

Figure 8 LSC analysis of intracellular generation of ROS and cytotoxicity. (a) Histogram of rhodamine (Rho) fluorescence in the presence or absence of CH (30 μM). RAW 264.7 cells were incubated with DHR (5 μM) for 10 min at 37°C. After washing, cells were incubated with or without CH (30 μM) for 20 min. (b) Determination of apoptotic (A)/necrotic(N) and viable (V) cells after incubation with CH (30 μM) for 20 min. Fluorescein isothiocyanate–annexin V (horizontal axis), propidium iodide (vertical axis). (c) Concentration-dependent increase of Rho fluorescence after CH treatment. Cells were incubated with 10, 20 or 30 μM CH for 20 min under the same conditions as in (a). Results are representative of four similar experiments. $^{*}P<0.05$, $^{**}P<0.01$.

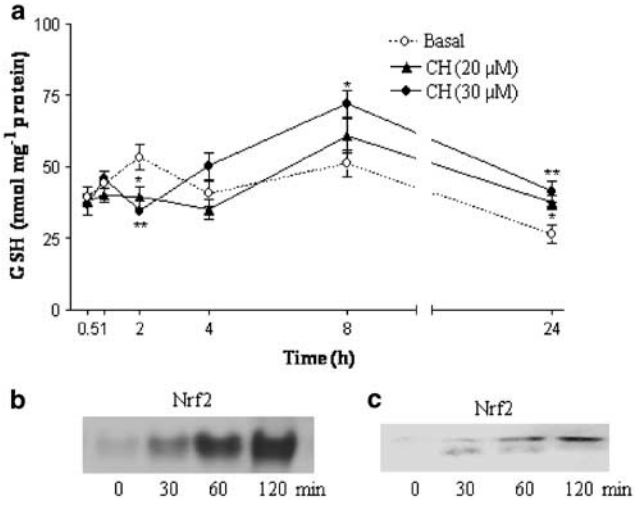

Figure 9 (a) Time course of CH modification of GSH levels. CH was incubated with cells at 20 and 30 μM for different times. Intracellular GSH levels were determined as indicated in Methods. $^{*}P<0.05$, $^{**}P<0.01$ with respect to basal. (b, c) Nrf2 activation. Cells were incubated with CH (30 μM) for different times and then nuclear and cytoplasmic fractions were obtained. (b) Nrf2–DNA binding in nuclear fractions. EMSA was performed as indicated in Methods. (c) Nrf2 translocation to the nucleus. Western blot analysis was performed as indicated in Methods. Results are representative of three separate experiments.

for different time periods resulted in a small decrease in GSH levels at 2 h, followed by a sustained increase up to 24 h of CH treatment.

Activation of Nrf2

We next determined the ability of CH to act on Nrf2, a transcription factor activated by oxidative stress with a relevant role in HO-1 induction. EMSA experiments indicated that CH is able to increase Nrf2–DNA binding (Figure 9b). We also assessed the influence of this chalcone derivative on the translocation of Nrf2 protein to the nucleus. Figure 9c shows that CH augmented Nrf2 translocation in a time-dependent manner.

Discussion

The anti-inflammatory effects of CH can be related to inhibition of NO production *in vitro* and *in vivo* (Herencia *et al*., 1999). In this paper we provide evidence that CH controls NO production in activated macrophages *via* the inhibition of iNOS expression acting at a transcriptional level, and that this effect is mediated by blocking NF-κB function. We have also investigated the mechanisms of NF-κB inhibition by this chalcone derivative. iNOS is induced by many inflammatory stimuli including the bacterial endotoxin LPS, which binds to the LPS-binding protein to interact with CD14 followed by activation of several signal transduction pathways. In macrophages, NF-κB is mainly formed by a p50/p65 heterodimer constitutively present as an inactive cytoplasmic complex by binding to IκB proteins. Cellular stimulation by cytokines or LPS results in the phosphorylation of inhibitory proteins by IKKs and degradation, allowing the translocation of NF-κB to the nucleus to bind specific sequences (Baldwin, 1996; Abraham, 2000). Our results indicate that CH inhibits the activation pathway of NF-κB in intact RAW 264.7 macrophages by targeting IκB-α degradation. Nevertheless, our results do not support an inhibitory effect of CH on proteasome activity since this compound did not increase the expression of the phosphorylated form of IκB-α, in contrast to the proteasome inhibitor MG-132. In addition, the phosphorylation of this inhibitory protein and IKK activity were not inhibited by CH, but this compound reduced NF-κB–DNA binding in cytoplasmic fractions after cell free activation of NF-κB. CH would be able to interact directly with NF-κB, resulting in a diminished access of the NF-κB–IκB complex to the proteasome, and thus this transcription factor would be retained in an inactive state in the cytoplasm. As a consequence, NF-κB translocation into the nucleus, DNA binding and transcriptional activity would be inhibited.

A number of studies have demonstrated that intracellular redox levels can regulate NF-κB signal transduction in a cell-specific manner (Bonizzi *et al*., 2000). Therefore, the activation of this transcription factor can be blocked by structurally unrelated antioxidants (Ma *et al*., 2003). Modification of the redox state of cells leads to a change in the thiol status, which plays a key role in regulating NF-κB activation. Therefore, not only could high intracellular thiol levels act by scavenging oxidants, but they could also modify protein folding or activation resulting in inhibition of kinases involved in phosphorylation of IκB or the transport of activated NF-κB

to the nucleus (Staal *et al*., 1990). Phenolic groups in the chalcone skeleton provide the basis for a free radical scavenging activity that may participate in the inhibition of NF-κB activation by these molecules (Cheng *et al*., 2001). Nevertheless, we have previously established that CH is devoid of direct antioxidant activity (Herencia *et al*., 1998), and in this study the effect of this compound on NF-κB occurred in the presence of ROS generation. Thus, our results do not support the idea that inhibition of NF-κB by CH can be due to antioxidant effects. Other mechanisms may underlie the modification of the NF-κB pathway by CH, such as a direct modification of thiol groups in relevant proteins. Indeed, there are cysteine residues in the DNA-binding domain of p50 (Cys62) and p65 (Cys38) susceptible to redox modification or alkylation, which results in impairment of NF-κB–DNA binding (Toledano *et al*., 1993; Chen *et al*., 1998). A similar mechanism has been suggested for NF-κB inactivation by the chemopreventive agent sulforaphane (Heiss *et al*., 2001).

On the other hand, we have shown that CH is an inducer of HO-1 in macrophages and that this effect can be related to the activation of transcription. A number of transcription factors could participate in HO-1 induction. In particular, Nrf2 plays a key role in the regulation of antioxidative proteins including HO-1 (Alam *et al*., 1999). This transcription factor mediates the induction of phase 2 enzymes by phenolic antioxidants, a detoxification mechanism that would account for chemoprotection against some carcinogens and toxins (Ma & Kinneer, 2002). Nrf2 remains in the cytoplasm bound to proteins such as Keap1 and, upon exposure to oxidative stress, dissociates and translocates into the nucleus to activate the transcription of target genes (Itoh *et al*., 1999). It is accepted that subtoxic concentrations of ROS could function as an intracellular second messenger (True *et al*., 2000; Arnold *et al*., 2001). We have shown that CH increases intracellular levels of oxidative species, although no detectable cell injury was observed at the concentrations and incubation times used. Thus, this could be one mechanism to activate Nrf2 transcription by this chalcone derivative.

Some phenolic compounds possessing α,β-unsaturated carbonyl groups such as curcumin and caffeic acid phenethyl ester have been reported as potent HO-1 inducers (Motterlini *et al*., 2000; Scapagnini *et al*., 2002) due to the inactivation of the Nrf2–Keap1 complex (Balogun *et al*., 2003), and other electrophiles have been shown to cause Nrf2 nuclear accumulation and stabilization (Itoh *et al*., 2003). Similarly, the α,β-unsaturated carbonyl moiety of CH may react with free sulfhydryl groups of GSH and cysteine residues in proteins. Our results suggest that oxidation of critical protein sulfhydryl residues is either directly or indirectly involved in HO-1 induction by CH. The target for this interaction may be biological sensor molecules such as the Nrf2–Keap1 complex.

GSH plays an important role in the detoxification of xenobiotics and protection against oxidative damage. The reduction in GSH levels is a mechanism of HO-1 induction by oxidants (Ryter & Choi, 2002). Our results indicate that CH causes a small decrease in GSH levels followed by an increase. This could be explained by the fact that γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, is subjected to an inhibitory feedback mechanism and also to transcriptional regulation (Meister & Anderson, 1983). Nevertheless, the reduction of GSH levels by CH is significant at 2 h, later than Nrf2 activation, suggesting that modification of GSH is not relevant for HO-1 induction at least at earlier times. It should be noted that the sustained elevation of intracellular GSH levels by CH at later times would favor a cellular protective effect by this compound.

It has been reported recently that products derived from HO-1 activity such as CO can inhibit NF-κB activity (Lee *et al*., 2003). In our experiments, we have observed that HO-1 activity induced by CH did not precede NF-κB inhibition by this agent. In addition, HO inhibitors did not modify the effect of CH on NF-κB. Our data thus suggest that the inhibition of NF-κB by CH is not mediated by HO-1 activity, although the interaction of this chalcone derivative with both pathways may depend on similar chemical properties.

In summary, this study shows that the anti-inflammatory compound CH simultaneously induces a cytoprotective response (HO-1) and downregulates an inflammatory pathway (NF-κB) with a mechanism of action different from antioxidant chalcones.

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