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Repeated Nrf2 stimulation using sulforaphane protects fibroblasts from ionizing radiation

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ABSTRACT

Most of the cytotoxicity induced by ionizing radiation is mediated by radical-induced DNA double-strand breaks. Cellular protection from free radicals can be stimulated several fold by sulforaphane-mediated activation of the transcription factor Nrf2 that regulates more than 50 genes involved in the detoxification of reactive substances and radicals. Here, we report that repeated sulforaphane treatment increases radioresistance in primary human skin fibroblasts. Cells were either treated with sulforaphane for four hours once or with four-hour treatments repeatedly for three consecutive days prior to radiation exposure. Fibroblasts exposed to repeated-sulforaphane treatment showed a more pronounced dose-dependent induction of Nrf2-regulated mRNA and reduced amount of radiation-induced free radicals compared with cells treated once with sulforaphane. In addition, radiation- induced DNA double-strand breaks measured by gamma-H2AX foci were attenuated following repeated sulforaphane treatment. As a result, cellular protection from ionizing radiation measured by the 5-ethynyl-2′-deoxyuridine (EdU) assay was increased, specifically in cells exposed to repeated sulforaphane treatment. Sulforaphane treatment was unable to protect Nrf2 knockout mouse embryonic fibroblasts, indicating that the sulforaphane-induced radioprotection was Nrf2-dependent. Moreover, radioprotection by repeated sulforaphane treatment was dose-dependent with an optimal effect at 10 uM, whereas both lower and higher concentrations resulted in lower levels of radioprotection. Our data indicate that the Nrf2 system can be trained to provide further protection from radical damage.

Introduction

It has become clear in recent years that the cellular clearance of radicals and other reactive molecules is regulated, in part, by the transcription factor Nuclear factor– erythroid– 2- related factor 2 (Nrf2), a member of the cap 'n' collar basic leucine zipper transcription factor family (Moi et al., 1994). Normally, Nrf2 is kept inactive in the cytoplasm by its repressor protein kelch- like ECH-associated protein 1 (Keap1) (Itoh et al., 1999). Keap1 contains cysteine that reacts with oxidative and electrophilic radicals, leading to conformational changes and the release of Nrf2 (Kobayashi et al., 2006). Nrf2 translocates to the nucleus and binds to antioxidant response element (ARE), leading to promoter-specific transcription of a battery of defensive genes, including glutathione transferases (GSTs), catalase, heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase-1 (NQO1) (Itoh et al., 1997; Rushmore et al., 1991). The Nrf2-mediated adaptive response protects cells against acute and chronic damage caused by environmental stress, such as cigarette smoke (Iizuka et al., 2005; Rangasamy et al., 2004), solar radiation (Hirota et al., 2005), diesel exhaust (Aoki et al., 2001; Li et al., 2013) and benzo[a]pyrene (Ramos-Gomez et al., 2001).

Several phytochemicals, including sulforaphane, are known to induce Nrf2 (Li et al., 2012; Zhang et al., 1992), thereby increasing the resistance to radicals and other reactive chemicals. Sulforaphane is an isothiocyanate present in cruciferous vegetables like broccoli and has a potential role in modulating phase II enzymes. Sulforaphane induces modifications in Keap1 cysteine residues allowing activation of the Nrf2 - ARE pathway and subsequent up regulation of cytoprotective enzymes (Dinkova-Kostova et al., 2002; Hu et al., 2011).

Interestingly, this sulforaphane-induced protection is lost in Nrf2-deficient mice (Xu et al., 2006), underscoring the critical importance of the Nrf2 system in this process. In addition to its chemopreventive properties (Fahey and Talalay, 1999), sulforaphane is also an inducer of apoptosis in tumour cells, thereby enhancing tumour radiosensitivity (Kotowski et al., 2011; Sawai et al., 2013; Yu et al., 2009). Activation of Nrf2 could therefore be a way to modulate the effect of radiotherapy.

Abbreviations: AP, apurinic/apyramidinic; ARE, antioxidant response element; CM-H_2DCFDA, 5-(and-6) chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate; DSBs, double-strand breaks; EdU, 5-ethynyl-2′-deoxyuridine; GST, glutathione transferase; HO1, heme oxygenase1; Keap1, kelch-like ECH-associated protein 1; MEF, mouse embryonic fibroblasts; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor-erythroid-2-related factor 2; ROS, reactive oxygen species; SSBs, single-strand breaks; tBHQ, tert-butylhydroquinone.

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Ionizing radiation (IR) represents a potent carcinogen as well as an important tool in radiation therapy due to its ability to induce DNA damage. IR triggers DNA damage, for the most part through the generation of free radicals (Quintiliani, 1986; Riley, 1994) that cause a variety of DNA lesions, including oxidized bases, apurinic/apyramidinic (AP) sites, single-strand breaks (SSBs), DNA double-strand breaks (DSBs), DNA- DNA and DNA- protein cross links (von Sonntag, 1991; Ward, 1981). Several lines of evidence indicate that the cytotoxic effect of IR is mainly mediated by the DSBs that are produced (Olive, 1998; Ward, 1990).

Since IR induces DNA damage and cytotoxicity by the production of radicals it is possible that Nrf2 activity will influence radiosensitivity. Previous studies have produced conflicting results in this regard and indicate that single stimulations with the relatively weak Nrf2 activator sulforaphane failed to protect from IR-induced toxicity (McDonald et al., 2010), whereas the strong Nrf2 activator bardoxolone methyl was protective (Kim et al., 2012). Therefore, the extent of Nrf2 activation could influence its effect on IR-induced cytotoxicity.

We have shown previously that repeated sulforaphane stimulation resulted in a stronger Nrf2 response, compared with single stimulation (Bergstrom et al., 2011). For this reason, we tested the hypothesis that repeated daily treatments with sulforaphane protect human cells from the toxic effects of ionizing radiation.

Materials and methods

Cell culture. All cell cultures were kept in a humidified atmosphere at 5 % CO_2, 37 °C. Primary human skin fibroblasts (CRL-2091, ATCC) were cultured in Minimum Essential Medium (MEM) Eagle's medium, supplemented with 10 % foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (all from Sigma-Aldrich), 1 mM sodium pyruvate and 0.1 mM MEM non-essential amino acids (Life technologies). Nrf2 knockout (Nrf2−/−) and Nrf2 wild type (Nrf2 +/+) mouse embryonic fibroblasts (MEFs) (Higgins et al., 2009) were kindly provided by Dr. John D. Hayes at the University of Dundee, UK. Cells were cultured in 0.1% (w/v) gelatin-coated plates in Iscove's Modified Dulbecco's Medium (IMDM, Life technologies), containing 10 % FBS, 10 ng/ml human recombinant epidermal growth factor (Life technologies), 1 x insulin/transferrin/selenium (Life technologies) and 100 U/ml penicillin–100 μg/ml streptomycin.

Pretreatment with sulforaphane. L-sulforaphane (Enzo Life Sciences) was dissolved in dimethyl sulphoxide (DMSO) to stock solutions of 100 mM and stored at −70 °C. Working concentrations were freshly prepared in culture medium and added to confluent monolayers. The final DMSO concentration was kept at 0.1 % for all samples, including controls. After four hours of incubation, the sulforaphane-containing medium was removed and replaced by fresh growth medium. For repeated stimulation, the treatment procedure was repeated as four hours per day for three consecutive days. The treatment procedure is briefly illustrated in Fig. 1.

Stock solutions of curcumin and tert-butylhydroquinone (tBHQ) (Sigma Aldrich) were prepared in DMSO. Working concentrations were prepared and the treatment conditions were the same as described above.

Irradiation. Cells were exposed to ionizing radiation using Gammacell®3000Elan (Best Theratronics) at a dose rate of 128 mGy/s.

Cell survival. Cell survival was measured using the flow cytometry-based 5-ethynyl-2′-deoxyuridine (EdU) assay (Buck et al., 2008). In short, three hours after irradiation, the cells were re-seeded into appropriate plates to provide enough surface area for cell division. After 48 hours of incubation, 10 μM EdU was added to the medium and the cells were incubated for another 16 hours to allow EdU incorporation into newly synthesized DNA. The cells were then harvested by trypsinization and washed with PBS wash buffer with 1 % bovine serum albumin (BSA, Sigma Aldrich), followed by fixation (4 % paraformaldehyde for 15 min, RT in the dark), permeabilization (0.1 % saponin–PBS wash buffer for 30 min, RT in the dark), and staining using Click-IT reaction mix (1 x PBS containing 2 mM $CuSO_4$, 2 μM Alexa Fluor® 647 azide and 10 mM sodium ascorbate for 30 min in the dark). EdU-labeled cells were detected using a BD Accuri C6 cytometer (BD Biosciences) and the cell survival results were calculated as the relative number of EdU-positive cells in treated samples normalized to the DMSO vehicle control.

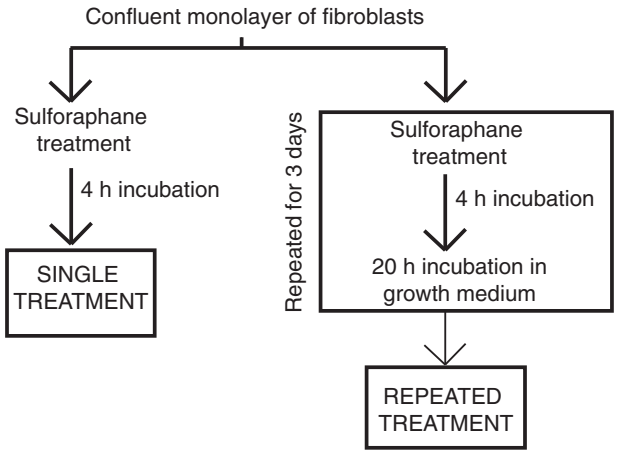

Fig. 1. Illustrative description of sulforaphane treatment in cells. Fibroblasts were treated with sulforaphane for single 4 h or 4 h each for three consecutive days represented as single treatment or repeated treatment respectively.

mRNA extraction & cDNA synthesis. mRNA was extracted and purified with oligo (d)T-covered magnetic beads using a MagAttract Direct mRNA M48 Kit (Qiagen). The cells were lysed directly in the well by addition of 360 μl Buffer MRL. mRNA was then extracted from the lysate on a GenoM-48 Robotic Workstation (Qiagen/Genovision), according to the manufacturer's instructions. Standard settings for mRNA extraction were used. cDNA was synthesized from 10 μl of purified mRNA in a buffer consisting of First Strand Buffer x 1, 10 mM dithiothretiol (DTT), 5 U/μl SuperScript™ II Reverse Transcriptase (all from Invitrogen), 1 U/μl Protector RNase Inhibitor, 20 pmole/μl Hexanucleotide Mix and 1 mM of each dNTP; Li-salt (Roche Diagnostics). The reaction was performed on a PTC-200 Peltier Thermal Cycler (MJ Research) (22 ºC for 10 min, 42 ºC for 45 min and 99 ºC for 3 min), in a total reaction volume of 20 μl.

Quantitative PCR. Quantitative PCR was performed using inventoried TaqMan® Gene Expression Assays with FAM reporter dye in TaqMan® Fast Universal PCR Master Mix according to protocol, but in a total reaction volume of 25 μl. QPCR reactions were carried out on Micro-Amp ™ 96-well optical microtitre plates on a 7900HT Fast QPCR System (Applied Biosystems), using standard settings for Standard Curve qPCR. TaqMan® Gene Expression Assays for the following genes were used: Heme oxygenase (decycling) 1 (human HMOX1; Hs00157965_m1 and mouse Hmox1; Mm00516005_m1), NAD(P)H dehydrogenase, quinone 1 (human NQO1; Hs00168547_m1 and mouse Nqo1; Mm01253561_m1) and polymerase (RNA) II (DNA directed) polypeptide A (human POLR2A; Hs00172187_m1 and mouse Polr2a; Mm00839493_m1). All primer pairs are designed to span exon-exon junctions to avoid amplification of genomic DNA. To exclude DNA contamination, PCR was also run on RNA prior to cDNA synthesis. cDNA was diluted 10 x prior to PCR and all samples were run in duplicates. PCR results were analysed with the SDS 2.3 software (Applied Biosystems) and the relative quantity was determined using the $\Delta\Delta C_T$