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Original Contribution

Protection of hydroquinone-induced apoptosis by downregulation of Fau is mediated by NQO1

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ABSTRACT

The *Fau* gene (Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)-associated ubiquitously expressed gene) was identified as a potential tumor suppressor gene using a forward genetics approach. Downregulation of *Fau* by overexpression of its reverse sequence has been shown to inhibit apoptosis induced by DNA-damaging agents. To address a potential role of Fau in benzene toxicity, we investigated the apoptotic effects of hydroquinone (HQ), a major benzene metabolite, in W7.2 mouse thymoma cells transfected with either a plasmid construct expressing the antisense sequence of *Fau* (rfau) or the empty vector (pcDNA3.1) as a control. HQ induced apoptosis via increased production of reactive oxygen species and DNA damage, measured using dihydroethidine (HE) staining and alkaline Comet assay, respectively, in W7.2 pcDNA3.1 cells. In contrast, when *Fau* was downregulated by the antisense sequence in W7.2 rfau cells, HQ treatment did not cause DNA damage and oxidative stress and these cells were markedly more resistant to HQ-induced apoptosis. Further investigation revealed that there was an upregulation of NAD(P)H: quinone oxidoreductase 1 (NQO1), a detoxification enzyme for benzene-derived quinones, in W7.2 rfau cells. Compromising cellular NQO1 by use of a specific mechanism-based inhibitor (MAC 220) and NQO1 siRNA resensitized W7.2 rfau cells to HQ-induced apoptosis. Silencing of Fau in W7.2 wild-type cells resulted in increased levels of NQO1, confirming that downregulation of Fau results in NQO1 upregulation which protects against HQ-induced apoptosis.

Introduction

Benzene is an environmental and occupational toxicant which is found in cigarette smoke, automobile exhaust, and petrochemical industries (reviewed in [1]). Benzene-induced myelotoxicity requires hepatic metabolism by cytochrome P450 2E1 to produce the phenolic metabolites including hydroquinone (HQ) and catechol [2]. The benzene metabolite hydroquinone inhibits proliferation of human T-lymphocytes and induces apoptosis in hematopoietic cells including human bone marrow $CD34^+$, HL-60 promyelocytic leukemia, and Jurkat T-lymphoblastic leukemia cells [3–5]. Accumulation of HQ in the bone marrow has been demonstrated and further oxidation *in situ* by myeloperoxidase or autoxidation produces the toxic 1,4-benzoquinone (BQ) [6]. The reactive BQ can bind to DNA, leading to DNA damage and apoptosis which are both precursors for hematopoietic disorders including acute myeloid leukemia and aplastic anemia [7]. However, BQ can be detoxified back to HQ by NAD(P)H: quinone oxidoreductase 1 (NQO1) (reviewed [2]).

NQO1 is a flavin-containing quinone reductase that maintains quinones and their derivatives in a reduced state, facilitating conjugation and subsequent excretion [8]. Many studies have reported that NQO1 reduces the toxic 1,4-benzoquinone to the less reactive HQ via two-electron reduction. This detoxification prevents unwanted one-electron reduction which results in the formation of reactive oxygen species (ROS) mediated by redox cycling of quinones (reviewed in [9]). NQO1 is a detoxifying enzyme for 1,4-benzoquinone in bone marrow cells and a high myeloperoxidase/NQO1 ratio has been suggested to be a predisposing factor for myelotoxicity as previously demonstrated in human bone marrow $CD34^+$ progenitor cells [2]. Moran et al. [10] have demonstrated that HQ treatment of KG1a human promyeloblastic cells resulted in the induction of NQO1 and subsequent protection against HQ-induced apoptosis. Recently, Siegel et al. [11] have demonstrated that NQO1 directly scavenges superoxide radicals which may confer protection against oxidative stress generated from benzene metabolism. NQO1 has also been implicated in maintaining p53 stability [12].

Various strategies have been utilised to identify the pathways which control cell death and survival [13]. Advances in cDNA

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library-based functional expression cloning have led to the discovery of novel genes which regulate cell death through apoptosis [13]. Our group and others have successfully identified *Fau* (Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)-associated ubiquitously expressed gene), a novel apoptosis-controlling gene and we have recently shown that downregulation of *Fau* confers resistance to HQ-induced cytotoxicity and apoptosis [14]. The full role of Fau in this process has not been completely characterised, although it has already been associated with post-transcriptional modification of the proapoptotic protein Bcl-G [15].

It is clearly possible that NQO1 may play a role in the protection of cells from HQ toxicity by rfau, but this hypothesis has not previously been tested. Specifically, in the present study the role of Fau in regulating HQ-induced apoptosis was further investigated using W7.2 mouse thymoma rfau cells where *Fau* expression has been downregulated by insertion of *Fau* in the reverse, antisense orientation (i.e., rfau). Our results show that HQ-induced apoptosis occurred via DNA damage and ROS production in W7.2 cells, but these effects were abolished in the presence of rfau. Further investigation revealed that NQO1 protein was highly elevated in rfau-stable transfectants as compared to vector control cells (W7.2 pcDNA3.1). Inhibition of NQO1 enzymatic activity and protein expression restored the sensitivity of W7.2 rfau cells to HQ-induced toxicity. Our data demonstrate that the cytoprotection by rfau is mediated by NQO1 and this could potentially play a role in benzene toxicity.

Materials and methods

Cell culture

The W7.2 cells (clone W7.2c [16]), originally derived from mouse thymoma WEHI-105.726 were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine at 37 °C in a 5% CO_2 incubator. The amount of 200 μg/ml G418 (Gibco) was included in cultures of W7.2 rfau and W7.2 pcDNA3.1 to ensure retention of the transfected DNA.

Reagents and antibodies

Hydroquinone, propidium iodide, dihydroethidine (HE), menadione, ethidium bromide, trypan blue, low melting agarose (LMA), normal melting agarose (NMA), mouse monoclonal anti-β-actin antibody, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and dichlorophenol indophenol (DCPIP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Annexin V-FITC was purchased from Pharmingen (San Diego, CA). Mini protease inhibitor cocktail tablet was purchased from Roche Diagnostics (Indianapolis, IN USA). NQO1, anti-Fau and phospho-γH2AX antibody were purchased from Abcam (San Francisco, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody and HRP-conjugated goat anti-rabbit antibody were purchased from Cell Signalling Technology, Inc. (Beverly, MA). MAC220 was synthesized and kindly provided by Dr. C.J Moody as previously described [17].

Establishment of stable clones

The generation of the constructs pcDNA3.1/rfau in expression vectors and the establishment of stable clones expressing these constructs have been described in Mourtada-Maarabouni et al. [18].

Flow cytometric analysis of apoptosis by using annexin V-FITC/PI assay

Cells were treated with HQ (50 and 100 μM) for 14 h prior to apoptosis assessment. The measurement of apoptosis is based on phosphatidylserine (PS) exposure as described by Inayat-Hussain et al. [14]. Briefly, 1×10^6 cells were collected and resuspended in 150 μl annexin V buffer containing 2.5 μl FITC-conjugated annexin V and incubated for 15 min in the dark. Propidium iodide (10 μl of 50 μg/ml stock in PBS) was then added and samples were subjected to flow cytometric analysis using FACS CANTO II (BD Bioscience).

Flow cytometric analysis of reactive oxygen species using dihydroethidine

Generation of superoxide anion was determined as described by Chan et al. [19]. Briefly, 1 μl of 10 mM HE was added to 1 ml of treated cells (1×10^6 cells/ml) and further incubated for 15 min at 37 °C. Cells were then centrifuged at 220*g* for 5 min and resuspended in 1 ml PBS. Flow cytometry was performed using FACS CANTO II (BD Bioscience).

Alkaline Comet assay

The alkaline Comet assay was carried out using cells (1×10^6 cells/ml) treated in 6-well plates. Following incubation, 1 ml cell suspension was transferred to a tube for centrifugation (220*g* for 5 min at 4 °C). The supernatant was removed and pellet washed with Ca^{2+}/Mg^{2+}-free PBS and recentrifuged. The pellets left at the bottom were mixed thoroughly with 80 μl of 0.6% low melting agar (w/v). The mixture was then pipetted onto hardened 0.6% normal melting agar (w/v) as the first layer gel on the slide. Coverslips were placed to spread the mixture and slides were left on ice for low melting agar to solidify. Following the removal of the coverslips, the embedded cells were lysed in lysing buffer containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, and 1% Triton X-100 for 1 h at 4 °C. Slides were soaked in electrophoresis buffer solution for 20 min for DNA unwinding before electrophoresis at 300 mA, 25 V for 20 min. Subsequently, the slides were rinsed with neutralizing buffer for 5 min and stained with 50 μl (10 μg/ml) ethidium bromide solution. Slides were left overnight at 4 °C before analysis with a Leitz Laborlux epifluorescence microscope equipped with 515 nm barrier filter and 560 nm emission filter. Fifty cells per slide were scored and the parameter, tail moment (Tm), was analyzed using the Comet Assay III (Perceptive Instrument Ltd).

Western blot analysis

Samples (1×10^6 cells) were mixed with Laemmli's loading buffer, boiled for 5 min, and subjected to 12% SDS-PAGE at 130 V followed by electroblotting to nitrocellulose membranes for 2 h at 100 V. Membranes were blocked overnight with 5% nonfat milk or 5% (w/v) bovine serum albumin for phospho-protein in TBST at 4 °C. Subsequently, the blot was probed with anti-NQO1 antibody (Abcam, 1:1000), mouse monoclonal anti-Fau antibody (Abcam, 1:1000), and phospho-H2AX antibody (Abcam, 1:1000). The membranes were then rinsed and incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signalling Technology, 1:5000). Following the secondary antibody incubation, the membranes were rinsed and bound antibodies were detected using enhanced chemiluminescence according to the manufacturer's instructions (Perkin Elmer). The densities of specific bands were measured and normalised with the control band using ImageMeter 1.1.1 software.