



Original Contribution

Mechanisms of stress resistance in Snell dwarf mouse fibroblasts: Enhanced antioxidant and DNA base excision repair capacity, but no differences in mitochondrial metabolism

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ABSTRACT

Dermal fibroblasts from long-lived Snell dwarf mice can withstand a variety of oxidative and non-oxidative stressors compared to normal littermate controls. Here, we report differences in the levels and activities of intracellular antioxidant and DNA repair enzymes between normal and Snell dwarf mice fibroblasts cultured under a variety of conditions, including: 3% and 20% ambient O₂; the presence and absence of serum; and the addition of an exogenous oxidative stress. The only significant difference between normal and dwarf cells cultured in complete medium, at 20% O₂, was an approximately 40% elevation of glutathione peroxidase (GPx) activity in the mutant cells. Serum deprivation elicited increases in GPx in both genotypes, but these activities remained higher in dwarf mouse cells. Dwarf mouse cells deprived of serum and challenged with exposure to paraquat or hydrogen peroxide showed a generally greater upregulation of catalase and DNA base excision repair enzymes. As these toxins can interact with mitochondria to increase mitochondrial ROS production, we explored whether there were differences in mitochondrial metabolism between normal and dwarf mouse cells. However, neither mitochondrial content nor the apparent mitochondrial membrane potential differed between genotypes. Overall, the results suggest that superior hydrogen peroxide metabolism and a marginally greater DNA base excision repair capacity contribute to the stress resistance phenotype of Snell dwarf mouse fibroblasts.

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Introduction

Cellular resistance to physiological stress is correlated with animal lifespan within species [1–3] and between species [4,5]. Stress resistance is also greater in some states of metabolic quiescence that are associated with extended lifespan [6]. Both stress resistance and lifespan can be extended via genetic mutations [1,3,7–9], demonstrating the plasticity of the phenotype and allowing the identification of specific genes involved. In *C. elegans*, a series of insulin-like growth factor–1 (IGF-1) signaling pathway (*daf*) mutants have been studied to uncover the regulation of lifespan by this hormone. The basic architecture and function of this pathway appears to be conserved in mammals, as mice deficient in the IGF-1 receptor

(IGF-1R^{+/-}) are also stress resistant and long-lived [10], under some housing regimens. Several other strains of mice affected by mutations that occur upstream of this pathway are also long-lived. In Snell dwarf mice, a mutation within the *Pit-1* gene, which encodes a transcription factor required for the normal development of the anterior pituitary, results in deficient levels of growth hormone (GH), thyroid stimulating hormone (TSH) and prolactin (PRL) [11,12]. One of the most notable secondary effects of the *Pit-1* mutation is the reduction of basal levels of IGF-1 [13]. As a result, these mice have provided useful models for the study of mechanisms of stress resistance that confer longevity.

Dermal fibroblasts isolated from Snell dwarf mice can withstand levels of a variety of oxidative and non-oxidative stressors that are lethal to cells derived from their normal-sized littermates [2,9]. In addition, the Snell dwarf mouse fibroblasts are relatively resistant to the growth crisis and cell death induced by passage in standard culture conditions with atmospheric (20%) rather than physiological (3%) levels of O₂ [14]. Thus, dermal fibroblasts derived from the Snell dwarf mice are resistant to the oxidative stress that appears to drive growth crisis and transformation in mouse fibroblasts [15].

Abbreviations: ROS, Reactive oxygen species; MnSOD, manganese superoxide dismutase; CuZnSOD, copper-zinc superoxide dismutase; CS, citrate synthase; GPx, glutathione peroxidase; CAT, catalase; LDH, lactate dehydrogenase; AP, apurinic/apyrimidinic endonuclease; SD, serum deprivation; PQ, paraquat; H₂O₂, hydrogen peroxide; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.

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Less is known about the specific mechanisms through which cellular stress resistance is enhanced than the upstream signaling pathways that regulate the phenotype, and direct measurements of the levels and activities of potential proteins involved in the stress resistance mechanism have rarely been made.

Cellular oxidative stress resistance can be conferred by the antioxidant enzymes that detoxify reactive oxygen species (ROS) originating endogenously (via mitochondrial respiration) or exogenously. Cytosolic (CuZnSOD) and mitochondrial (MnSOD) superoxide dismutase isoforms convert superoxide radicals into hydrogen peroxide, which can subsequently be detoxified by glutathione peroxidase (GPx; found within various cellular compartments including mitochondria) and catalase (CAT; localized to peroxisomes).

In the event that ROS escape detoxification, however, DNA repair enzymes may play a critical role in preserving genomic integrity and thus conferring stress resistance. Base excision repair (BER) is the main pathway for repair of oxidative damage; it is also involved in repairing alkylative (caused by, for example, methyl methanesulfonate) and other forms of DNA damage. Though a number of subpathways are recognized to exist [16,17], two enzymes are thought to play central roles in BER. The apurinic/aprimidinic (AP) endonuclease processes abasic sites generated either by removal of damaged bases (via DNA glycosylase activity) or spontaneous base loss. AP endonuclease has been found to also possess the rate limiting activity of a 3'-phosphoesterase specifically following oxidative-induced DNA base loss [18]. Polymerase β is a small DNA polymerase dedicated to repair. Resynthesis of DNA at AP endonuclease-generated gaps is catalyzed by polymerase β . The activity of this enzyme is also particularly informative regarding overall BER activity as it possesses the rate limiting activity of a deoxyribosephosphatase (dRP) [17].

Here we have used Snell dwarf mouse fibroblasts to investigate the molecular mechanisms that allow these cells to resist oxygen toxicity. We explore two hypotheses: (1) that antioxidant enzyme activities are upregulated, and (2) that DNA BER enzyme activities are upregulated in the dwarf mouse fibroblasts. We addressed these questions via measurements of protein levels and activities of the key antioxidant and BER enzymes in Snell normal and dwarf fibroblasts cultured at 3% or 20% oxygen, in the presence and absence of serum, and with or without exposure to the pro-oxidants, paraquat (PQ) and hydrogen peroxide (H_2O_2). Possible differences between normal and dwarf mouse cells in mitochondrial content and activity were also accounted for.

Experimental procedures

Materials

Chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), Fisher Scientific (Mississauga, Ontario, Canada) and Bioshop (Burlington, Ontario, Canada). BioRad protein dye was purchased from BioRad laboratories (Hercules, California, USA). Prestained broad range protein marker was purchased from BioLabs (New England, Massachusetts, USA). MemCode reversible protein stain kit was purchased from Pierce Biotechnology (Rockland, Illinois, USA). MnSOD and CuZnSOD human antibodies were purchased from Stressgen (Victoria, British Columbia, Canada). Infrared dye-conjugated secondary antibodies to rabbit were purchased from Rockland Immunochemicals (Gilbertsville, Pennsylvania, USA). Oligonucleotides were purchased from Midland Certified Reagent Co. (Midland, Texas, USA). Purified human AP endonuclease and human polymerase β were purchased from Trevigen (Gaithersburg, Maryland, USA). MitoTrackerTM Green and MitoTrackerTM Red were purchased from Molecular Probes (now Invitrogen, Carlsbad, CA, USA).

Preparation of Snell Mice Dermal Fibroblasts

Tail skin biopsies from 8 Snell normal control mice (+/+ or dw/+ genotype) and 8 Snell dwarf mice (dw/dw homozygotes) were collected, diced and split into 2 samples; both were digested overnight in collagenase, one in an atmospheric O_2 incubator (20%), and the other in a 3% O_2 incubator as in [14]. Cultures were passaged on the same days, ensuring that all treatments were initiated at the same time for both growth conditions. For 3% and 20% O_2 , each cell line was split into four 100 mm tissue culture dishes ($\sim 6 \times 10^6$ cells/dish) and were treated as follows; A) 24 h attachment, complete media (confluent), B) treatment A plus 24 h serum deprivation, C) treatment A plus treatment B plus 2 h incubation with 15 μM hydrogen peroxide (H_2O_2), D) treatment A plus treatment B plus 24 hour incubation with 15 μM paraquat (PQ). The concentrations of H_2O_2 and PQ were chosen to provide a sublethal level of oxidative stress and therefore avoid the contribution of dead cells to the harvested sample. Following treatments, cells were rinsed with cold phosphate buffered saline (PBS); scraped, centrifuged and excess PBS was removed. Cell pellets were stored at $-80^\circ C$ for several months.

Preparation of Cell Lysates

Cell pellets were lysed by incubation in ice cold lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonylfluoride (PMSF), 40% glycerol and 0.5% NP40) for 1 h with periodic sonication (Ultrasonic Inc. Sonicator W-375; setting 3). Following incubation, cell lysates were centrifuged at 13,000 g ($4^\circ C$) for 10 min (Fisher Scientific accupspin™ MicroR). Protein concentration was determined using the Bradford technique with a BioRad protein kit. Whole cell lysates were stored at $-80^\circ C$.

Western Blots

Equal amounts of protein extract (20 μg) were separated via SDS-PAGE (5% stacking and 12% resolving gels) and electroblotted to a PVDF membrane. Equal protein transfer was verified with MemCode reversible protein stain following the manufacturer's instructions. Following blocking, membranes were incubated with MnSOD and CuZnSOD antibodies overnight in blocking buffer at $4^\circ C$. The membranes were visualized using the Odyssey infrared imaging system from LI-COR Biosciences (Lincoln, Nebraska, USA). Band signal intensities were measured for each sample and normalized to an internal standard (a single Dwarf sample cultured at 3% O_2 confluence). MnSOD and CuZnSOD protein levels were calculated from a standard curve constructed from the internal standard. Protein level quantification analysis was performed using Odyssey software version 1.0 as recommended by the manufacturer.

Antioxidant and Metabolic Enzyme Assays

All enzyme assays were performed at $30^\circ C$ using a Varian Cary 100 Bio UV-Visible Spectrophotometer. Conditions for the cytochrome c oxidase (COX) activity assay were: 25 mM potassium phosphate buffer with 0.5% Tween 20 (pH 7.2). 10 μg protein was added to obtain a background rate of absorbance change before the reaction was initiated with the addition of 50 μM fully reduced cytochrome c and the instantaneous change in absorbance was measured at 550 nm. Reduced cytochrome c was prepared by adding sodium dithionite to an aqueous solution of cytochrome c until fully reduced, then passing the solution through a Sephadex G-25 (Sigma-Aldrich) column to remove the excess dithionite. Lactate dehydrogenase (LDH) activity was measured in a solution containing 20 mM HEPES buffer (pH 7.3), 0.2 mM NADH, and 20–30 μg of protein to obtain a background absorbance. The reaction was initiated with the addition of 10 mM pyruvate after which a change in absorbance was measured at

340 nm. Glutathione peroxidase (GPx) activity was measured in a solution containing 50 mM potassium phosphate buffer (pH 7.0), 0.4 mM EDTA, 0.15 mM β -NADPH, 1 unit glutathione reductase, 1 mM glutathione and 5–10 μ g of protein to obtain a background absorbance. The reaction was initiated with the addition of 0.007% H_2O_2 and the instantaneous change in absorbance was measured at 340 nm. Catalase (CAT) activity was measured in a solution containing 25 mM potassium phosphate buffer and 33 mM H_2O_2 . The reaction was initiated with 60 μ g whole cell lysate and a change in absorbance was measured at 240 nm.

Base Excision Repair Assays

AP endonuclease activity was measured by incubating 100 ng protein with 1 pmol of [γ - ^{32}P]-tetrahydrofuran-containing 30-mer oligonucleotide (THF; Table 1). The % incision of the THF oligonucleotide was determined at 5, 10, 15 min at 37°C in a 10 μ l reaction buffer (50 mM HEPES (pH 7.5), 50 mM KCl, 100 μ g/ml BSA, 100 mM $MgCl_2$, 10% glycerol, and 0.05% Triton X-100). Reactions were terminated with the addition of 10 μ l formamide loading dye (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue) followed by incubation at 90°C for 10 min. Two controls were run in tandem with each AP endonuclease activity assay: an oligonucleotide without the abasic site analog (THF-control; Table 1); and the absence of cell lysate. A control assay with pure human AP endonuclease (0.05U) was also done under the same conditions (data not shown). Reaction products were resolved by electrophoresis at 15 W for 1 h 30 min in 20% polyacrylamide gels containing 7M urea. Gels were visualized with PhosphorImager (Fujifilm FLA-3000) and analyzed with ImageGuage™ software. AP endonuclease activity was quantified as the intensity of the product bands relative to the combined intensity of the product and substrate bands.

Polymerase β gap filling activity was measured by incubating 0.5 μ g protein with 1 pmol of an oligonucleotide with a single nucleotide gap (GAP; Table 1) for 10, 20, 30 min at 37°C, in a total volume of 10 μ l reaction buffer (40 mM HEPES, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.2 mg/ml BSA, 50 mM KCl, 1 mM DTT, 40 mM phosphocreatine, 100 μ g/ml phosphocreatine kinase, 3% glycerol, 2 mM ATP, 40 μ M dCTP, and 4 μ Ci [α - ^{32}P]dCTP). Reactions were terminated with the addition of 5 μ g proteinase K, 1 μ l 10% SDS and incubation at 55°C for 30 min. DNA was precipitated with 1 μ g glycogen, 4 μ l 11M ammonium acetate and 90% ethanol overnight (-20°C). Samples were washed with 75% ethanol, centrifuged (16,000 g and 4°C) and speed vacuumed. Dried DNA was then resuspended in 10 μ l formamide loading buffer and incubated at 90°C for 10 min. Two controls were run in parallel with each polymerase β activity assay: the identical oligonucleotide with no gap (GAP-control; Table 1); and exclusion of cell lysate (data not shown). In addition, on each gel an internal standard was included to control for inter-gel variation. A control assay with pure human polymerase β (0.25U) was also measured under the same conditions. Gels were visualized in a similar manner as above, however polymerase β activity was quantified as the increase in signal intensity due to incorporation of [^{32}P]dCTP.

Table 1
Oligonucleotides used to measure activities of BER enzymes

	Name	Sequence
AP endonuclease	THF Control	5' ATATACCGCGGCCGCCGATCAAGCTTATT 3'
	THF	5' ATATACCGCGG(THF)CGGCCGATCATCAAGCTTATT 3'
	THF Comp	5' AATAAGCTTGATGATCGGCCGCCGCGGTATAT 3'
Polymerase β	GAP Control	5' CTGCAGCTGATCGCCGTACGGATCCCCGGGTAC 3'
	GAP	15mer - 5' CTGCAGCTGATGCGC-OH 3'
		18mer - 5' P-TACGGATCCCCGGGTAC 3'
	GAP Comp	5' GTACCCGGGGATCCGTACGGCGCATCAGTGCAG 3'
	GAP Control	5' CATTGGGCCCCATAGGCATGCCGCTAGTCGACGTC 3'

THF, tetrahydrofuran; Comp, complementary sequence; GAP, doublestranded oligonucleotide containing single nucleotide gap.

Assessment of Mitochondrial Abundance and Membrane Potential

The mitochondrial content and membrane potential of fibroblasts from Snell dwarf and normal littermate controls were measured by flow cytometry using MitoTracker™ probes. Briefly, confluent cells grown at 20% O_2 were placed in complete and serum-free media for 24 hours, after which the cells were washed thoroughly in DMEM media, without serum, immediately prior to their incubation with MitoTracker™ Green (100 nM) and MitoTracker™ Red (250 nM) probes for 30 min. The cells were then washed twice with ice-cold PBS before being scraped into test tubes. The cells were analyzed on a Beckman-Dickson FACSCalibur with excitation at 488 nm and emission at 530 nm for MitoTracker™ Green and 585 nm for MitoTracker™ Red and gated for single cells.

Statistical Analysis

Data were analyzed by repeated measures two-factor ANOVA using Systat v.12. Post-hoc comparisons between means were done by Sidak's significant differences method. All data are presented as means $\pm$ standard error of the mean (SEM). A p-value of <0.05 was considered significant. Where p-values are close to, but greater than, 0.05 they are provided in the text.

Results

Antioxidant and BER enzymes in normal and dwarf mouse fibroblasts in complete and serum-free media

Fibroblasts from Snell dwarf mice are relatively resistant to the oxygen-mediated growth inhibition and replicative failure compared to cells from their normal littermates [14]. Overexpression of intracellular SOD isoforms confers stress resistance and prevents apoptotic cell death [19,20], and the activities of these enzymes are also negatively correlated with cellular senescence in culture [21], so we determined whether an upregulation of SOD activities might contribute to the stress resistance of dwarf mouse cells. However, there was no effect of genotype on MnSOD or CuZnSOD levels under any of the experimental conditions (Fig. 1). As MnSOD localizes exclusively within the mitochondrial matrix, differences in mitochondrial content of cells under different experimental conditions could affect MnSOD levels. We measured COX activity and used MitoTracker™ green fluorescence to estimate cellular mitochondrial content (see below). Even when standardized to mitochondrial content (standardized data not shown), there was no effect of genotype on MnSOD levels under any of the experimental conditions shown in Fig. 1. O_2 level and serum deprivation, however, did affect cellular MnSOD levels. MnSOD levels were approximately 2-fold higher in cells grown at 20% versus 3% O_2 (Fig. 1B; complete). This was partially a result of an apparent increase in cellular mitochondrial abundance based on COX activity (see below). On the other hand, neither O_2 level nor serum deprivation affected CuZnSOD protein levels in either normal or dwarf mouse cells (Fig. 1C).

Interestingly, greater resistance (higher LD₅₀) of Snell dwarf fibroblasts, compared to normal littermates, to exogenous stressors, such as PQ and H_2O_2 , is only evident in the absence of serum [2], because exogenous serum increases resistance dramatically in both Snell and control cell lines. We therefore investigated SOD levels in fibroblasts cultured in serum deprived (SD) media in the presence and absence of an additional oxidative stressor. There was no genotype-specific response to SD with respect to either MnSOD or CuZnSOD levels. However, SD led to a significant increase in MnSOD levels in both normal and dwarf fibroblasts (Fig. 1B). As SD had no apparent effect on mitochondrial content (see below), this suggests that the amount of MnSOD protein per mitochondrion increased approxi-

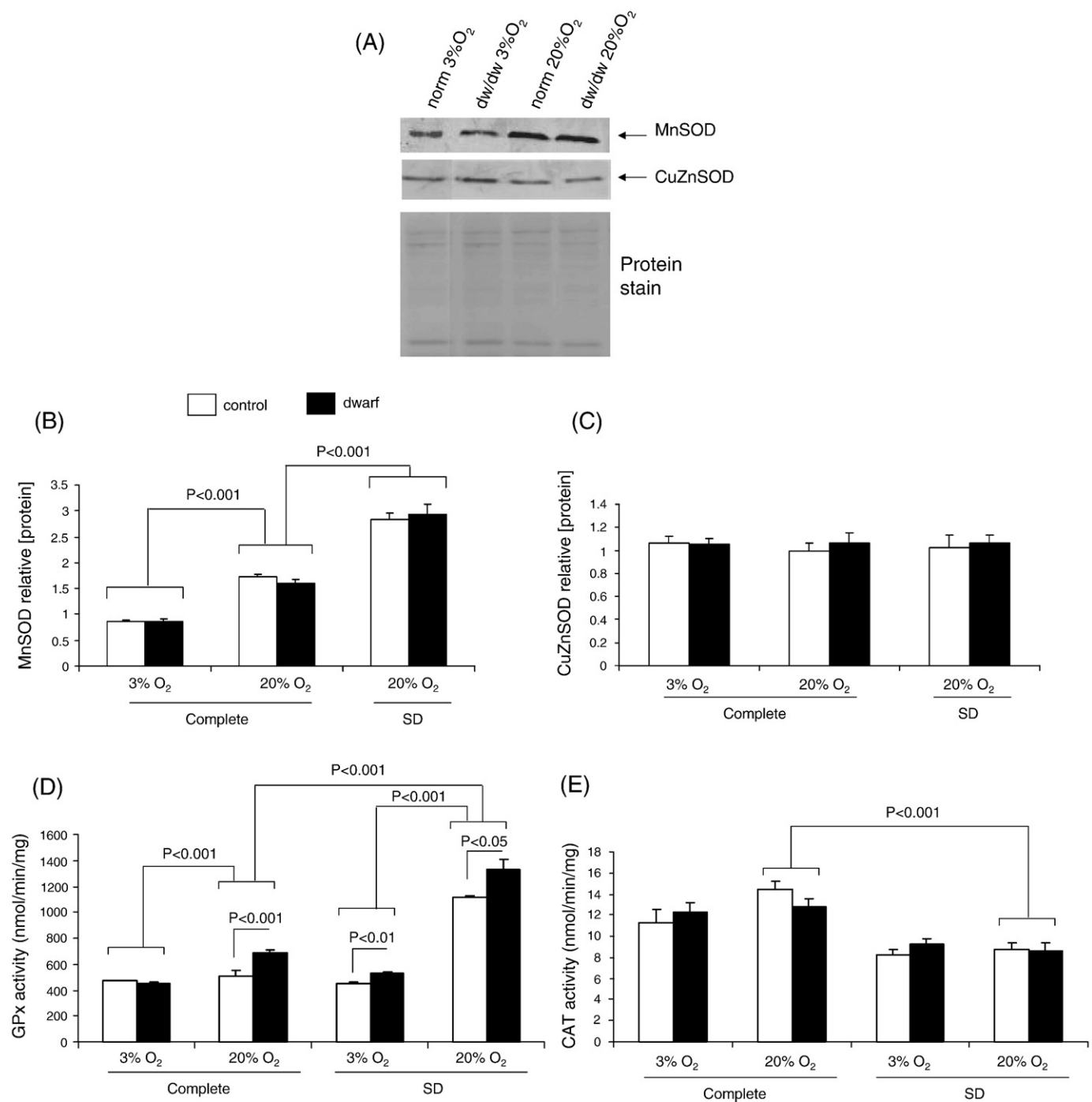


Fig. 1. Antioxidant protein levels and activities in normal and Snell dwarf mouse skin derived fibroblasts cultured at physiological (3%) and standard (20%) oxygen in complete and serum-free media. (A) Representative Western blots showing MnSOD and CuZnSOD expression. The lower panel shows the membrane stained for equal loading and transfer. (B) MnSOD relative protein concentration from normal (empty bars) and Snell dwarf (filled bars) mouse fibroblasts. (C) CuZnSOD relative protein levels in normal and Snell dwarf mouse fibroblasts. In (B and C) bars represent mean protein levels $\pm$ SEM measured in six independent cell lines for each genotype. (D) GPx activity of normal and Snell dwarf mouse fibroblasts. (E) CAT activity from normal and Snell dwarf mouse fibroblasts. In (D and E) bars represent mean enzyme activity $\pm$ SEM from eight independent cell lines measured in duplicate for each genotype. Statistical comparisons between means were by repeated measures ANOVA with Sidak post-hoc test.

mately 2- to 5-fold (Fig. 1B). In contrast, CuZnSOD protein levels were unaffected by SD (Fig. 1C).

Intracellular detoxification of H₂O₂ is catalyzed primarily by GPx and CAT. While the latter enzyme is localized to peroxisomes, isoforms of GPx are distributed in various subcellular compartments, including mitochondria [22]. Genetic disruption of GPx1 induces characteristics of senescence in cultured fibroblasts [23], suggesting it may also play a role in mediating resistance to senescence. Also, mice overexpressing the phospholipid hydroperoxide, GPx4, are protected from cellular

injury during ischemia-reperfusion stress [24]. In contrast to the SODs, GPx activity was affected by genotype ($p < 0.01$), being elevated in dwarf mouse cells under several experimental conditions (Fig. 1D). In addition, GPx activity was also affected by O₂ level, and the interaction between genotype and O₂ level was significant ($p < 0.001$), indicating that the normal and dwarf mouse fibroblasts differed in their ability to upregulate GPx in response to increasing O₂ levels. In contrast, there was no effect of either genotype or oxygen level on CAT activity, though it was reduced by SD in cells of both genotypes (Fig. 1E).

Because cumulative DNA damage has been associated with aging and cellular senescence, we hypothesized that enhanced DNA repair in the Snell dwarf fibroblasts may increase their resistance to detrimental DNA oxidative damage. BER is the major pathway of DNA oxidative damage repair in active cells, and so we measured two key enzymes within this pathway using *in vitro* assays. AP endonuclease activity, which catalyzes the processing of abasic sites in DNA, did not differ between genotypes under any of the conditions shown in Fig. 2(A, B). However, it was significantly affected by experimental condition, being reduced by SD (versus complete medium) in cells grown at 20% O₂. Polymerase β catalyzes the incorporation of a nucleotide in the gap produced by AP endonuclease activity. The interaction between genotype and O₂ level for polymerase β activity was almost significant ($p=0.08$), suggesting that normal and dwarf cells may differ in their ability to upregulate polymerase β activity in response to oxidative stress. Also, in complete medium, dwarf mouse cells cultured at 3% O₂ had approximately 70% greater polymerase β activity than those of normal mice (Fig. 2D).

There were also significant effects of experimental condition on polymerase β activity (Fig. 2C, D), with effects of both O₂ and SD. As we have observed in other mammalian fibroblasts [25], polymerase β activity appeared to be elevated (approximately 80%) in normal mouse fibroblasts grown in complete medium at 20% O₂ versus 3% O₂ (Fig. 2D; complete). However, this effect did not reach statistical significance in the current set of comparisons.

Effects of exogenous oxidants on antioxidant and BER enzymes

Following SD for 24 h, Snell normal and dwarf fibroblasts were exposed to sublethal concentrations of oxidants; either 15 μ M PQ for 24 h or 15 μ M H₂O₂ for 2 h. These concentrations were determined based upon previous studies of lethal doses [4,14]. PQ exposure slightly, but significantly, reduced MnSOD protein levels in both normal and dwarf mouse fibroblasts (Fig. 3A) grown at 20% O₂. Under this experimental condition, MnSOD protein levels were almost 10% higher in dwarf mouse, versus normal, cells. PQ exposure did not affect CuZnSOD protein levels (Fig. 3B). Although there was no effect of genotype on GPx activity under these conditions, both PQ and H₂O₂ exposure significantly reduced GPx activity in both normal and dwarf fibroblasts (Fig. 3C). There was also a significant interaction between genotype and experimental condition ($p<0.05$) in respect to CAT activity, which appears to have been driven by higher CAT activity in dwarf relative to normal cells following exposure to either PQ or H₂O₂. However, only the H₂O₂ effect reached statistical significance ($p<0.05$; Fig. 3D).

Genotype also affected AP endonuclease activity in SD cells in the presence or absence of exogenous stressors ($p<0.05$; Fig. 4A) with dwarf cells having apparently higher activities under all experimental conditions, though only in the instance of H₂O₂ exposure was the dwarf versus normal comparison statistically significant. In contrast, polymerase β activity was not affected by genotype in SD cells in the presence or absence of exogenous stressors, though a trend toward

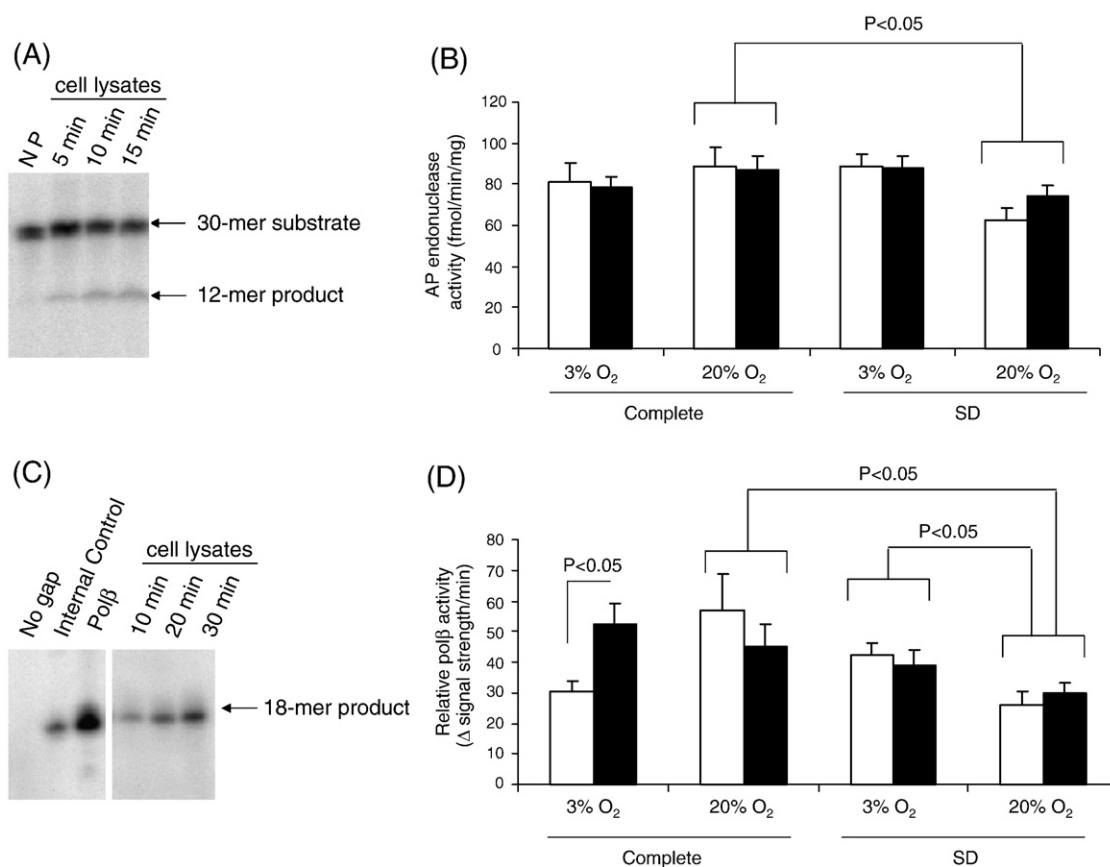


Fig. 2. Base excision repair activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at physiological (3%) and standard (20%) oxygen conditions in complete and serum-free media. (A) Representative gel showing AP endonuclease activity, measured in whole cell lysates (100 ng) containing 1 pmol THF-containing double-stranded oligonucleotide and incubated for 5, 10, 15 min. NP = no protein control (for summary of experimental controls see Materials and Methods). (B) AP endonuclease activity of normal and dwarf Snell mice skin fibroblasts. (C) Representative gel showing measurement of polymerase β activity in whole cell lysates (0.5 μ g). Incorporation of [³²P]dCTP into GAP oligonucleotide (see Table 1) is visible as the progressive accumulation of an 18-mer oligonucleotide, at 10, 20, 30 min pol β =0.25U pure human polymerase β . (D) Relative polymerase β activity in normal and Snell dwarf mouse skin fibroblasts. In (B) and (D) bars represent mean activity $\pm$ SEM measured in lysates from eight independent cell lines for each genotype; in (B) measurements were done in duplicate. Identity of bars and statistical analysis as in Fig. 1.

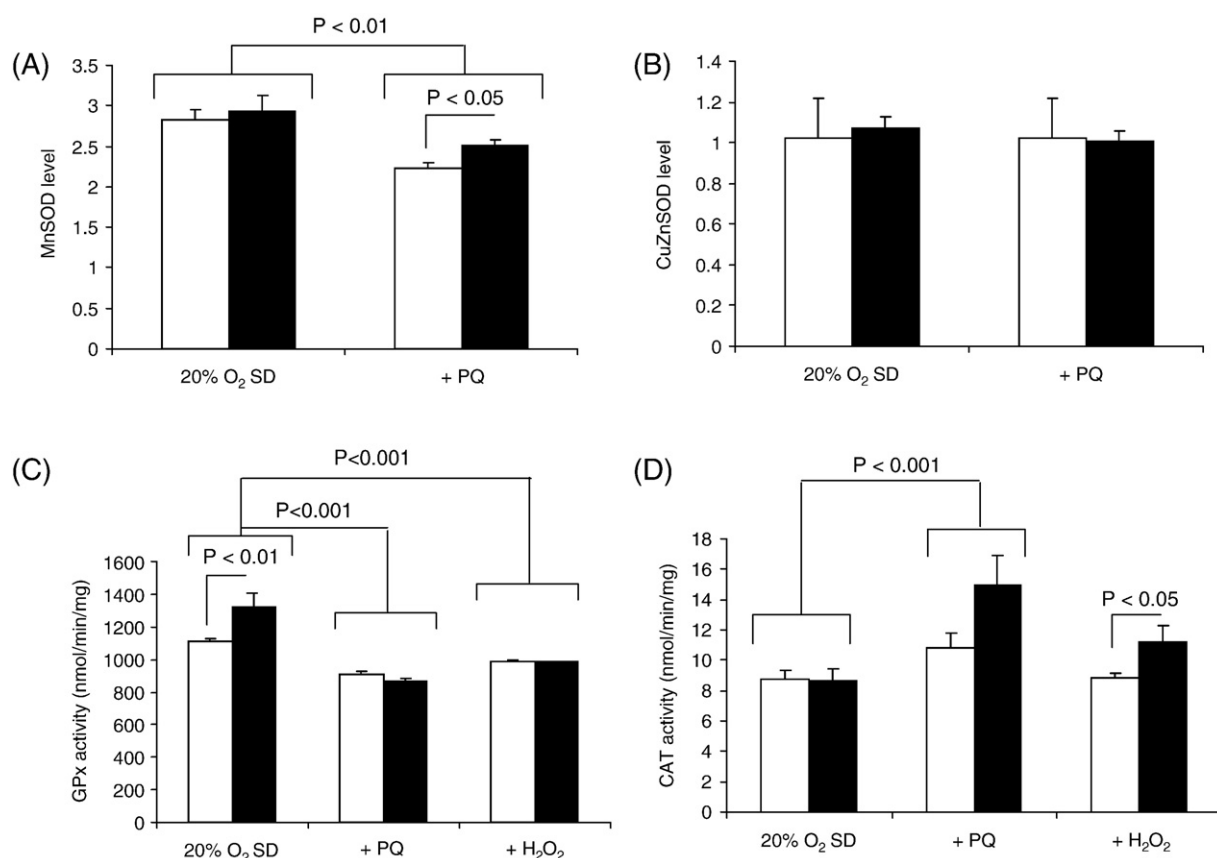


Fig. 3. Effect of paraquat and hydrogen peroxide on antioxidant protein levels and activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at standard (20%) oxygen conditions in serum-free media. (A) MnSOD relative protein concentration. (B) CuZnSOD relative protein concentration. In (A and B) bars represent mean SOD protein levels $\pm$ SEM from six samples for each genotype. (C) GPx activity of normal and dwarf Snell mice fibroblasts. (D) CAT activity from normal and Snell dwarf mouse fibroblasts. In (C) and (D), bars represent mean enzyme activity $\pm$ SEM from eight samples for each genotype, measured in duplicate. Identity of bars and statistical analysis as in Fig. 1.

higher activities in the dwarf cells was evident ($p=0.08$; Fig. 4B). Polymerase β activity however was affected by the presence of exogenous oxidants ($p<0.05$); there is a suggestion that dwarf cells may be more responsive to H₂O₂ treatment, but the interaction between genotype and stress was not significant.

Mitochondrial and fermentative metabolism in normal and dwarf mouse fibroblasts

Previous studies have shown that fibroblasts derived from Snell dwarf mice are more resistant to stressors that interact directly with mitochondria, such as PQ. This raises the possibility that the superior stress resistance of dwarf mouse fibroblasts could be due to reduced mitochondrial activity, such that the impact of metabolic and oxidative poisons that involve mitochondrial activation is less. To investigate this possibility, we analyzed mitochondrial content and activity under several of the experimental conditions studied above. Using COX activity as a proxy of mitochondrial number/activity, the only apparent effect of genotype was that this was lower in dwarf compared to normal fibroblasts grown to confluence in serum containing media at 20% O₂, dwarf cells ($p<0.05$); Fig. 5A).

Total cellular mitochondrial content and mitochondrial inner membrane potential were also estimated in both normal and dwarf mouse fibroblasts using the fluorescent probes MitoTracker™ Green (total mitochondria) and MitoTracker™ Red (membrane potential). These results are shown in Fig. 5(B, C), and indicate no significant differences between dwarf and normal fibroblast mitochondrial content or membrane potential in cells grown in the presence or absence of serum. The ratio of the membrane potential signal to the total mitochondria signal (Fig. 5D; mean membrane potential per

mitochondrion), was also calculated. However, this value also did not differ between genotypes, under either experimental condition investigated. The addition of 10 μ M FCCP, which reduces or abolishes the inner membrane potential, served as a negative control, and this ratio was also not different between normal and dwarf cells (data not shown). Taken together with the COX data, this suggests that mitochondrial activity of normal and dwarf mouse fibroblasts is similar under most experimental conditions.

Fibroblasts in culture rely partially on glycolytic pathways to meet energy requirements, even in the presence of relatively high O₂ levels. Therefore we also measured the activity of lactate dehydrogenase (LDH), an indicator of anaerobic metabolic flux, as an indirect test of whether dwarf and normal mouse fibroblasts differed in their reliance on fermentative metabolism for ATP production. In complete media, there was a significant effect of genotype on LDH activity, which was approximately 15% lower in dwarf mouse cells growing at either 3% or 20% O₂. LDH activities were lower in both genotypes at 20% O₂, which is expected as fibroblasts upregulate oxidative phosphorylation under these conditions [26]. Exposure to exogenous oxidative stressors affected LDH activity similarly in both genotypes ($p<0.001$; Fig. 5C).

Discussion

Fibroblasts cultured from dermal tissue of Snell dwarf mice are resistant to multiple forms of stress [2,9,27] and oxygen-induced growth crisis or replicative failure [14]. We initially hypothesized that the oxidative stress resistance of dwarf mouse fibroblasts might be mediated by a systematic upregulation of antioxidant enzymes to reduce ROS levels, and DNA repair defenses, to remove oxidative damage. The experimental results reported here are partially sup-

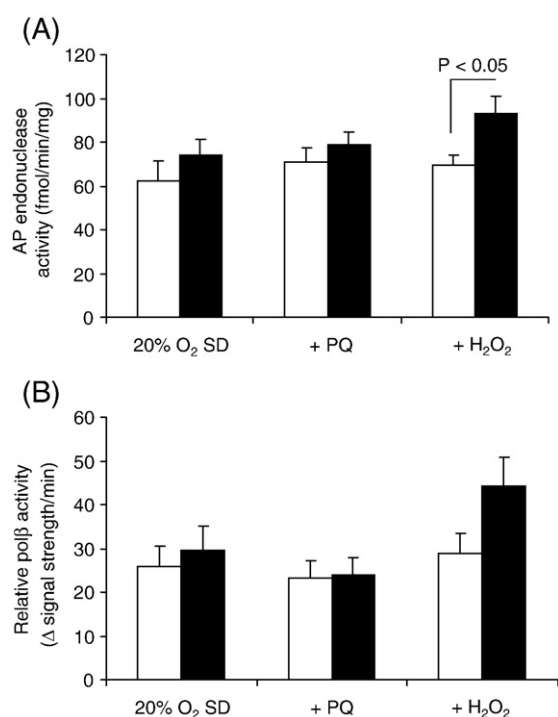


Fig. 4. Effect of paraquat and hydrogen peroxide on base excision repair activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at standard (20%) oxygen conditions in serum-free media. (A) AP endonuclease activity. (B) Polymerase β activity; bars represent mean activity $\pm$ SEM from eight independent cells line for each genotype; in (A) measurements were done in duplicate. Identity of bars and statistical analysis as in Fig. 1. PQ = paraquat; see Materials and Methods for details of oxidant exposure.

portive of this hypothesis. Interestingly, some molecular targets identified in other models of extended longevity were not differentially expressed in Snell dwarf cells. Also, our data support the contribution of multiple, relatively subtle, differences in metabolism, antioxidant status and DNA BER capacity to the overall phenotype of stress resistance.

MnSOD has been identified as a molecular mediator of animal longevity in a wide variety of contexts. It is positively correlated with stress resistance in fibroblasts from mammalian species of widely ranging lifespans (compare [4] and [25]), upregulated in long-lived type 5 adenylyl cyclase mice [3], in *C. elegans* *daf-2* mutants [28], and by the polyphenol resveratrol [29,30], which promotes longevity in some animals [reviewed in 31]. Transgenic overexpression of MnSOD is effective in preventing or limiting mitochondrial permeability transition and cell death under a variety of stressful conditions [32–34]. Despite this association of MnSOD activity with longevity in disparate biological contexts, we found limited evidence to support a role for this enzyme in the stress resistant phenotype of the dwarf mouse cells. MnSOD protein levels were found to be higher in dwarf compared to normal fibroblasts only following PQ exposure.

Conversely, experimental conditions, including O₂ level and SD, significantly affected MnSOD levels in both the normal and dwarf mouse cells. For example, growth of cells at 20% O₂ caused a 2-fold increase in MnSOD levels over cells grown at 3% O₂. This appears to have been partially caused by mitochondrial proliferation in fibroblasts exposed chronically to atmospheric oxygen, an effect which has been reported previously [26]. The effect of elevated (i.e. greater than atmospheric) oxygen tensions on mitochondrial biogenesis has also been demonstrated in vivo [35,36]. An induction of MnSOD in response to SD was also observed in both normal and dwarf mouse cells. This appeared to represent a true increase in the level of this enzyme per mitochondrion, as apparent cellular mitochondrial content did not differ between cells cultured in complete media

compared to those cultured in SD media under atmospheric oxygen conditions. This elevation of MnSOD levels is consistent with reports that increased MnSOD activity regulates the transition into quiescence in mouse embryonic fibroblasts [37]. Elevation of MnSOD activity can trigger a transition from proliferative growth to replicative senescence in culture [37,38].

The above results suggest that elevation of MnSOD makes only a minor contribution to the stress resistant phenotype of dwarf mouse fibroblasts. Similarly, CuZnSOD, which confers enhanced stress resistance in mammals [33,34], can extend longevity in *Drosophila* (reviewed in [39]), and is associated with replicative senescence in culture [21], was unaffected by either experimental treatment or genotype in our experiments. Taken together, this suggests that differences in superoxide dismutation are not a major factor underlying differences in stress resistance between normal and Snell dwarf fibroblasts.

Interestingly, however, there was more evidence that enzymatic detoxification of H₂O₂ was greater in Snell dwarf fibroblasts compared to those from normal littermates, and this could be important in conferring stress resistance. Elevated GPx activities may also contribute to the resistance of dwarf mouse cells to in vitro growth crisis when grown at 20% O₂ [14]. The importance of GPx in this context is illustrated by experiments in which genetic disruption of *GPx1* elicits characteristics of senescence in cultured fibroblasts [23]. The elevation of GPx activity in Snell dwarf cells deprived of serum may also be important in imparting resistance to oxidative stress. GPx activity has been positively correlated with cellular stress resistance in a number of studies. For example, *GPx1* $-/-$ mice are susceptible to paraquat exposure [40] and ischemia/reperfusion brain injury [41]. Similarly, MEFs cultured from *GPx1* deficient mice have increased levels of apoptotic cell death under standard culture conditions and following exposure to H₂O₂ [23]. In contrast, *GPx1* overexpression protects mouse fibroblasts from UV-induced DNA damage [42] and protects murine neuronal cells from H₂O₂-induced cell death [43], perhaps by inhibiting cytochrome c release from mitochondria [44].

There are multiple GPx isoforms (reviewed in [45]) and, although GPx1 is the most ubiquitous, abundant, and active towards reduction of hydrogen peroxide, the phospholipid hydroperoxide glutathione peroxidase (GPx4) appears to play a similar role in promoting stress resistance. Lung fibroblasts isolated from *GPx4* heterozygous mice are susceptible to H₂O₂-mediated cell death [46]. *GPx4* overexpression reduces ischemia/reperfusion associated cardiac dysfunction [47] and protects mouse fibroblasts from oxidant-induced apoptosis [48]. Mitochondria isolated from *GPx4* transgenic mice are resistant to diquat exposure, perhaps due to increased protection of the mitochondrial inner membrane and the maintenance of membrane potential [49]. We were unable to detect GPx4 activity in our cell lysates. It is, however, likely that the differences in total GPx activity we observed were due primarily to GPx1, based on the fact that fibroblasts have extremely low GPx4 activity [46], and negligible GPx activity towards H₂O₂ is measurable in *GPx1* $-/-$ mice [40].

Interestingly, differences in GPx activity between normal and Snell dwarf cells disappeared following exposure to PQ or H₂O₂, when activity in cells from both genotypes was reduced. Lower GPx activities in both normal and dwarf cells are consistent with oxidative stress-induced inactivation of GPx1, which has been previously reported in pulmonary microvascular endothelial cells exposed to paraquat [50]. Interpretation of this reduction in GPx activity may also be complicated by the possibility that PQ and/or H₂O₂ may have injured the cells, causing loss of cytosolic material. In any case, the initially higher GPx activity of dwarf mouse cells prior to oxidant exposure may be a significant factor in their subsequent survival.

While there was no effect of genotype on CAT activity in cells grown in serum-containing media, CAT activity was significantly higher in dwarf mouse cells following exposure to H₂O₂ in serum-free media, and showed a similar (but not significant) trend in cells

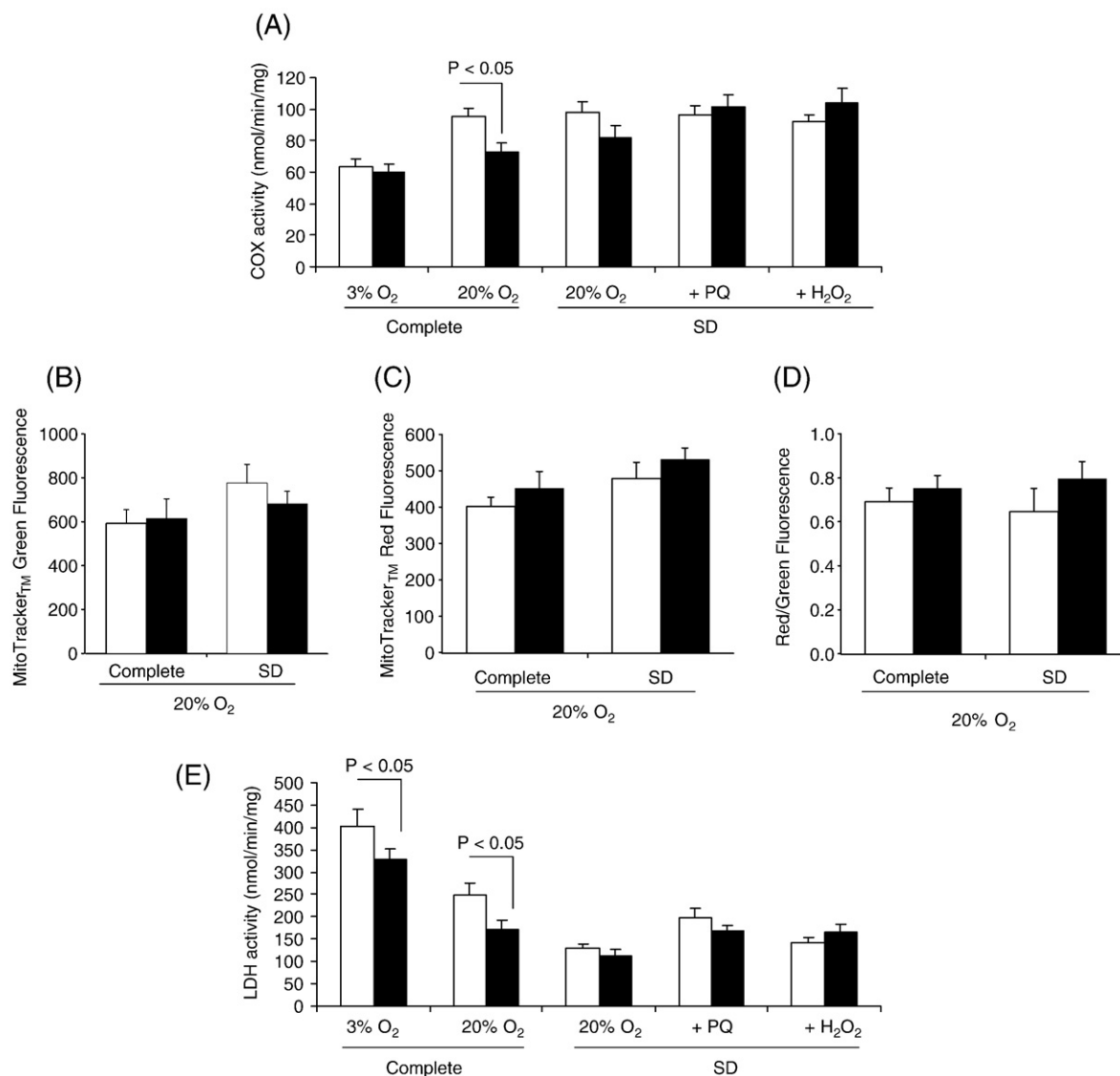


Fig. 5. Mitochondrial content and membrane potential in normal and Snell dwarf mouse skin derived fibroblasts cultured in complete and metabolically stressful conditions. (A) Cytochrome c oxidase (COX) activity; (B) Mitochondrial content as measured by fluorescence of MitoTracker GreenTM binding in normal (empty bars) and dwarf (filled bars) fibroblasts in complete media or following 24 h SD. (C) Mitochondrial membrane potential as measured by fluorescence of MitoTracker RedTM binding in cells in complete media or following 24 h SD. (D) The ratio of mitochondrial membrane potential to mitochondrial content (mean membrane potential per mitochondrion). (E) Lactate dehydrogenase (LDH) activity. (A) and (E) are means $\pm$ SEM of eight independent cell lines for each genotype, measured in duplicate. (B–D) are means $\pm$ SEM of 4 experiments. Differences between experimental conditions are not indicated in A and E, though there were significant effects of O₂ level, SD and exogenous oxidants on CS and LDH activities. LDH activity was significantly greater in dwarf, compared to normal mouse cells growing in complete media (Effect of genotype by ANOVA; $p < 0.05$).

exposed to PQ. While the contribution of CAT activity to oxidative stress resistance is likely to be less significant than that of GPx, owing to its lower specific activity under all conditions, this does suggest that Snell dwarf mouse cells have a generally superior ability to detoxify H₂O₂ via multiple pathways. This may be a significant factor in the multiplex stress resistance of the Snell dwarf fibroblasts. As even stressors that are not directly oxidative can induce redox stress secondarily [51], this capacity could affect resistance to multiple toxins.

Dermal fibroblasts derived from Snell dwarf mice are more resistant than their normal littermates to multiple genotoxic agents, including cadmium, paraquat, hydrogen peroxide, UV light, and DNA methylating agents [2,9]. Most of these compounds can produce lesions that are substrates for DNA BER, and therefore increased resistance to such stressors could be a direct result of greater DNA BER activity. Reduced polymerase β and AP endonuclease activities are associated with reduced BER and increased DNA damage, mutation and cell death caused by oxidizing and alkylating agents [52–54]. However, we did not observe constitutively elevated BER enzyme

activities in Snell dwarf cells under all experimental conditions, though some important differences between genotypes were noted. Perhaps most interesting was the apparently greater ability of the dwarf mouse cells (compared to controls) to increase AP endonuclease and polymerase β ($p = 0.08$) activities in response to H₂O₂ exposure. This suggests that the dwarf mouse cells are better able to upregulate BER in response to H₂O₂-induced stress.

The BER results support the contention that dwarf mouse cells might possess superior DNA repair capacity, at least under some experimental conditions. It has previously been shown that Snell dwarf mouse dermal fibroblasts repair UV-induced DNA lesions more rapidly than normal mouse fibroblasts, indicating enhanced nucleotide excision repair (NER) in the former [55]. Taken together, the BER and NER results suggest that that dwarf mouse cells possess an enhanced ability to repair a wide variety of DNA lesions, and that this contributes to their stress resistance. The participation of other DNA repair pathways, such as mismatch repair (MMR) and double strand break (DSB) repair would also be interesting targets of further

investigation in the Snell dwarf model, particularly as enzymes involved in MMR and DSB repair are consistently identified in proteomic surveys of the cellular stress response [51].

It is interesting that, in addition to their oxidative stress resistance, Snell dwarf fibroblasts are resistant to several metabolic stressors [27]. The breadth of this stress resistant phenotype points to the potential involvement of mitochondria, organelles that have been broadly connected to aging and cell death (reviewed in [56]). Molecules such as PQ (see [57]) achieve their toxicity primarily via interactions with mitochondria. Also, mitochondria are known to undergo adaptive remodeling in such disparate models of extended longevity as $p^{66shc/-}$ mice [58] and caloric restriction [59,60], and such remodeling could alter the activation of metabolic and oxidative stressors. We considered, therefore, that similar mitochondrial remodeling might occur in the Snell dwarf context.

COX activity as well as MitoTracker™ fluorescence measured in both normal and dwarf cells under different experimental conditions revealed no differences in mitochondrial content or membrane potential between genotypes. This suggests that the dwarf fibroblasts were no less reliant than normal fibroblasts on oxidative phosphorylation for the maintenance of ATP pools, and nor were they in general less metabolically active. The parallel measurements of LDH, an indicator of anaerobic metabolism, suggested few apparent differences in the recruitment of anaerobic pathways, though LDH activity was slightly reduced in dwarf cells (relative to normal) growing in complete media. The significance of this observation is not known, but the results generally indicate that if Snell dwarf fibroblasts are less metabolically active, the difference lies primarily in reduced fermentative metabolism. On the other hand, dwarf mouse cells replicate more rapidly and reach higher densities in culture than those of normal mice [14], which also argue against the idea that dwarf cells have lower rates of anabolic metabolism. It may nonetheless be interesting in the future to examine aspects of mitochondrial function such as ROS production and propensity to undergo permeability transition and/or apoptotic release of cytochrome c, as these properties are known to be modulated in cells of other long-lived mouse mutants (eg [58]).

In summary, Snell dwarf mouse fibroblasts appear to maintain levels of metabolic activity similar to those of their normal littermates. The capacity to dismutate superoxide radicals is also similar in normal and dwarf mouse cells. Potentially important differences in dwarf mouse cells, relative to normal mouse cells, included elevated activities of H_2O_2 detoxifying enzymes, and an ability to induce DNA BER activities in response to H_2O_2 exposure. Taken together with the results of previous studies, these results are not consistent with the existence of a single mechanism conferring stress resistance to the Snell dwarf dermal fibroblasts. Rather, they suggest that relatively subtle differences in a number of cellular processes contribute to the stress resistant phenotype.

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