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Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38

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ABSTRACT

Dietary flavonoid quercetin has been suggested as a cancer chemopreventive agent, but the mechanisms of action remain unclear. This study investigated the influence of quercetin on p38-MAPK and the potential regulation of the nuclear transcription factor erythroid-2p45-related factor (Nrf2) and the cellular antioxidant/detoxifying defense system related to glutathione (GSH) by p38 in HepG2 cells. Incubation of HepG2 cells with quercetin at a range of concentrations (5–50 μM) for 4 or 18 h induced a differential effect on the modulation of p38 and Nrf2 in HepG2 cells, 50 μM quercetin showed the highest activation of p38 at 4h of treatment and values of p38 similar to those of control cells after 18 h of incubation, together with the inhibition of Nrf2 at both incubation times. Quercetin (50 μM) induced a time-dependent activation of p38, which was in concert with a transient stimulation of Nrf2 to provoke its inhibition afterward. Quercetin also increased GSH content, mRNA levels of glutamylcysteine-synthetase (GCS) and expression and/or activity of glutathione-peroxidase, glutathione-reductase and GCS after 4 h of incubation, and glutathione-*S*-transferase after 18 h of exposure. Further studies with the p38 specific inhibitor SB203580 showed that the p38 blockage restored the inhibited Nrf2 transcription factor and the enzymatic expression and activity of antioxidant/detoxificant enzymes after 4 h exposure. In conclusion, p38-MAPK is involved in the mechanisms of the cell response to quercetin through the modulation of Nrf2 and glutathione-related enzymes in HepG2 cells.

1. Introduction

Quercetin (3,3′,4′,5,7-pentahydroxylflavone) is a flavonoid, one of the most abundant polyphenolic groups in plants (fruits and vegetables) [1], which can be extensively metabolized during absorption in the small intestine and in the liver [2,3]. Many studies have demonstrated that quercetin exerts potential anticarcinogenic activities, since it is a potent inhibitor of tumor initiation *in vivo* [4], is a powerful radical scavenger able to prevent or delay conditions which favor cellular oxidative stress [5,6], possesses anti-proliferative activities against tumor cells *in vitro* [4,7,8], and induces apoptosis in different cancer cell lines by modulating a number of key elements in cellular signal transduction pathways linked to the apoptotic cell death [1,7–12].

Two possible mechanisms have been suggested to explain the chemoprotective effects of quercetin. The first includes the removal of preinitiated cells from damaged tissues through cell cycle arrest and apoptosis [10–13]. Alternatively, the modulation of metabolizing enzymes has been proposed as another chemopreventive mechanism that can block carcinogen activation (inhibition of cytochrome P450) or enhance the detoxification of activated carcinogens (activation of phase II detoxifying enzymes) [14]. Thus, quercetin is found to interact with the cellular defense system such as NADPH:quinone oxidoreductase, iNOS, monooxygenase, COX, xanthine oxidase, lipoxygenase, hemeoxygenase-1, etc. [8,15–17]. In this regard, the induction of antioxidant/detoxifying enzymes as well as glutathione (GSH) levels provides significant biological mechanisms for protection against toxic effects of endogenous reactive oxygen species (ROS) and exogenous carcinogens and/or their reactive intermediates [16,18–22].

Nuclear factor-erythroid 2p45-related factor-2 (Nrf2) plays a central role in the induction of antioxidant and detoxifying enzymes through its binding to the antioxidant response element

Abbreviations: AKT/PKB, protein kinase B; AP-1, activator protein-1; ARE, antioxidant response element; CHX, cycloheximide; DAPI, 6-diamidino-2-phenylindole; DTT, dithiothreitol; ERK, extracellular regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCS, glutamylcysteine synthetase; GPx, glutathione peroxidase; GR, glutathione reductase; GRB2, growth factor receptor-bound protein 2; GSH, glutathione; GST, glutathione *S*-transferase; JNK, *c-jun* amino-terminal kinase; Keap-1, Kelch-like ECH-associated protein-1; MAPK, mitogen-activated protein kinase; MCB, monochlorobimane; NAC, *N*-acetyl-cysteine; Nrf2, nuclear transcription factor erythroid 2p45 (NF-E2)-related factor; PARP, poly(ADPribose)polymerase; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species.

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(ARE) [23,24]. Nrf2 is sequestered in the cytoplasm as an inactive complex with its cytosolic repressor Kelch-like ECH associated protein-1 (Keap-1). The dissociation of Nrf2 from Keap-1 is crucial for its nuclear translocation, followed by binding to DNA and activation of cytoprotective genes [23]. To date, multiple signaling kinases related to cell survival/proliferation have been reported to regulate Nrf2, which include extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase (JNK), phosphatidylinositol-3-kinase (PI3K) and protein kinase C (PKC) [21,23]. Indeed, the phosphorylation of Nrf2 by these different kinases at multiple sites seems to be an important mechanism in Nrf2-mediated ARE activation and in regulating the stability of this transcription factor [25], but few data have been reported on Nrf2 regulation and p38 MAPK.

p38 MAPK consists of four isoforms: p38α, p38β, p38γ and p38δ. p38α is the most abundant and ubiquitously expressed family protein and has a well established role in stress response and inflammation [26]. Once p38-MAPK is phosphorylated and activated, it phosphorylates and/or activates downstream substrates (kinases, transcriptional factors, etc.), resulting in various cellular responses such as proliferation, apoptosis, cell cycle arrest, and inflammation [26]. In line with this, up-regulation of the hemeoxygenase-1 detoxification enzyme via Nrf2 has been reported to involve p38-MAPK in renal cells [27]. Similarly, Yao et al. [17] have demonstrated that p38 and ERK mediated quercetin-derived Nrf2 translocation into nuclei and subsequent induction of hemeoxygenase-1 activity.

Although different studies have consistently demonstrated that quercetin is a strong inducer of GSH content and antioxidant/detoxificant enzymes, the detailed upstream signaling mechanisms are still unclear. In the present study, quercetin modulation of p38 in relation to Nrf2 function and the role of p38 on the regulation of cellular antioxidant/detoxifying defense system related to glutathione have been analyzed.

2. Materials and methods

2.1. Materials and chemicals

Quercetin, SB203580, cycloheximide (CHX), gentamicin, penicillin G and streptomycin were purchased from Sigma Chemical (Madrid, Spain). Anti-phospho-p38 recognizing phosphorylated Thr180/Tyr182 (9216) and anti-β-actin (4697) were obtained from Cell Signalling Technology (Izasa, Madrid, Spain). Anti-p38α (sc-535), anti-Nrf2 (C-20, sc-722), anti-Nrf2 (H-300, sc-13032), anti-PARP (sc-7150) and anti-GRB2 (sc-255) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Materials and chemicals for electrophoresis were from BioRad (BioRad Laboratories S.A., Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Biowhittaker Europe (Lonza, Madrid, Spain), respectively.

2.2. Cell culture and quercetin treatment

Human hepatoma HepG2 cells were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain), supplemented with 2.5% Biowhitaker fetal bovine serum (FBS) and the following antibiotics: gentamicin, penicillin and streptomycin (50 mg/L). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Cells were seeded and routinely grown in DMEM-F12 medium and 2.5% FBS, but they were changed to serum-free medium 24 h before the assay in order to avoid the influence of the growth factors contained in the FBS on the results. To study the time-course effects of quercetin, cells were treated with 50 μM quercetin and then harvested at different times (0, 10, 30, 60, 120, 240 and 1080 min).

In the experiments with quercetin and the selective pharmacological inhibitor of p38, cells were preincubated with SB203580 (10 μM) for 2 h prior to 50 μM quercetin treatment for 240 min or 1080 min.

To analyze the effect of quercetin and p38 inhibition on Nrf2 stabilization, HepG2 cells were incubated with SB203580 for 2 h before incubating with 50 μM quercetin for 1080 min and then were treated with 100 μg/mL CHX for 1 h.

2.3. Fluorescence microscopy

Fluorescence assay was performed as previously described [28]. Briefly, HepG2 cells were seeded (25,000 cells/well were used to cary out the assay) on glass coverslips with DMEM F-12 supplemented with FBS for 24 h and changed to serum-free medium 24 h before the assay. After incubation with quercetin for the indicated times, cells were washed with PBS at room temperature and then fixed with 3.7% paraformaldehyde for 10 min at room temperature. Nuclei were visualized by using DAPI staining. The coverslips were mounted in Vectashield and images were taken with a Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss Microimaging GmbH, Munich, Germany) at 63× magnification. AxioVisionRel 4.6 software was used for the analysis of the images obtained.

2.4. Preparation of total cell lysates

To detect p38 and phospho-p38, cells were lysed at 4 °C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.1% Triton X-100, 200 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). The supernatants were collected, assayed for protein concentration by using the Bio-Rad (Bio-Rad, Madrid, Spain) protein assay kit according to the manufacture's specifications, aliquoted and stored at −80 °C until used for Western blot analyses.

2.5. Preparation of nuclear and cytosolic extracts

To analyze Nrf2, cells were resuspended at 4 °C in 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF (buffer A), allowed to swell on ice for 10 min, and then vortexed for 10s. Samples were centrifuged at 10,000*g* for 2 min and the supernatant containing the cytosolic fraction was stored at −80 °C. The pellet was resuspended in cold buffer B (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2.5 μg/mL leupeptin, 2.5 μg/mL aprotinin) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation at 13,000*g* for 10 min at 4 °C, and the supernatant fraction containing nuclear protein extract was stored at −80 °C. Proteins were measured using the Bradford reagent (Bio-Rad, Madrid, Spain) following the recommendations of the supplier.

2.6. Western blot analysis

Equal amounts of proteins (100 μg) were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) filters (Protein Sequencing Membrane, Bio-Rad, Madrid, Spain). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit immunoglobulin (GE Healthcare, Madrid, Spain) or peroxide-conjugated anti-mouse immunoglobulin only for p-p38