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Activation of the Nrf2-antioxidant system by a novel cyclooxygenase-2 inhibitor furan-2-yl-3-pyridin-2-yl-propenone: implication in anti-inflammatory function by Nrf2 activator

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Abstract

Furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) is a novel synthetic compound and has demonstrated anti-inflammatory activity by inhibiting cyclooxygenase-2 (COX-2). It is widely accepted that reactive oxygen species (ROS) generated by activated inflammatory cells can exacerbate inflammation. In this study, the potential antioxidative efficacy of FPP-3 has been investigated in murine cells. FPP-3 increased the expression of multiple antioxidative enzymes, including NAD(P)H:quinone oxidoreductase 1 (Nqo1), γ-glutamylcysteine ligase (GCL) and heme oxygenase-1 (HO-1), by facilitating the nuclear translocation of nuclear factor-erythroid 2-p45-related factor 2 (Nrf2). Inducibility of antioxidant proteins such as HO-1 were lost in *nrf2*-deficient murine fibroblasts. As a result of enhanced cellular antioxidative capacity, elevation of NF-κB-driven reporter gene expression by lipopolysaccharide was attenuated by FPP-3 treatment in murine fibroblasts. Furthermore, FPP-3 treatment inhibited UVA-mediated induction of COX-2 in murine keratinocytes. Our current study suggests that FPP-3, which has been developed as a novel COX-2 inhibitor, has antioxidative properties by activating the Nrf2-ARE pathway. The dual function of this compound may provide a better strategy to block/attenuate the inflammation process and to alleviate ROS-associated inflammatory complications.

Introduction

Inflammation is marked by increased expression of inflammatory proteins such as cyclooxygenase-2 (COX-2) and by over-production of reactive oxygen species (ROS) from activated inflammatory cells. Cyclooxygenases are key enzymes and catalyse the conversion of arachidonic acid to prostaglandins and ROS (Lee et al 2003a). COX-1 is constitutively expressed in tissues, whereas COX-2 is highly inducible in response to pro-inflammatory cytokines, oncogenes and tumour promoters (Oshima et al 1996; Subbaramaiah et al 1996; Basu et al 2005). The expression of COX-2 is primarily regulated by nuclear factor-κB (NF-κB), which plays a crucial role in the early stages of acute inflammatory reactions (Lawrence et al 2001; Makarov 2001). It is known that elevated expression of COX-2 in the skin is associated with the inflammatory response following UV exposure (Akunda et al 2007).

Oxidative stress contributes to various pathological conditions including inflammation and cancer (Symons & King 2003; Bartsch & Nair 2004). Increased levels of ROS during inflammation can prime the innate immune cells and lead these cells to have increased responsiveness to inflammatory mediators. Therefore, suppression of ROS levels has been implicated in alleviation of the acute inflammatory response and reduction of the incidence of inflammatory complications (Rangasamy et al 2004; Li & Nel 2006; Thimmulappa et al 2006a). One of the defence systems against ROS is the coordinated induction of the antioxidative genes (e.g. γ-glutamylcysteine ligase (GCL), glutathione reductase (GR)) and phase 2 detoxifying enzymes (e.g., NAD(P)H:quinone oxidoreductase 1 (Nqo1), glutathione S-transferases). Regulation of both basal and inducible expression of these protective genes is mediated mainly by *cis*-acting antioxidant

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response element (ARE). The nuclear factor-erythroid 2-p45-related factor 2 (Nrf2) is found to be a critical element to transactivate the ARE-driven genes via heterodimerization with small Maf or bZIP proteins (Kwak et al 2004; Kobayashi & Yamamoto 2005; Kensler et al 2007). Kelch-like ECH associated protein 1 (Keap1) is known to regulate the intracellular localization of Nrf2 by binding to the N-terminal domain of this protein (Itoh et al 1999). Exposure of cells to chemopreventive or electrophilic chemical inducers disrupts the Keap1/Nrf2 binding and results in the translocation of Nrf2 into the nucleus. Recent studies show that Keap1 is also involved in proteasome-mediated degradation of Nrf2 in quiescent cells by acting as an adaptor protein of Cul3-based E3 ubiquitin ligase complex (McMahon et al 2003; Kobayashi et al 2004).

Accumulating evidence shows that disruption of *nrf2* in mice induces marked inflammatory injury following exposure to lipopolysaccharide (LPS), carageenan and cigarette smoke (Itoh et al 2004; Rangasamy et al 2004; Thimmulappa et al 2006a). One of the Nrf2-regulated genes, heme oxygenase-1 (HO-1), is known to contribute to the Nrf2-mediated anti-inflammatory effect. HO-1 inhibits the expression of pro-inflammatory cytokines (Keyse & Tyrrell 1989; Otterbein & Choi 2000) and increased HO-1 levels by 15-deoxy-$\Delta^{12,15}$-prostaglandin J_2 can suppress LPS-induced production of tumour necrosis factor-α (TNF-α) and nitric oxide (NO) in murine macrophages (Lee et al 2003b). These reports suggest the potential anti-inflammatory role of small molecules which can activate the Nrf2-antioxidative system.

Furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) is synthesized as a novel propenone compound in an attempt to develop dual inhibitors of COX-2 and 5-lipoxygenase (5-LOX) (Jahng et al 2004). FPP-3 has shown an anti-inflammatory efficacy in several in-vitro and in-vivo studies (Lee et al 2004, 2006). Here we show that the anti-inflammatory efficacy of FPP-3 may be mediated in part through activation of the Nrf2-antioxidant system in murine cells. FPP-3 effectively increased the expression of multiple antioxidant genes, including HO-1, in an Nrf2-dependent manner. Elevated inflammatory markers following exposure of cells to LPS and UV radiation was attenuated by FPP-3 pretreatment. These results imply that FPP-3 has a dual effect on inflammation by enhancing the Nrf2-antioxidant pathway as well as by inhibiting COX-2 activity directly.

Materials and Methods

Materials

FPP-3 and its derivatives were provided by Dr Eung-Seok Lee (Yeungnam University, Gyeongsan-si, South Korea). MAPK pathway inhibitor anthra[1,9-*cd*]pyrazol-6(2*H*)-one-1,9-pyrazoloanthrone (SP600125) and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) were purchased from Calbiochem (La Jolla, CA). 2′-Amino-3′-methoxyflavone (PD98059) was purchased from Promega (Madison, WI). Antibodies recognizing Nrf2 and β-tubulin were obtained from Santa Cruz Biotechnology (St Cruz, CA). Reporter plasmid containing the Nqo1 ARE was a gift from Dr Nobunao Wakayashi (Johns Hopkins University, MD). The NF-κB reporter plasmid was obtained from Panomics Inc. (Redwood city, CA).

Cell culture

Murine keratinocyte PE cells (Yuspa et al 1986) were maintained in Eagle's Minimal Essential Medium (Cambrex Bio Science, Walkersville, MD) containing 10% Chelex (Bio-Rad, Hercules, CA)-treated fetal bovine serum (Invitrogen, Carlsbad, CA), 0.05 mM $CaCl_2$ and 1% penicillin/streptomycin (Hyclone, Logan, UT). Murine embryonic fibroblasts (MEFs) from wild-type and *nrf2*-disrupted mice (Kwak et al 2003) were maintained in Iscove's Modified Dulbeccos's Medium (Hyclone) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone).

UV irradiation

Before UV irradiation, cells were incubated with vehicle or FPP-3 salt (2.5 μM) for 2 or 18 h, and washed with phosphate-buffered saline (PBS). A thin layer of PBS-covered cells were placed 15 cm below the UV source and exposed to UVA by using UV lamp (VL-215LM, Vilber Lourmat, Cedex1, France) at a wavelength of 365 nm. The amount of UVA exposure on cells was 1350 μW cm^{-2}. Cells were irradiated for different time periods (60, 120, 300 and 480 s) and recovered in the complete medium for 6 h.

Total RNA extraction and RT-PCR analysis

Total RNAs were isolated from cells using a Trizol reagent (Invitrogen) following treatment of cells with FPP-3 or its HCl salt (1.25, 2.5 and 5 μM). For the synthesis of cDNAs, reverse transcriptase reaction was performed by incubating 200 ng of total RNA with reaction mixture containing nuclease-free water, 10×PCR buffer (200 mM Tris-HCl (PH 8.4), 500 mM KCl), 50 mM $MgCl_2$, 100 mM dNTPs, 0.5 μg μL^{-1} oligo $(dT)_{12-18}$ and 200 U μL^{-1} Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). PCR amplification for each gene was performed with a thermal cycler (Bio-Rad) and amplification conditions were 27–30 cycles of 40 s at 95°C, 30 s at 56°C and 40 s at 72°C. Primers were synthesized by Integrated DNA Technology (Coralville, IA) or Bioneer (Daejeon, South Korea): catalytic subunit of GCL (GCLC), 5′-ATGATGCCAACGAGTCTGAC-3′ and 5′-CGCCTTTGCAGATGTCTTTC-3′; modulatory subunit of GCL (GCLM), 5′-AGGAGCTTCGGGACTGTATT-3′ and 5′-TGGGCTTCAATGTCAGGGAT-3′; glutathione reductase (GR), 5′-GGCATGATAAGGTACTGAGA-3′ and 5′-TTCGTCTACTAGGATGTGGC-3′; β-actin, 5′-GCAGAAGGAGATTACTGCTC-3′ and 5′-CTAGAAG-CACTTGCGGTGCA-3′; HO-1, 5′-AGGTGTCCAGA-GAAGGCTT-3′ and 5′-ATCTTGCACCAGGCTAGGCA-3′; COX-2, 5′-GGAGCTTCCTGATTCAAAAG-3′ and 5′-CTCTACCTGAGTGTCTTTGA-3′. PCR products were resolved on 1.2% agarose gels and the images were captured by using Visi Doc-It Imaging system (UVP, Upland, CA).

Preparation of nuclear extracts

Cells were treated with 2.5 μM FPP-3 salt for indicated time periods (30 min to 6 h) and nuclear proteins were extracted as described previously (Dignam et al 1983). Briefly, crude nuclear fractions were obtained by lysing the cells with homogenization buffer (2 M sucrose, 1 M HEPES, 2 M $MgCl_2$, 2 M KCl, 30% glycerol, 0.5 M EDTA, 1 M DTT, protease inhibitor mixture and 10% NP40) and followed by centrifugation at 12 000 *g* for 15 min.

Immunoblot analysis

Cell lysates were loaded on 6% or 12% SDS-polyacrylamide gels and separated by electrophoresis. Proteins on gels were transferred to nitrocellulose membrane (Whatman, Dassel, Germany) and membranes were blocked with 5% skimmed milk in TPBS buffer (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4 and Tween-20 2 mL L^{-1}) for 1 h. Following antibody incubation, detection was carried out using the Enhanced Chemiluminescence Reagent (Amersham Biosciences, Bucks, UK).

DNA transfection and luciferase activity measurement

Cells were transfected with plasmids at 50% confluency by using Welfect-Ex Plus Transfection Reagent (WelGene Inc., South Korea). Briefly, cells were incubated with transfection complex containing 0.5 μg DNA, 0.05 μg pRLtk control plasmid and the transfection reagent (Welfect-Ex 2 μg and Enhancer-Q 1.5 μg) in serum- and antibiotic-free medium. Complete medium with 10% FBS was added 4 h after transfection and cells were further incubated overnight. After recovering cells in the complete medium for 6 h, cells were treated with vehicle, FPP-3 salt (2.5 μM) or pharmacological inhibitors MAPK kinase for 18 h. Luciferase activity from *Firefly* and *Renilla* were determined in cell lysates by using Dual-Luciferase Assay kit (Promega) with a 20/20^n luminometer (Turner Biosystems, Sunnyvale, CA).

Statistical analysis

Statistical significance was determined by paired *t*-test or Kruskal–Wallis test followed by Dunn's multiple comparison test (GraphPad Prism4, San Diego, CA).

Results

FPP-3 increased the ARE-driven reporter gene expression by elevating nuclear level of Nrf2 in murine keratinocyte

First, we investigated the efficacy of several propenone derivatives to activate the ARE in murine keratinocyte PE cells (Figure 1A). These propenone derivatives were synthesized as dual inhibitors of COX-2 and 5-LOX, and were found to inhibit

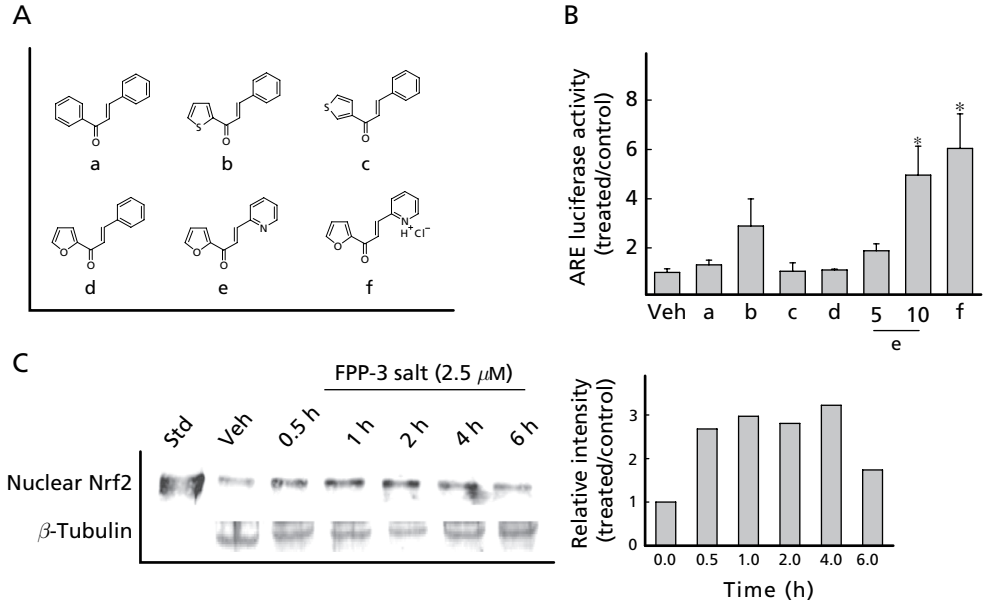

Figure 1 Activation of Nrf2 by propenone derivatives in murine keratinocytes. A. Chemical structure of propenone derivatives: a, 1,3-diphenyl propenone ((E)-chalcone); b, (E)-3-phenyl-1-(thiophen-2-yl)prop-2-en-1-one; c, (E)-3-phenyl-1-thiophen-3-yl)prop-2-en-1-one; d, (E)-1-(furan-2-yl)-3-phenylprop-2-en-1-one; e, (E)-1-(furan-2-yl)-3-(pyridine-2-yl)prop-2-en-1-one (FPP-3); f, FPP-3 HCl salt. B. Effects of propenone derivatives on luciferase reporter activity from the human Nqo1 ARE. Briefly, cells were plated on 24-well plates and transfected with the ARE luciferase plasmid. Cells were then treated with vehicle (Veh, DMSO) or propenone derivatives (a–d, 5 μM; FPP-3 (e), 5 or 10 μM; FPP-3 salt (f), 5 μM) for 18 h and ARE-driven luciferase activity was measured by using Dual Assay System. Values are mean ± s.e. from four independent experiments. $^*P < 0.05$ compared with vehicle-treated control group. C. Effect of FPP-3 HCl salt on levels of nuclear Nrf2 in murine keratinocytes. Cells were treated with vehicle or FPP-3 salt (2.5 μM) for different time periods (0.5, 1, 2, 4 or 6 h) and obtained nuclear extracts from cells were used for immunoblot analysis of Nrf2 and β-tubulin. Bar graph represents relative intensities of nuclear Nrf2 levels.

LPS-stimulated prostaglandin formation in murine macrophage RAW264.7 cells (Lee et al 2004). To examine the effect of these compounds on the Nrf2 pathway, the reporter plasmid containing the ARE core sequence from the human *Nqo1* gene was transfected into PE cells and luciferase activity was measured following incubation of cells with vehicle (dimethyl sulfoxide, DMSO) or propenone derivatives (5 or 10 μM) for 18 h. Among several derivatives of propenone, FPP-3 (compound e) increased luciferase activity by 2- and 5-fold at a concentration of 5 and 10 μM, respectively (Figure 1B). The hydrochloride salt of FPP-3 (compound f) also increased the ARE-driven luciferase activity by 6-fold at a concentration of 5 μM. Other propenone derivatives showed marginal or no increase in the ARE-luciferase activity. These results indicate that FPP-3 and its salt can effectively activate the ARE activity in murine keratinocytes. In accordance with the ARE activation, the nuclear level of Nrf2 was also increased following incubation with FPP-3 salt for 30 min to 4 h (Figure 1C). Elevated nuclear Nrf2 levels returned to normal when cells were incubated with FPP-3 for more than 6 h. These results suggest that FPP-3 and its salt can facilitate nuclear accumulation of Nrf2 and subsequent activation of the ARE.

FPP-3 elevated the expression of multiple antioxidant genes including HO-1 without involvement of the MAPK cascades

Next, we examined effect of FPP-3 on the expression of Nrf2-regulated antioxidant genes in murine keratinocytes. Cells were incubated with vehicle (DMSO), FPP-3 (2.5 μM) or FPP-3 salt (1.25 and 2.5 μM) for 18 h, and transcript levels for antioxidant genes were monitored by using RT-PCR analysis. FPP-3 increased transcript levels for Nqo1, GSH-synthesizing enzymes (GCLC and GCLM) and GR following 18 h incubation (Figure 2A). The expression of HO-1 was also elevated by FPP-3 incubation; however, induction patterns were different from those of other genes. An enhanced level of HO-1 transcript was observed at 4 h after addition of FPP-3 (Figure 2B), while no increase was observed in the 18-h incubation group (data not shown). These results indicate that FPP-3 can

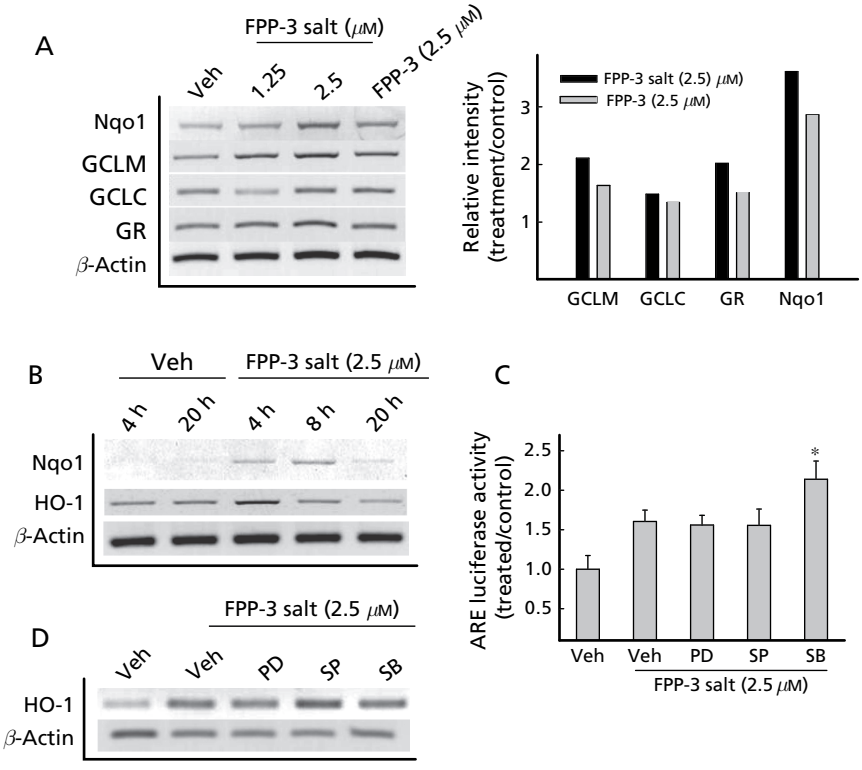

Figure 2 Induction of Nrf2-regulated genes by FPP-3 and effect of inhibitors of the MAPK cascades in murine keratinocytes. A. Transcript levels of Nrf2-target genes in murine keratinocytes following treatment with FPP-3. Cells were incubated with vehicle (Veh, DMSO), FPP-3 (2.5 μM) or FPP-3 salt (1.25 or 2.5 μM) for 18 h and total RNAs were extracted. Transcript levels for Nqo1, catalytic/modulatory subunits of GCL (GCLC, GCLM), GSH reductase (GR), β-actin were measured by RT-PCR analysis. Similar results were obtained in 3 or 4 separate experiments. Bar graph represents relative intensities of gene expression following incubation with 2.5 μM of FPP-3 and its salt form. B. Transcript levels for Nqo1 and HO-1 following incubation with vehicle (Veh) or FPP-3 salt for 4, 8, or 20 h. C. Effect of pharmacological inhibitors of MAPKs on the ARE activation by FPP-3 salt. Cells transfected with the Nqo1 ARE-containing reporter plasmid and incubated with vehicle (Veh) or FPP-3 salt (2.5 μM) alone or together with pharmacological inhibitors of MAPKs (ERK inhibitor (PD98059, 20 μM), JNK inhibitor (SP600125, 20 μM) or p38 kinase inhibitor (SB203580, 20 μM)). ARE-driven luciferase activity was measured 18 h after incubation. Values are mean ± s.d. of three independent experiments. $^*P < 0.05$ compared with vehicle-treated control group. D. Transcript levels for HO-1 following co-incubation with MAPK inhibitors. Cells were incubated with vehicle (Veh), or FPP-3 salt (2.5 μM) alone or together with pharmacological inhibitors of MAPKs (ERK inhibitor, PD98059 (20 μM); JNK inhibitor, SP600125 (20 μM); p38 kinase inhibitor, SB203580 (20 μM)) for 4 h. Similar results were obtained in three independent experiments.

increase the expression of multiple Nrf2-target genes including HO-1 in murine keratinocytes. The Nrf2 pathway is known to be regulated by the phosphorylation cascades (Huang et al 2000; Bloom & Jaiswal 2003; Cullinan et al 2003). Based on this, we examined the potential involvement of MAPKs in the activation process of Nrf2 by FPP-3 in PE cells. The reporter plasmid containing the Nqo1 ARE was transfected into PE cells and cells were incubated with FPP-3 salt (2.5 μM) alone or together with pharmacological inhibitor of extracellular signal-related kinase (ERK) (PD98059, 20 μM), c-Jun N-terminal kinase (JNK) (SP600125, 20 μM) or p38 kinase (SB203580, 20 μM) for 18 h. The ARE-driven luciferase activity, which was elevated by FPP-3 incubation, was not affected by co-incubation with these pharmacological inhibitors of MAPKs (Figure 2C). A similar result was obtained with HO-1 mRNA levels (Figure 2D). When cells were incubated with FPP-3 salt alone or together with pharmacological inhibitors of MAPKs (PD98059, SP600125 and SB203580) for 4 h, the inducible level of HO-1 by FPP-3 was not significantly attenuated by these MAPK inhibitors. These results suggest the MAPK pathways are not involved in the FPP-3-mediated activation process of Nrf2 in murine keratinocytes.

Induction of HO-1 by FPP-3 is mediated through Nrf2

Next, we studied the inducibility of Nrf2-target genes in murine embryonic fibroblasts (MEFs) from wild-type and *nrf2*-disrupted mice. When wild-type MEFs were incubated with 2.5 μM FPP-3 for indicated time periods (2, 8 and 18 h), transcript levels for Nrf2-target genes were increased. Levels for Nqo1 and GCLC were elevated from 2 h to 18 h, while increase in transcript level for HO-1 was detected only at an early incubation time point (2 h) (Figure 3A). In accordance with an increased expression of Nrf2-target genes, accumulation of nuclear Nrf2 was also observed in wild-type MEFs (Figure 3B). Nuclear Nrf2 level was increased following incubation of cells with FPP-3 for 30 min and remained elevated until 6 h. Next, inducible ARE-driven luciferase activity was monitored following FPP-3 incubation in wild-type and *nrf2*-deficient MEFs. Luciferase activity from the Nqo1 ARE was increased by FPP-3 in wild-type MEFs, while no increase was observed in *nrf2*-deficient MEFs (Figure 3C). Furthermore, induction of HO-1 by FPP-3 and FPP-3 salt (2.5 and 5 μM) in MEFs was completely lost in the absence of *nrf2* (Figure 3D). These results show that FPP-3 has antioxidative efficacy in murine cells through enhancing the expression of multiple antioxidative genes such as HO-1, and this modulation is mediated primarily through Nrf2.

FPP-3 suppressed the increase of inflammatory markers in response to LPS and UV radiation

Exposure of cells to LPS and UV radiation can trigger the inflammatory process by enhancing the expression of NF-κB-target genes. Enhanced ROS generation by these challenges is known to be associated with the exacerbation of inflammation.

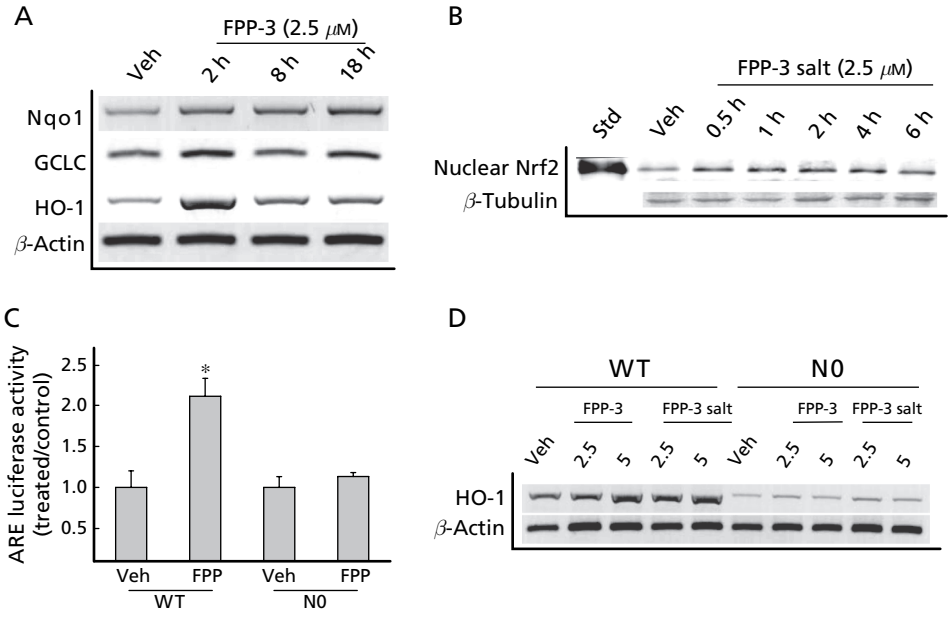

Figure 3 Induction of antioxidative genes by FPP-3 is dependent on Nrf2. A. Increases in the expression of antioxidative genes in murine embryonic fibroblasts (MEFs). Wild-type MEFs were incubated with vehicle (Veh) or FPP-3 (2.5 μM) for 2, 8 or 18 h, and total RNAs were extracted for RT-PCR analysis of Nqo1, GCLC and HO-1. B. Effect of FPP-3 salt on levels of nuclear Nrf2 in wild-type MEFs. Cells were incubated with vehicle (Veh) or FPP-3 salt (2.5 μM) for indicated time periods (0.5, 1, 2, 4 or 6 h) and nuclear proteins were extracted for immunoblot analysis. Similar results were observed in 2 or 3 different experiments. C. Effect of FPP-3 on ARE-driven luciferase activity in MEFs from wild-type (WT) and *nrf2*-disrupted mice (N0). Cells were transfected with the reporter plasmid containing the human Nqo1 ARE and were incubated with vehicle (Veh) or FPP-3 (2.5 μM) for 18 h. Values are mean ± s.d. from four independent experiments. $^*P < 0.05$ compared with vehicle-treated control group. D. Transcript levels for HO-1 in MEFs with wild-type (WT) and *nrf2*-disruption (N0) following treatment with FPP-3. Cells were incubated with vehicle, FPP-3 or FPP-3 salt (2.5 or 5 μM) for 2 h and isolated total RNAs were used for RT-PCR analysis. Similar results were found in three independent experiments.

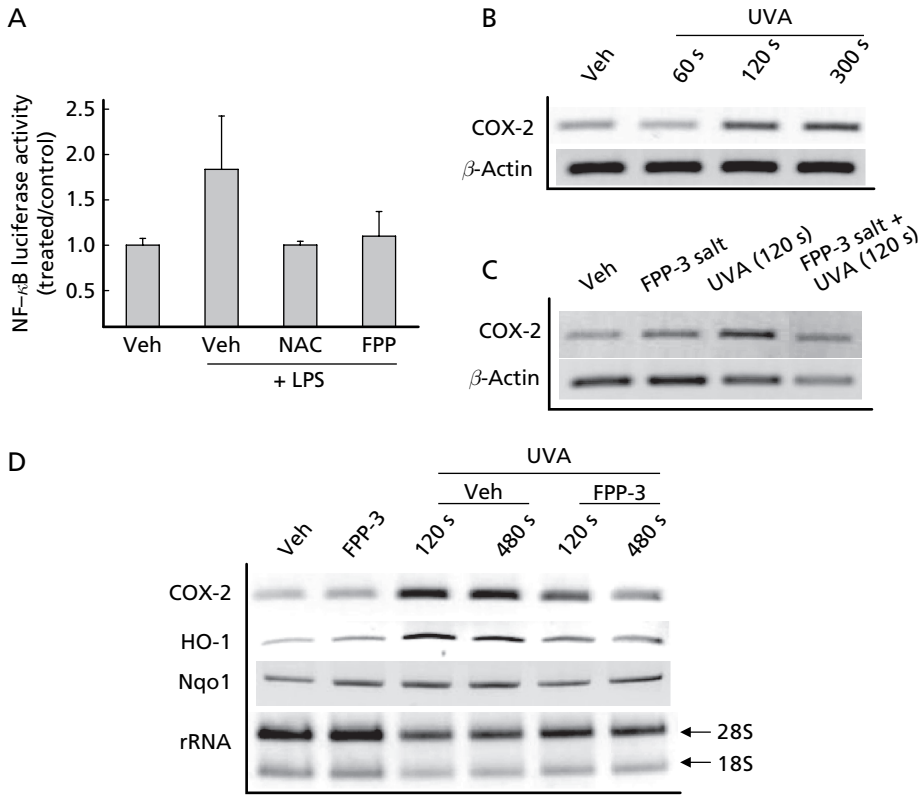

Figure 4 Suppression of LPS- and UVA-induced inflammatory markers by FPP-3. A. Effect of FPP-3 and *N*-acetylcysteine (NAC) on LPS-mediated NF-κB activation in wild-type MEFs. Cells were transfected with the luciferase reporter plasmid containing NF-κB response element, and were incubated with vehicle (Veh), NAC (500 mM) or FPP-3 (2.5 μM) for 18 h. Then, cells were washed with PBS and LPS (10 μg mL^{-1}) incubation was followed for a further 18 h. Values are mean ± s.d. from three independent experiments. B. Transcript levels for COX-2 following exposure of cells to UVA. Murine keratinocyte PE cells were irradiated by UVA (1,350 μW cm^{-2}) for indicated time periods (60, 120 or 300 s) in the presence of a thin layer of PBS. Cells were then recovered for 6 h in the complete medium and total RNAs were extracted for RT-PCR analysis of COX-2 mRNA. C, D. Effect of FPP-3 salt on UVA-induced COX-2 expression in murine keratinocytes. Cells were pre-incubated with vehicle (Veh) or FPP-3 salt (2.5 μM) for 2 h (C) or 18 h (D), and were exposed to UVA radiation (1,350 μW cm^{-2}) for indicated times. Cells were recovered for 6 h and total RNAs were isolated for RT-PCR analysis of COX-2, HO-1 and Nqo1. Similar results were obtained in three independent experiments.

Next, the effect of FPP-3 on the expression of inflammatory markers was studied in murine cells following LPS or UV exposure. First, we examined the effect of FPP-3 on LPS-mediated NF-κB activation in wild-type MEFs. Cells were transfected with the reporter plasmid containing the NF-κB response element and further incubated with LPS (10 μg mL^{-1}) for 18 h. Measured luciferase activity from the NF-κB response element was found to increase following LPS treatment, and this increase was effectively inhibited by a GSH precursor *N*-acetyl cysteine (NAC, 500 mM). This result implies the involvement of ROS generation in the activation process of NF-κB following LPS challenge. In a similar pattern to NAC treatment, pre-incubation of cells with FPP-3 for 18 h also attenuated NF-κB stimulation in these cells (Figure 4A). Second, we examined the effect of FPP-3 on UVA-induced COX-2 expression in murine keratinocytes. When cells were irradiated by UVA, the expression of COX-2 was increased significantly in an exposure-time-dependent manner (Figure 4B). To examine the effect of FPP-3, cells were pre-incubated with FPP-3 for 2 or 18 h, and then exposed to UV irradiation. Our results indicate that the increase in COX-2 levels by UVA was attenuated in FPP-3 pre-treated cells (Figure 4C, 4D). Alteration of COX-2 expression by short-time incubation of FPP-3 might imply that this compound can directly interfere with COX-2 expression, not through activating the Nrf2 pathway. However, involvement of activated Nrf2 in COX-2 repression by FPP-3 can be still hypothesized. Increase in HO-1, which is known to be a potent anti-inflammatory molecule, was rapidly inducible by FPP-3 (Figure 2B, 3A). Furthermore, it has been observed that inducible expression of HO-1 and Nqo1 by UVA-associated stress was suppressed in the 18 h pre-incubation group. These results suggest that FPP-3 may participate in the process of inflammation through activation of Nrf2.

Discussion

Enhanced ROS generation during the process of inflammation plays a beneficial role in innate immunity and cell signalling. However, excess levels of ROS are often associated with tissue damage and exacerbation of inflammation by enhancing responsiveness of immune cells to inflammatory mediators. In particular, the NF-κB pathway induces the expression

of potent pro-inflammatory mediators, including TNF-α and IL-8, in response to ROS (Lee et al 2004). In fact, ROS have been found to be involved in UV- and asthma-associated inflammatory processes (Clydesdale et al 2001; Rangasamy et al 2005). Thus, inhibition or neutralization of excess ROS using antioxidants is known to suppress the inflammatory signalling. The cellular antioxidant system is largely regulated by transcription factor Nrf2. Genes encoding multiple phase 2 metabolizing enzymes, GSH-synthesis/maintenance enzymes and small antioxidants, such as thioredoxin, are known to be downstream genes of Nrf2 (Kwak et al 2004; Kensler et al 2007). Accumulating lines of evidence show that Nrf2 plays an important role in modulating acute inflammation. *Nrf2*-disrupted mice have been shown to be more sensitive to allergen-induced airway inflammation, carrageenan-induced pleurisy, cigarette-smoke-induced emphysema, dextran-sulfate-mediated colitis and sepsis following LPS exposure (Itoh et al 2004; Rangasamy et al 2004, 2005; Li & Nel 2006; Thimmulappa et al 2006a, b; Osburn et al 2007). The Nrf2 pathway is known to modulate the expression of cytokines and chemokines by suppressing levels of ROS (Rangasamy et al 2004; Thimmulappa et al 2006b). Another strong linkage between the Nrf2-antioxidant system and its anti-inflammatory activity can be obtained from the function of HO-1. HO-1 is the rate-limiting enzyme in haem catabolism. It catalyses the degradation of haem and produces iron, carbon monoxide and biliverdin (Otterbein & Choi 2000). HO-1 has a strong antioxidative potential through several mechanisms—firstly, HO-1 effectively removes haem, which is one of the most dangerous oxidant in the body and, secondly, HO-1 mediates the final production of bilirubin and carbon monoxide, which are strong endogenous antioxidants in animals. Furthermore, generated carbon monoxide is known to function as a strong anti-inflammatory component. The expression of pro-inflammatory cytokines was inhibited by HO-1 induction. Increased HO-1 expression by 15-deoxy-$\Delta^{12,15}$-prostaglandin J_2 could suppress the production of TNF-α following treatment with LPS in murine macrophages (Lee et al 2003b). As a consequence, exposure of *ho-1*-deficient mice to endotoxin leads to increased pro-inflammatory cytokine secretion and high mortality compared with wild-type mice (Poss & Tonegawa 1997; Lee et al 2003b; Kapturczak et al 2004). The expression of both basal and inducible HO-1 is primarily regulated by Nrf2 through the ARE located on its promoter (Kwak et al 2004). These reports suggest that pharmacological intervention using Nrf2 activators might be beneficial in alleviating acute inflammation and its pathological complications such as cancer. Dinkova-Kostova et al (2005) have shown that there is a linear correlation between the potency of Nrf2 activation and the anti-inflammatory effect of a series of synthetic triterpenoid analogues. LPS-induced inflammatory response and mortality in mice were effectively suppressed by a strong Nrf2 activator triterpenoid analogue and these protective effects were lost in the absence of *nrf2* (Thimmulappa et al 2006b).

FPP-3 has been developed as an enzyme inhibitor of COX-2 and 5-LOX (Jahng et al 2004). FPP-3 inhibited LPS-stimulated production of NO and TNFα in cultured murine macrophages (Lee et al 2004) and suppressed carrageenan-induced paw oedema formation in rats (Lee et al 2006). As the chemical structure of this compound possesses an activated Michael acceptor, which is known to be one of the chemical categories activating Nrf2, we investigated the effect of this compound on the Nrf2 pathway in murine cells. FPP-3 induced antioxidant genes, including Nqo1, GCLC, and HO-1, and accumulated nuclear Nrf2 in murine fibroblasts and keratinocytes. Activation of the ARE and induction of HO-1 mRNA were lost in *nrf2*-deficient fibroblasts, which indicates that the molecular target of FPP-3 is Nrf2. Enhanced cellular antioxidative capacity following FPP-3 treatment prevented LPS-mediated NF-κB activation in murine fibroblasts and UVA-induced COX-2 expression in murine keratinocytes. These results suggest that COX-2 inhibitor FPP-3 can activate the Nrf2-antioxidant pathway and the dual effect of FPP-3 on COX-2 and Nrf2 may provide a better strategy to control inflammation and its complications.

Signalling pathways involved in the process of Nrf2 activation by FPP-3 remain undiscovered. The Nrf2 pathway is known to be regulated by the kinase cascades under different conditions. Protein kinase C (PKC), MAPKs, phosphatidylinositol 3-kinase (PI3K) and PKR-like endoplasmic reticulum kinase (PERK) have been implicated in the process of Nrf2 activation by redox chemicals and cancer-preventive chemicals (Huang et al 2000; Bloom & Jaiswal 2003; Cullinan et al 2003; Kwak et al 2004). A recent study by Hwang et al (2008) demonstrated that the induction of Nqo1 by FPP-3 was blocked by pharmacological inhibitors of PKC and PI3K in breast cancer cells. However, in our study, inducible expression of the ARE reporter gene and HO-1 by FPP-3 was not altered by pharmacological inhibitors of ERK, JNK and p38 kinase in murine keratinocytes. This discrepancy can be explained by different observations showing that the signalling pathways involved in the process of Nrf2 activation might vary depending on cell type and chemicals. Our previous study demonstrated that chemopreventive 3*H*-1,2-dithiole-3-thione activates the Nrf2 pathway through the extracellular signal-regulated kinase (ERK) signalling pathway in murine keratinocyte PE cells; however, no involvement was observed in murine hepatoma hepa1c1c cells (Manandhar et al 2007). The probable molecular mechanism for Nrf2 activation by FPP-3 in PE cells would be on the Michael acceptor within its chemical structure. The sulfhydryl residues in Keap1 protein are regarded as a redox-sensor regulating the nuclear translocation of Nrf2, and these residues can be modified by Michael acceptor-containing chemicals (Dinkova-Kostova et al 2001; Wakabayashi et al 2004). Therefore, it can be hypothesized that FPP-3 might interact directly with Keap1 and mediate accumulation of Nrf2 within the nucleus by releasing Nrf2 from Keap1.

Conclusion

Inflammation is accompanied by increased generation of ROS. Increased ROS is associated with activation of the NF-κB pathway, which can lead to the enhanced expression of inflammatory mediators such as COX-2. Now, it is widely accepted that inhibition of excess ROS generation during inflammation can suppress the inflammatory response and its complications. Pharmacological intervention with small molecules,

which can activate the Nrf2-antioxidant system, might be particularly beneficial in the fight against ROS during inflammation. Our current study shows that a novel COX-2 inhibitor, FPP-3, enhances the expression of multiple antioxidant genes such as HO-1 through the Nrf2 pathway. The antioxidative property of this compound appears to participate in the suppressive effects on LPS- and UVA-activated inflammatory signalling. Therefore, the dual function of this compound may effectively control the process of inflammation by blocking inflammatory mediators directly, as well as by alleviating oxidative stress at the site of inflammation.

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