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Research Paper

Nuclear trapping of inactive FOXO1 by the Nrf2 activator diethyl maleate

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ABSTRACT

Diethyl maleate (DEM), a thiol-reactive α,β-unsaturated carbonyl compound, depletes glutathione (GSH) in exposed cells and was previously shown by us to elicit a stress response in *Caenorhabditis elegans* that, at lower concentrations, results in enhanced stress resistance and longer lifespan. This hormetic response was mediated through both the Nrf2 ortholog, SKN-1, and the forkhead box O (FOXO) family transcription factor DAF-16. As FOXO signaling is evolutionarily conserved, we analyzed here the effects of DEM exposure on FOXO in cultured human cells (HepG2, HEK293). DEM elicited nuclear accumulation of GFP-coupled wild-type human FOXO1, as well as of a cysteine-deficient FOXO1 mutant. Despite the nuclear accumulation of FOXO1, neither FOXO1 DNA binding nor FOXO target gene expression were stimulated, suggesting that DEM causes nuclear accumulation but not activation of FOXO1. FOXO1 nuclear exclusion elicited by insulin or xenobiotics such as arsenite or copper ions was attenuated by DEM, suggesting that DEM interfered with nuclear export. In addition, insulin-induced FOXO1 phosphorylation at Thr-24, which is associated with FOXO1 nuclear exclusion, was attenuated upon exposure to DEM. Different from FOXO-dependent expression of genes, Nrf2 target gene mRNAs were elevated upon exposure to DEM. These data suggest that, different from *C. elegans*, DEM elicits opposing effects on the two stress-responsive transcription factors, Nrf2 and FOXO1, in cultured human cells.

1. Introduction

The intracellular tripeptide thiol, glutathione (γGlu-Cys-Gly; GSH), is generally known as a crucial contributor to cellular antioxidant defense systems as well as to xenobiotic metabolism [1]. It was therefore previously tested for its role in the regulation of stress resistance and life span in the model organism, *Caenorhabditis elegans*. In line with its role in antioxidant defense, a decreased stress resistance of *C. elegans* was originally expected to result from GSH depletion. Unexpectedly, however, a non-linear relationship between GSH levels and stress resistance was observed, indicating that a moderate decrease of GSH in *C. elegans* may in fact enhance, rather than diminish, resistance against oxidative stress [2].

Diethyl maleate (DEM), an α,β-unsaturated carbonyl compound that is frequently used for the depletion of cellular GSH, and a known stimulator of Nrf2 signaling in mammalian cells [3–5], also elicited an increase in stress resistance in *C. elegans* [2]. Not only SKN-1, the ortholog of Nrf2, but also DAF-16, the *C. elegans* ortholog of mammalian FOXO transcription factors, was demonstrated to contribute to this effect [2].

FOXO transcription factors are evolutionarily conserved major regulators of cellular metabolic processes, including fuel metabolism, antioxidant defense and cell death [6,7]. Insulin, through phosphoinositide 3′-kinase (PI3K)-dependent stimulation of FOXO phosphorylation by the Ser/Thr-kinase Akt, causes inactivation and nuclear exclusion of three of the four mammalian FOXO isoforms (FOXOs 1, 3 and 4) [8]. As FOXOs were previously demonstrated in mammalian cells to be regulated not only by insulin, but also by stressful stimuli, such as by hydrogen peroxide [9] or the thiophilic agents Cu^{2+} and arsenite [10,11], we asked whether exposure to the thiol depleting agent DEM would result in a similar modulation of FOXO signaling in mammalian cells, and whether this occurs with consequences for FOXO target gene expression that are similar to the observations in *C. elegans*.

We here demonstrate that indeed DEM affects FOXO1 subcellular localization in cultured human cells, but that the consequences of DEM exposure differ from those observed in *C. elegans* as FOXO-dependent

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gene expression was not elicited by a mere depletion of GSH. Rather, we propose that DEM interferes with nucleocytoplasmic shuttling of FOXOs.

2. Materials and methods

2.1. Materials

SP600125 was purchased from Selleck Chemicals (Houston, TX, USA); all other chemicals were purchased from Sigma-Aldrich (Munich, Germany) and Carl Roth (Karlsruhe, Germany) unless stated otherwise. Primers were obtained from Life Technologies (Darmstadt, Germany).

2.2. Cell culture, transfection, plasmids

HepG2 human hepatoma cells and HEK293 human embryonic kidney cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). HEK293 cells were held in Dulbecco's modified Eagle's medium (DMEM, low glucose; Sigma, Munich, Germany, Cat# D6046), supplemented with 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma); HepG2 cells were held likewise, with non-essential amino acids (in MEM; Sigma, Cat# M7145) added to the medium. Cells were maintained at 37 °C in a humidified atmosphere with 5% (v/v) CO_2. For DNA binding assays (Fig. 3B) DMEM with 4500 mg/l glucose was used (Sigma, Cat# D6429), and for experiments in Figs. 4B-D, 5A-B and 6A, HepG2 cells were held in DMEM with 4500 mg/l glucose and 2 mM glutamine (Sigma-Aldrich) supplemented with 10% (v/v) FCS (PAA, Etobicoke, ON, Canada), penicillin/streptomycin and non-essential amino acids.

Transfection of HepG2 cells was performed using GenJet (SignaGen Laboratories, Rockville, MD, USA) or nanofectin (PAA, Etobicoke, ON, Canada) according to the manufacturers' instructions. For transfection of HEK293 cells TurboFect reagent (Thermo Scientific, Waltham, MA, USA) was used according to the manufacturer's recommendations.

Plasmids containing cDNA encoding human wildtype FOXO1 [FOXO1(WT)] and human FOXO1 with all seven cysteines mutated into serines [FOXO1(C#1-7S)] coupled to GFP were generated as described previously [12]. Briefly, wildtype and mutated FOXO1 sequences were cloned into two different GFP vectors, pcDNA-DEST53 (Invitrogen) or pEGFP-C1 (Clontech), yielding pcDNA-GFP-hFOXO1(WT) and pEGFPC1-hFOXO1(WT), respectively, as well as the mutant versions, pcDNA-GFP-hFOXO1(C#1-7S) and pEGFPC1-hFOXO1(C#1-7S).

2.3. Analysis of subcellular FOXO1 localization

HepG2 or HEK293 cells transfected with plasmids coding for human FOXO1 variants N-terminally coupled to GFP were analyzed by fluorescence microscopy following transfection (usually approx. 24 h post-transfection). Visibly GFP-positive cells were grouped into three categories with respect to the predominant subcellular localization of the GFP signal ("cytoplasmic", "cytoplasmic/nuclear" or "nuclear"). Time-course analyses of subcellular relocalization (Fig. 5C) were performed as follows: HepG2 cells were grown on 6-channel-microscopy slides with a coverslip bottom ("µ-Slides VI 0.4"; ibidi, Martinsried, Germany, Cat# 80606) covered with collagen. 16–22 h following transfection of cells with GFP plasmids (GenJet reagent, added already to the cell suspension prior to their application to slides), cells were exposed to insulin and/or DEM (or the respective solvent controls). The microscopy slides were placed in an incubator box (okolab, Ottaviano, Italy) and held at 37 °C/5% CO_2 while fluorescence microscopic images were taken at multiple time points (Nikon Eclipse Ti fluorescence microscope).

2.4. C. elegans maintenance and treatment

The TJ356 zIs356 [daf-16p::daf-16a/b::GFP + rol-6] *C. elegans* strain was provided by the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, USA), which is supported by the National Institutes of Health-Office of Research Infrastructure Programs. *E. coli* strain OP50 was also received from CGC. Nematodes were grown, maintained and treated at 20 °C on nematode growth medium (NGM) agar plates spotted with *E. coli* OP50 as food source, as previously described [2]. Stock solutions of diethyl maleate (DEM) were prepared in DMSO. DEM or the solvent control (0.1% DMSO) were added directly to the agar during preparation of plates. 24 h after synchronization, nematodes of the transgenic strain TJ356 stably expressing a DAF-16::GFP fusion protein were transferred to NGM agar plates containing the respective compound or solvent control for an additional 24 h. Subsequently, around 40 L3 larvae of each group were placed on microscope slides coated with 3% agarose, anaesthetized with 10 mM sodium azide, and covered with coverslips. Cellular localization of DAF-16 was analyzed by fluorescence microscopy on an Axio Observer D1 fluorescence microscope (Zeiss, Göttingen, Germany) using appropriate filters (ex. 472 ± 30 nm, em. 520 ± 35 nm).

2.5. Western blotting

Western blotting was performed according to standard procedures [11], using the following primary antibodies: phospho-Jun (Ser63), phospho-SAPK/JNK (Thr183/Tyr185) Rabbit mAb (98F2), phospho-FoxO1 (Thr24)/FoxO3a (Thr32), FoxO1 (C29H4) Rabbit mAb (all from Cell Signaling Technology, Danvers, MA, USA), GAPDH (Millipore, Billerica, MA, USA). Incubation with secondary antibody [horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or HRP-coupled anti-mouse IgG (GE-Healthcare, Piscataway, USA)] was followed by detection using chemiluminescent HRP substrate. Images were acquired using an ImageQuant LAS 4000 mini system (GE Healthcare Bio-Sciences).

2.6. Determination of glutathione levels

GSH was determined by HPLC (Jasco, Gross-Umstadt, Germany) after derivatization of thiols with orthophtaldialdehyde (OPA) and fluorometric detection as described [2]. Cells grown to approx. 75% confluence in cell culture dishes (growth area: 58 cm^2) were scraped off the dishes in 1 mL of ice-cold 0.01 N HCl. Suspensions were frozen at − 80 °C for approx. 20 h, thawed, resuspended 10 times, then centrifuged to separate debris (4 °C, 18,000 × *g*). After centrifugation, aliquots of 50 µl were prepared from the supernatant and stored at − 80 °C until further use. Four independent experiments were performed, and two different aliquots of the same supernatant were determined in each of the experiments for each data point. Protein content was determined from another of the aliquots in a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Proteins were precipitated by addition of 25 µl of cold 2 N perchloric acid to 50 µl of the supernatant, followed by incubation on ice for 1 min. This mixture was neutralized by addition of 200 µl of 0.5 M sodium phosphate buffer (pH 7.0), followed by centrifugation for 10 min at 4 °C. 50 µl of the neutralized supernatant was used for derivatization with 50 µl of OPA [0.15 M in 0.1 M sodium borate, pH 9]. Separation was performed by gradient elution on a ZORBAX Bonus RP column (4.6 × 250 mm; Agilent) at a flow rate of 1 mL/min. Eluents were (A) 98% of 50 mM sodium acetate (pH 7)/2% acetonitrile (VWR, Fontenay-sous-Bois, France) and (B) 80% acetonitrile/20% 50 mM sodium acetate (pH 7.0). Peaks were detected at 420 nm after excitation at 340 nm. GSH was normalized to protein content of the respective sample. Glutathione disulfide levels were measured analogously, after chemical masking of GSH using N-ethyl maleimide (NEM), followed by blocking residual NEM and reduction of GSSG to GSH, which was then detected as above.