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Constitutive MAP-kinase activation suppresses germline apoptosis in NTH-1 DNA glycosylase deficient *C. elegans*

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ABSTRACT

Oxidation of DNA bases, an inevitable consequence of oxidative stress, requires the base excision repair (BER) pathway for repair. *Caenorhabditis elegans* is a well-established model to study phenotypic consequences and cellular responses to oxidative stress. To better understand how BER affects phenotypes associated with oxidative stress, we characterised the *C. elegans nth-1* mutant, which lack the only DNA glycosylase dedicated to repair of oxidative DNA base damage, the NTH-1 DNA glycosylase.

We show that *nth-1* mutants have mitochondrial dysfunction characterised by lower mitochondrial DNA copy number, reduced mitochondrial membrane potential, and increased steady-state levels of reactive oxygen species. Consistently, *nth-1* mutants express markers of chronic oxidative stress with high basal phosphorylation of MAP-kinases (MAPK) but further activation of MAPK in response to the superoxide generator paraquat is attenuated. Surprisingly, *nth-1* mutants also failed to induce apoptosis in response to paraquat. The ability to induce apoptosis in response to paraquat was regained when basal MAPK activation was restored to wild type levels. In conclusion, the failure of *nth-1* mutants to induce apoptosis in response to paraquat is not a direct effect of the DNA repair deficiency but an indirect consequence of the compensatory cellular stress response that includes MAPK activation.

1. Introduction

Base Excision Repair (BER) is an evolutionarily conserved pathway that removes a wide range of modified DNA bases. BER is the most important pathway for repair of oxidised DNA bases, an inevitable consequence of oxidative stress. As oxidative stress is known to accompany aging and a wide range of human age-related diseases, it can be expected that BER would be important for maintaining health during aging. This, however, has been difficult to firmly demonstrate. Lesion specificity in BER is provided by the initial step wherein modified bases are detected and excised by DNA glycosylases [1]. In mammalian cells, there are several DNA glycosylases that recognise and remove oxidised DNA bases. DNA glycosylases have overlapping substrate specificities, and the lack of adverse phenotypes of DNA-glycosylase deficient mouse models has been explained by extensive redundancy [2]. Thus, to further understand the role of BER in general, and DNA glycosylases in particular, in counteracting deleterious effects of oxidative stress, there is a need to study the consequence of BER defects in other animal models.

The nematode *Caenorhabditis elegans* is an established animal model for studies of oxidative stress and DNA damage responses [3]. Work in *C. elegans* also has the potential to uncover phenotypes associated with BER deficiency that may be masked in mammals due to extensive redundancy because the *C. elegans* genome encodes only two DNA glycosylases, UNG-1 [4–6] and NTH-1 [7]. Other *C. elegans* BER enzymes include two AP-endonucleases, EXO-3 [8] and APN-1 [9], of which

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EXO-3 is homologous to the mammalian APE1 enzyme [10]. There is no ortholog of DNA polymerase β but BER has been demonstrated in *C. elegans* [11] and likely utilizes DNA polymerase θ or a replicative DNA polymerase [12].

The endonuclease III homolog NTH-1 is the only representative of DNA glycosylases known to repair oxidative DNA base damage in *C. elegans*. Like its mammalian counterpart, NTH-1 has activity on a range of oxidised pyrimidines, like thymine glycol, 5-hydroxymethyluracil (5-hmU), and 5-formyluracil (5-foU), but also on 8-oxoG when base paired with G [7]. A null mutant, *nth-1(ok724)* which carries a deletion spanning exons 2 through 4 of the *nth-1* gene, showed normal mean and maximum lifespan [7,13], no hypersensitivity to the oxidizing agents H_2O_2 or paraquat but a mild sensitivity to juglone [13]. Previously, Hunter and co-workers [14] failed to observe any difference in *in vivo* repair of nuclear or mitochondrial DNA in *nth-1* mutants following treatment with H_2O_2. Loss of NTH-1 did, however, suppress the reproductive deficit of *exo-3* mutants [10], supporting that NTH-1 functions as a DNA glycosylase *in vivo* and generates AP-sites, which are substrates for EXO-3 in the germline.

Here, we show that *nth-1* mutants exhibit chronic oxidative stress and mitochondrial dysfunction and fail to mount an apoptotic response to the superoxide generator paraquat. The inability to induce apoptosis was not a direct consequence of the DNA repair defect. Instead, failure to induce germline apoptosis was a consequence of compensatory cellular stress response that includes elevated basal phosphorylation of the mitogen-associated stress activated kinase (MAPK) PMK-1 and JNK-1. These results show that phenotypes in DNA-repair mutants that could be interpreted as direct consequences of the repair defect may instead be indirect consequence of cytoprotective stress responses that compensate for DNA repair deficiency.

2. Materials and methods

2.1. Strains and culture conditions

All strains were maintained at 20 °C as described [15] unless otherwise stated. The reference strain Bristol N2 and RB877 *nth-1*(*ok724)III*, unc-58(e665)X, MD701 *($P_{lim\text{-}7}$ced-1:gfp),* and CL2166 [*dvIs19[pAF15(gst-4:gfp:NLS)]* III] [16] strains were kindly provided by the *Caenorhabditis* Genetic Centre (University of Minnesota, St Paul, MN, USA). All strains were backcrossed at least 6 times into the N2 strain. The RB877 strain is referred to as *nth*-1 in subsequent sections. The transgenic strains *nth-1*;*($P_{lim\text{-}7}$ced-1:gfp)* and *nth-1*;*unc-58* were generated for this work. *Escherichia coli* strains OP50 and HT115(DE3) for RNAi studies, and BK2118 (*tag alkA)* were used as food sources. For RNAi experiments, worms were maintained for one generation on Nematode Growth Medium (NGM) plates containing 2 mM IPTG (isopropyl b-D-1-thiogalactopyranoside) seeded with *Escherichia coli* HT115(DE3). All experiments were performed at 20 °C unless otherwise stated.

2.2. Chemicals and antibodies

Paraquat (PQ), methyl viologen dichloride hydrate, was purchased from Sigma. The following commercially available antibodies were used for immunofluorescence and western blotting: phosphorylated PMK-1 (T180/Y182) (Cell signalling D3F9), phosphorylated JNK-1(T183/Y185) (NOVUS NBP1-72242), phosphorylated H3 (Ser 10) (Santa Cruz Biotechnology Inc,) and actin (Abcam ab1801). The specificity of the antibodies detecting phosphorylated PMK-1 and JNK-1 were validated by immunostaining after RNAi mediated depletion of PMK-1 and JNK-1 (Supplementary Fig. S3). Secondary antibodies were Alexa Fluor® 488 conjugate anti-rabbit (Invitrogen), enhanced chemiluminescence (ECL) anti-rabbit IgG horseradish peroxidase (HRP)-linked whole antibody (Thermo Scientific), and anti-rabbit IgG HRP-linked (Santa Cruz).

2.3. DNA damage induced apoptosis

Synchronized [$P_{lim\text{-}7}$*ced-1:gfp*] and *nth-1*(*ok724*);[$P_{lim\text{-}7}$*ced-1:gfp*] L4 hermaphrodites were exposed to 400 μM paraquat on NGM plates, or 5 mM *N*-ethyl-*N*-nitrosourea (ENU) as a positive control. Germ-cell corpses were identified by the presence of a GFP outline around the cells undergoing apoptosis in the pachytene region 24 h post-treatment. The number of germ-cell corpses per gonad arm was scored under a Zeiss Axioplan microscope equipped with a 63xPlan-Apochromate 1.4 NA objective and standard *epi*-fluorescence filters. Data are given as averages of at least 15 animals per genotype from three independent experiments.

2.4. Gene expression analyses

Transcriptional activation of *C. elegans* ced-13 and *egl-1* was measured in synchronized N2 and nth-1 L4 hermaphrodites after treatment with paraquat, as described above. For total RNA extraction, worms were disrupted in TRIZOL with 0.7 mm zirconia/silica beads (Biospec Products) using a Mini-Beadbeater 8 (Biospec Products) at maximum speed for 30 s. cDNA synthesis was performed using iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. Quantitative reverse transcriptase PCR (qRT-PCR) was performed with SYBR Green supermix (Bio-Rad). *ced-13* and *egl-1* transcript levels were normalized to an internal tubulin (*tbg-1*) control. *sod-3* transcript levels were normalized to Y45F10D.4 control. Primers (Eurogentech) with the following sequences were used: for *egl-1* (5-CCTCAACCTCTTCGGATCTT-3) and (5-TGCTGATCTCAGAGTCATCAA-3); for *ced-13* (5-GCTCCCTGTTTATCACTTCTC-3) and (5-CTGGCATACGTCTTGAATCC-3); for *tbg-1* (5-AAGATCTATTGTTCTACCAGGC-3) and (5-CTTGAACTTCTTGTCCTTGAC-3); for *sod-3* (5-gtcgcttcaaatcagttcagc-3) and (5-gttcttgtcaagtgatccgaca-3); for Y45F10D.4 (5- CTAAGGATGGTGGAGAACCTTCA-3) and (5-CGCGCTTAATAGTGTCCATCAG-3).

As a control of RNAi efficiency, mRNA expression levels were measured after three generations of feeding N2 animals on *E. coli* expressing RNAi, targeting the indicated genes. The primers were as follows (sense strand): *xpa-1* forward: (5-CTTGGTGGCGGATTCTGTGA-3), reverse: (5- TCCCAAAGCCAACTGTCCAT-3); *xpc-1* forward: (5-TTTCCCCATCCAAACGTGCT-3); reverse: (5- AGGGCTTGATATTGGTCGTCG-3); *csb-1* forward: (5-GATGCCAAGAGAGCCAGGAA −3); reverse: (5-GAATAATGGGAGCAAATGCGGT-3).

2.5. Immunohistochemistry

Animals were collected 24 h after treating synchronized L4 hermaphrodites with 400 μM paraquat. Germlines were dissected on poly-L-lysine-coated slides in egg buffer (25 mM HEPES, pH 7.4, 118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) supplemented with 0.1% Tween-20 and 0.2 mM levamisol. Germlines were fixed in 4% formaldehyde for 5 min at room temperature and freeze-cracked in liquid nitrogen. Next, the germlines were fixed in 1:1 acetone:methanol for 10 min at −20 °C, washed in PBS-T (1 x PBS, 0.1% Tween-20) for 5 min, followed by 30 min incubation with image-IT FX signal enhancer (Invitrogen) and 30 min blocking in PBS-TB (1 x PBS, 0.1% Tween-20, 0.5% BSA). The slides were incubated with primary antibody overnight at 4 °C, washed three times for 10 min in PBS-T, followed by incubation with the secondary antibody at room temperature for 2 h. Finally, the germlines were washed three times for 10 min in PBS-T and mounted with 7 μl mounting solution containing ProLong Gold (Thermo Fisher) and 0.5 μg/ml DAPI (Sigma). Primary antibodies were used at the following dilutions: pPMK-1 (1:200), pJNK-1 (1:200), pH3 (1:400), and RAD-51 (1:20 000) (a gift from Sarit Smolikove). The secondary antibody used to detect was Alexa 488- conjugated anti-rabbit at 1:1500 dilution and cy3 at 1:1000 dilution.