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Original Contribution

2′,5′-Dihydroxychalcone-induced glutathione is mediated by oxidative stress and kinase signaling pathways

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ABSTRACT

Hydroxychalcones are naturally occurring compounds that continue to attract considerable interest because of their anti-inflammatory and antiangiogenic properties. They have been reported to inhibit the synthesis of the inducible nitric oxide synthase and to induce the expression of heme oxygenase-1. This study examines the mechanisms by which 2′,5′-dihydroxychalcone (2′,5′-DHC) induces an increase in cellular glutathione (GSH) levels using a cell line stably expressing a luciferase reporter gene driven by antioxidant-response elements (MCF-7/AREc32). The 2′,5′-DHC-induced increase in cellular GSH levels was partially inhibited by the catalytic antioxidant MnTDE-1,3-IP^{5+}, suggesting that reactive oxygen species (ROS) mediate the antioxidant adaptive response. 2′,5′-DHC treatment induced phosphorylation of the c-Jun N-terminal kinase (JNK) pathway, which was also inhibited by MnTDE-1,3-IP^{5+}. These findings suggest a ROS-dependent activation of the AP-1 transcriptional response. However, whereas 2′,5′-DHC triggered the NF-E2-related factor 2 (Nrf2) transcriptional response, cotreatment with MnTDE-1,3-IP^{5+} did not decrease 2′,5′-DHC-induced Nrf2/ARE activity, showing that this pathway is not dependent on ROS. Moreover, pharmacological inhibitors of mitogen-activated protein kinase (MAPK) pathways showed a role for JNK and p38MAPK in mediating the 2′,5′-DHC-induced Nrf2 response. These findings suggest that the 2′,5′-DHC-induced increase in GSH levels results from a combination of ROS-dependent and ROS-independent pathways.

Hydroxychalcones (HCs) are both the biosynthetic precursors of flavonoids and the end-products associated with a variety of biological activities [1–3]. Their anti-inflammatory and antiangiogenic properties continue to attract considerable interest [4,5]. Along with flavonoids, HCs can generate oxidative stress by interfering with the mitochondrial respiratory chain and by inducing glutathione (GSH) efflux through multidrug resistance-associated proteins (MRPs) [6–8]. Whereas flavonoids such as chrysin and apigenin induce GSH efflux through MRP1, HC-induced GSH efflux is mediated by the breast cancer resistance protein (BCRP/ABCG2) [9–12]. Another relevant difference between HCs and chrysin is that HCs also seem to induce a rebound in intracellular GSH (iGSH) levels [13].

Cells adapt to increased levels of reactive oxygen species (ROS) by inducing the expression of a series of phase II antioxidant enzymes, including heme oxygenase-1 (HO-1) and the enzymes involved in the synthesis of GSH [14–16]. This antioxidant adaptive response is mediated by several transcriptional pathways, including NF-E2-related factor-2 (Nrf2) and activator protein-1 (AP-1) [17–19]. However, a number of protein kinase signaling pathways have been shown to trigger both the Nrf2 and the AP-1 pathways independent of ROS overproduction [20,21]. Several studies have examined the mechanism of HC-induced HO-1 expression, but not of HC-induced GSH synthesis [22–25]. The nonhydroxylated chalcone (Fig. 1) has been shown to trigger the Nrf2-mediated response [22], and studies with 2′-hydroxychalcone (2′-HC; Fig. 1) have associated HO-1 expression with the phosphatidylinositol 3-kinase (PI3K) pathway and AP-1 activation [23–25]. The involvement of ROS in these pathways remains unclear.

The aim of this study was to examine the mechanisms by which 2′,5′-dihydroxychalcone (2′,5′-DHC; Fig. 1) induces elevated cellular

Abbreviations: AP-1, activator protein-1; ARE, antioxidant-response element; BCRP, breast cancer resistance protein; BSO, L-buthionine sulfoximine; 2′,5′-DHC, 2′,5′-dihydroxychalcone; ERK, extracellular-signal-regulated kinase; GCLC, glutamate–cysteine ligase catalytic subunit; GCLM, glutamate–cysteine ligase regulatory subunit; GSH, reduced glutathione; HO-1, heme oxygenase-1; 2′-HC, 2′-hydroxychalcone; HC, hydroxychalcone; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MnTDE-1,3-IP^{5+}, manganese(III) *meso*-tetrakis(*N*,*N*′-diethylimidazolium-2-yl)porphyrin; MRP, multidrug resistance-associated protein; NAC, *N*-acetylcysteine; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; Nrf2, NF-E2-related factor-2.

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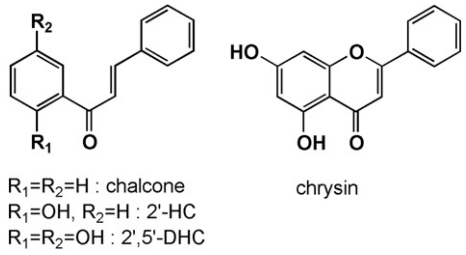

Fig. 1. Structures of chalcone, 2′-hydroxychalcone (2′-HC), 2′,5′-dihydroxychalcone (2′,5′-DHC), and the flavonoid chrysin.

levels of GSH using a transformed breast cancer cell line stably expressing a luciferase reporter gene driven by antioxidant-response elements (MCF-7/AREc32). A powerful tool to study the involvement of oxidative stress in cellular processes is the catalytic antioxidant manganese(III) *meso*-tetrakis(*N*,*N*′-diethylimidazolium-2-yl)porphyrin (MnTDE-1,3-IP^{5+}), a member of a series of cationic manganese porphyrins that dismute superoxide and hydrogen peroxide, scavenge peroxynitrite, and inhibit lipid peroxidation [26–28]. As shown using MnTDE-1,3-IP^{5+}, 2′,5′-DHC triggered a ROS-dependent activation of the JNK pathway, which was associated with both the AP-1- and the Nrf2-mediated antioxidant transcriptional responses. However, the activation of the Nrf2/ARE pathway appeared to be independent of ROS and involved both JNK and p38MAPK pathways.

Materials and methods

Chemicals and reagents

Chalcone, 2′-HC, and 2′,5′-DHC were purchased from Indofine Chemicals Co. (Hillsborough, NJ, USA). Chrysin, L-buthionine sulfoximine (BSO), L-glutathione, pyruvate (sodium salt), ATP, acivicin, *N*-ethylmorpholine, phenylmethanesulfonyl fluoride (PMSF), sulfosalicylic acid, phosphoric acid, *meta*-phosphoric acid, sodium phosphate (monobasic), Triton X-100, EDTA, NADH, NADPH, K_2HPO_4, KH_2PO_4, Hepes, KCl, $MgCl_2$, sucrose, D-mannitol, dimethyl sulfoxide, and dimethyl formamide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tris–HCl, NaCl, 2-mercaptoethanol, and methanol were purchased from Fisher (Pittsburgh, PA, USA). SDS was purchased from Bio-Rad Laboratories (Hercules, CA, USA). MitoSOX was obtained from Molecular Probes (Eugene, OR, USA). Phosphate-buffered saline (PBS) was obtained from Cellgro (Herndon, VA, USA). Protease inhibitor cocktail tablets supplemented with EDTA were obtained from Roche Diagnostics (Indianapolis, IN, USA). SB203580, wortmannin, and U0126 were purchased from Biomol (Plymouth Meeting, PA, USA). SP600125 was purchased from Calbiochem (San Diego, CA, USA). MnTDE-1,3-IP^{5+} was prepared as previously described in U.S. Patent 6,544,975B1 and was a kind gift from Aeolus Pharmaceuticals (Mission Viejo, CA, USA).

Cell lines and culture conditions

Transformed breast cancer cells stably expressing MCF-7/AREc32, referred to as AREc32 cells in this study, were obtained from Dr. C. Roland Wolf (University of Dundee, UK) and were grown in DMEM (low glucose) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (10,000 units; Cellgro), and Geneticin (400 mg/500 ml) at 37 °C and in a 5% CO_2-supplemented air atmosphere [29]. 16HBE cells, a transformed human bronchial epithelial cell line, were grown in minimal essential medium α + Glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 1% pen/strep. A Gal4 reporter system (Stratagene, La Jolla, CA, USA) was used to measure the transactivational activity of c-Jun as described previously [30]. This reporter assay uses a luciferase reporter gene driven by four copies of the Gal4 regulatory sequence (pGal4-TK-Luc) and an expression vector for the chimeric protein Gal4–c-Jun, which consists of the DNA-binding domain of Gal4 and the transactivation domain of c-Jun. Transient transfection of these plasmids into cultured 16HBE cells was carried out using LipofectAMINE 2000 reagent (Invitrogen–Life Technologies, Carlsbad, CA, USA). A constitutively active *Renilla* luciferase (pRL-TK-luc) was included to correct for transfection efficiency and to account for nonspecific effects of treatments on luciferase activity.

Assessment of cytotoxicity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay is commonly used to measure cancer cell survival, yet it has revealed artifacts when measuring the cytotoxicity of pro-oxidant agents [31]. Another simple method to evaluate drug-induced cytotoxicity is using membrane integrity as an index, which can be assessed by monitoring the release of cytosolic lactate dehydrogenase (LDH). AREc32 cells were grown in 24-well plates and LDH activity was measured after 48 h treatment in the culture medium and cell lysates (50 mM Hepes, 0.5% Triton X-100, pH 7) using a plate reader format as previously described [32]. Briefly, 5 µl of cell culture supernatant or lysate was incubated with 0.24 mM NADH in a Tris/NaCl, pH 7.2, buffer in 96-well plates for 5 min at 25 °C. The reaction was started by the addition of 9.8 mM pyruvate and the consumption of NADH was followed at 340 nm for 5 min at 30 °C. Percentage LDH release was calculated using the following equation: supernatant LDH/(supernatant LDH + lysate LDH) × 100.

Flow cytometry and microscopy

MitoSOX is an analog of hydroethidine and is routinely used to detect mitochondrial ROS by flow cytometry [33]. The results obtained with this method remain qualitative rather than quantitative [34]. The oxidation products of MitoSOX were detected using the FL2 channel. Briefly, AREc32 cells were grown in 24-well plates and treated cells (approximately 5×10^4) were exposed to 5 µM MitoSOX for 20 min. The supernatant was removed and the cells were scraped in 0.5 ml ice-cold PBS, centrifuged at 2000 *g* for 15 min, and resuspended in 0.5 ml ice-cold PBS. Cells were analyzed within 30 min using a FACSCalibur flow cytometer (Becton–Dickinson Biosciences, San Jose, CA, USA). The total number of gated cells counted was 10,000. Microscopy images were obtained directly from the culture plate after MitoSOX treatment and replacement of the culture medium with PBS using an Evos-fl microscope (Advanced Microscopy Group, Bothell, WA, USA).

Intracellular levels of GSH

Intracellular GSH levels were determined by HPLC with electrochemical detection [35]. Cultured AREc32 cells from 24-well plates were washed once with 1 ml of PBS, resuspended in 0.5 ml of PBS, and sonicated. Twenty-five microliters of 10% *meta*-phosphoric acid was then added to the samples (1% v/v final concentration), the samples were centrifuged at 20,000 *g* for 10 min, and the supernatants were used for HPLC analysis. The HPLC column used was a Synergi 4u Hydro-RP 80A (150 × 4.6 mm) from Phenomenex (Torrance, CA, USA) and the mobile phase was sodium phosphate buffer (125 mM sodium phosphate monobasic, pH adjusted to 3 with phosphoric acid) and 0.9% methanol. The flow rate was 0.5 ml/min. The retention time for GSH under these conditions was 7.0 min. The HPLC instrument was from ESA, Inc. (Chelmsford, MA, USA) and was equipped with an autosampler (Model 540) and a CoulArray detector (Model 5600A). The potential applied was +0.75 V vs H/Pd electrode, and the injection volume was 5 µl. The remaining 0.1 ml sample was used to measure protein content using Coomassie Plus protein assay reagent (Thermo Scientific, Rockford, IL, USA).