

Multiplex stress resistance in cells from long-lived dwarf mice

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ABSTRACT

Mutations that extend nematode longevity by interference with IGF-I/insulin sensing pathways also lead to resistance to multiple forms of stress. Here, we report that skin-derived fibroblasts from Snell dwarf mice, already known to show increased longevity and delayed aspects of aging, are resistant to multiple forms of cellular stress, including UV light, heat, paraquat, H₂O₂, and the toxic metal cadmium. The findings suggest that increases in cellular resistance to stress may mediate extended longevity in mammals.

Key words: aging • cellular stress resistance • fibroblasts • dwarf mouse

In Snell dwarf mice, mutation of the pituitary transcription factor (Pit)-1 gene leads to declines in growth hormone and IGF-I, thyroid hormones, and prolactin. This altered hormonal profile leads to dwarfism, deceleration of several aspects of aging, and a 40% increase in life span (1, 2). However, the mechanism by which the altered hormonal profile leads to life extension is unknown.

Life-extending mutants that perturb an insulin/IGF-1 pathway in the nematode *Caenorhabditis elegans* show increased resistance to multiple forms of stresses, including oxidative stress (3–5), heat (6), UV stress (7), and the heavy metals cadmium and copper (8). It has been proposed that resistance to multiple forms of stresses is the cause of life extension (7, 9). Indeed, life span can also be extended either by exposure to mild heat stress, which induces a temporary increase in stress resistance (6), or by reducing oxidative stress through mimetics of superoxide dismutase (SOD) in *C. elegans* (10).

The aim of the current study was to test the hypothesis that the Snell dwarf mutation, already known to extend life span and delay aspects of aging in mice, also renders cells resistant to a variety of forms of lethal stress. We found that skin-derived fibroblasts from the dwarfs were indeed resistant to a variety of cellular injuries caused by UV light, heat, paraquat, H₂O₂, and the toxic metal cadmium. Thus, the altered hormonal profile in the dwarfs causes long-lasting physiological changes in cells, leading to multiplex stress resistance. The results support the theory that modulation of cellular stress resistance regulates life span in mammals.

MATERIALS AND METHODS

General procedure

Tail skin biopsies were taken from dw/dw mice bred as the progeny of (DW/J × C3H/HeJ)-dw/+ females and (DW/J × C3H/HeJ)F1-dw/dw males; these sires had been previously treated with growth hormone and thyroxine to increase body size and fertility. Initial cultures were split sixfold or ninefold to create second passage cells, and twofold or threefold dilutions were made at each subsequent passage. Third or fourth passage fibroblasts were used for assessment of cytotoxicity. Cell survival was measured by a test based on metabolic conversion of the dye WST-1 (Roche Applied Science, Indianapolis, IN), using the method specified by the vendor. Cells from each mouse were tested in triplicate at each dose of stressor, and LD₅₀ was calculated as described subsequently.

Assessment of cytotoxicity after exposure to stress

Approximately 30,000 trypsinized cells were plated in triplicate onto a 96-well microtiter plate in Dulbecco's modified Eagle's medium (high-glucose variant; Gibco-Invitrogen, Carlsbad, CA) supplemented with 20% heat-inactivated fetal bovine serum, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Sigma, St. Louis, MO) and 0.25 µg/ml of fungizone (Biowhittaker-Cambrex Life Sciences, Walkersville, MD). After a 16- to 18-h overnight incubation in complete medium, the cells were incubated for ~27 h (ranging from 24 to 30 h) in serum-free DMEM supplemented with 2% bovine serum albumin (BSA; Sigma). Cells were exposed to one of the stresses over a range of doses indicated in the figure legends. For UV stress, the cells in the 96-well plates were washed with phosphate-buffered saline (PBS) and then irradiated with UV light (254 nm at 5.625 J/m²/s) in 100 µl of Dulbecco's PBS (Biowhittaker-Cambrex Life Sciences). Cell survival was measured 18 h later by a test based on metabolic conversion of WST-1. Cells from each mouse were tested in triplicate at each dose of stressor. In [Figure 2](#), survival is given as a proportion to the survival of cells in parallel cultures not exposed to the stress. For assessment of resistance to H₂O₂, paraquat, and cadmium (Sigma), the cells in the 96-well plates were washed and exposed to the stress agent for 6 h. For the heat stress assay, serum-starved cells were treated briefly with trypsin to release them from the plates. Approximately 30,000 cells were incubated for various intervals at 42°C in a PTC-100 programmable thermal controller (MJ Research, Waltham, MA). The heat-exposed cells were then plated onto 96-well plates coated with collagen type 1 (BD Labware, Bedford, MA) to promote cell attachment to the plates, followed by incubation for 18 h. The cells were incubated at 37°C at 5% CO₂ in air before measurement of survival.

Calculation of LD₅₀ and statistical analysis

At each dose of stress, mean survival was calculated for the triplicate wells. The mean survival at each dose was then used to determine the LD₅₀ for each mouse by probit analysis, using NCSS software (NCSS, Kaysville, UT). Probabilities were calculated by two-way ANOVA, using genotype and assay date as predictor variables and LD₅₀ as the dependent variable; the *P* value shown represents the null hypothesis that genotype has no effect on LD₅₀.

Quantification of cell cycle

Flow cytometry analysis was performed to determine the percentage of the cells at each cell cycle. Cells were trypsinized and collected before and after the serum starvation. Approximately 1×10^6 cells were fixed with ice cold ethanol and then were stained with 50 $\mu\text{g}/\text{ml}$ of propidium iodide in the presence of 100 $\mu\text{g}/\text{ml}$ of RNase A (type I-A). The stained cells were analyzed by flow cytometric analysis. Cells (10,000–20,000) were used to calculate the percentage of each cell cycle phase.

Thymidine uptake assay for fibroblasts

As in the stress assay, fibroblasts were plated onto 96-well microtiter plates and incubated for 20–24 h. Cells were incubated for another 27 h in the serum-free DMEM supplemented with 2% BSA and were then exposed to stress protocols as described previously. Cells were then allowed to recover in complete DMEM for 24 h. ^3H -thymidine was added to each well (0.5 μCi in 10 μl) in complete DMEM solution. After a 4-h incubation, cells were washed with PBS, released by trypsinization, and harvested onto glass-fiber filters. Incorporation of ^3H -thymidine was measured by scintillation counting.

RESULTS

Fibroblasts were grown from tail skin of young dwarf (dw/dw) mice and from their phenotypically normal littermates (dw/+). Third or fourth passage cells were arrested at the G0/G1 phase by a 27-h period of serum starvation and were then exposed to agents that induce cellular injury.

[Figure 1](#) shows LD₅₀ values for fibroblasts from dwarf and control mice in responses to each of five forms of stress. Dwarf cells were significantly more resistant to each stress compared with control cells. The effects on LD₅₀ ranged from a 45% change for UV light to a 180% change for cadmium, and all were significant by ANOVA at $P < 0.01$. Additional details of the statistical analysis are shown in [Table 1](#). Dose-response curves also showed that cells from the dwarf mice were relatively resistant to each stress over a wide dose range ([Fig. 2](#)).

The experiments shown in [Figure 1](#) and [2](#) were conducted using normal and dwarf mice produced as the progeny of (DW/J \times C3H/HeJ)-dw/+ females and (DW/J \times C3H/HeJ)F1-dw/dw males. To see whether the effect of the dw/dw genotype could be reproduced on a different genetic background, we compared three dwarf and three control littermates bred as the progeny of DW/J-dw/+ females and (C3H/HeJ \times DW/J)F1-dw/dw males, using UV light, hydrogen peroxide, and cadmium as stress agents. In this experiment, each set of six mice was tested by each of three independent investigators. Mean LD₅₀ values for the dwarf mice were higher than those for control mice for each agent for each investigator (data not shown). We conclude that the effect of the dw/dw genotype can be documented on at least two genetic backgrounds.

The WST-1 conversion assay used for [Figure 1](#) and [2](#) relies on mitochondrial activity as a surrogate for cellular survival. To rule out the possibility that the effects seen in dwarf mice represented some peculiarity in mitochondrial responsiveness, we conducted a series of experiments in which cells were exposed to UV light, hydrogen peroxide, and cadmium; were

allowed to recover for 24 h; and were then tested for proliferative ability by a thymidine uptake test. Results are shown in [Figure 3](#), which also shows, for comparison, a parallel assay in which the cells were given the same 24-h recovery period, in serum-containing medium, before WST-1 testing. Both approaches confirmed the findings of [Figure 1](#) and [2](#) by showing that the resistance of dwarf cells to these three stresses is seen regardless of the method used to assess cell viability.

In initial pilot experiments, we had noted that differences in stress resistance between normal and dwarf cells were not apparent unless the exposure to stress was preceded by an interval of culture in serum-free medium (as in the standard protocol shown in [Fig. 1](#), [2](#), and [3](#)). To provide a formal test of the effect of serum-free medium on stress sensitivity, we tested three cultures of dwarf fibroblasts and three of control fibroblasts in parallel, with or without a 27-h exposure to serum-free medium before exposure to UV, peroxide, or cadmium. The results are shown in [Table 2](#). For each of the three agents tested, there was a significant difference between dwarf and normal mice in the standard protocol ($P < 0.05$ in each case by unpaired t test) but no significant difference in LD_{50} for any of the agents when the serum starvation step was omitted. The table shows that the effect of serum starvation is to increase the sensitivity of the cells to the cytotoxic effects of each form of stress (lower LD_{50} levels) and that the increase in sensitivity is more dramatic in the normal than in the control cultures.

To see whether dwarf and normal fibroblasts differed in their position in the cell cycle, either before or after serum starvation, we used flow cytometric methods to estimate the fraction of cells in G1/G0, S, and G2/M phases. Results are shown in [Table 3](#), as means \pm SD of two independent experiments. Dwarf and normal fibroblasts do not differ in cell cycle position when tested under these conditions. Serum starvation leads, as expected, to some decrease in the proportion of cells in the S and G2 phases, but the cultures are not fully quiescent (G1/G0 fractions, 81–83%) even after the serum starvation step.

DISCUSSION

Our results show that fibroblasts from Snell dwarf mice are more resistant than cells of control mice to each of five different forms of cellular stress when tested after a 27-h period of serum starvation. This result can be documented using either of two tests for cellular viability, one based on mitochondrial activity and the other on incorporation of thymidine into DNA, and can be seen in *dw/dw* mice on either of two segregating genetic backgrounds. The differences between dwarf and control cells do not seem to reflect alterations in distribution of the cells with respect to the cell cycle. The data in [Table 2](#) suggest that the differences between dwarf and normal cells reflect differences in the speed, or extent, to which serum withdrawal leads to increases in sensitivity to multiple forms of stress. We are currently examining candidate proteins (by immunoblotting) and candidate genes (by gene expression screening) to try to develop greater insight into the molecular basis for this differential response to serum withdrawal.

Our findings have several interesting implications. First, they support the idea that the extended longevity and delayed aging of Snell dwarf mice may reflect increased resistance of cells to stress. Note that a mutation at the *p66-shc* locus that renders mouse fibroblasts resistant to UV-induced apoptosis also extends longevity in homozygous mice (11). It will be worthwhile to delineate the steps that connect cellular stress resistance to its pathological result, disease

resistance, in these and other models of decelerated aging. Second, the data suggest that resistance to multiple varieties of stress may be coordinately regulated by hormone-sensitive pathways in mice. Defining the molecular basis for this coordinate control may lead to important insights into the relation of cellular differentiation to organismal aging, and provide biochemical targets for potential interventions. Ames dwarf mice, similar in many respects to the Snell dwarfs, have higher levels of catalase in liver and kidney compared with nondwarf controls (12), and it will be of interest to determine what other forms of cellular defense may be bolstered in these and other cells in dwarf mice. Third, the data suggest that the stress-resistance property of slow-aging mice extends to cells, such as dermal fibroblasts, that may play little role in resistance in most forms of lethal disease but that are easily accessible and thus useful for tests of genetic and pharmacological anti-geric interventions. Fourth, the parallelism between the worm and mouse results suggests that the connections between cellular stress resistance and modulation of life history trajectories may have ancient evolutionary roots.

ACKNOWLEDGMENTS

We thank Maggie Vergara for technical assistance. The work was supported by NIH grants P30-AG08808, T32-GM07315-21, and T32-AG00114.

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Received January 9, 2003; accepted May 7, 2003.

Table 1**Stress resistance of serum-starved fibroblasts from normal and Snell dwarf mice**

Stress	Strain	LD ₅₀ ^a	Number of mice	P value	Increase
		Mean ±SD		ANOVA	
UV light	Normal	110 ± 19 (J/m ²)	10	–	–
	Dwarf	160 ± 29 (J/m ²)	13	< 0.0001	45%
H ₂ O ₂	Normal	92 ± 23 (μM)	6	–	–
	Dwarf	227 ± 20 (μM)	8	0.002	147%
Paraquat	Normal	1.28 ± 1.13 (mM)	7	–	–
	Dwarf	2.04 ± 0.90 (mM)	10	0.01	53%
Cadmium	Normal	4.5 ± 3.8 (μM)	12	–	–
	Dwarf	12.6 ± 10.8 (μM)	15	0.007	180%
Heat	Normal	14.1 ± 9.4 (min)	8	–	–
	Dwarf	28.6 ± 8.6 (min)	11	0.006	102%

^aAverage LD₅₀ across the set of mice tested.

Table 2**Comparison of serum-starved and serum-exposed cultures for stress resistance^a**

Stress (units)	Strain	LD₅₀ Serum Starved	LD₅₀ Not Starved
UV light (J/m ²)	Normal	13.8 ± 8.8	72 ± 10
	Dwarf	40.7 ± 4.1	100 ± 6.7
H ₂ O ₂ (μM)	Normal	50 ± 16	353 ± 79
	Dwarf	135 ± 34	409 ± 41
Cadmium (μM)	Normal	1.3 ± 0.7	25 ± 2.2
	Dwarf	3.5 ± 0.4	29 ± 3.0

^aEach value is given as mean LD₅₀ ±SE for three individual mice tested in parallel. Serum-starved cultures were treated to the same protocol used for **Figure 1** and **Figure 2**, that is, with a 27-h interval of incubation in serum-free medium before stress exposure. Not starved cultures were exposed to stress without a preceding 27-h incubation in serum-free medium.

Table 3**Percentage of the cells at each cell-cycle phase before and after 27 h of serum starvation^a**

Cell cycle and viability	Normal littermates		dw/dw	
	Before starvation (%)	Serum Starved (%)	Before starvation (%)	Serum Starved (%)
G1/G0	75.5 ± 2.9	82.8 ± 6.7	74.8 ± 4.0	81.0 ± 3.7
S	15.1 ± 0.7	12.4 ± 3.7	15.8 ± 1.0	13.7 ± 2.8
G2	9.4 ± 2.5	4.9 ± 0.4	9.5 ± 1.3	5.4 ± 1.2
Dead cells	2.0 ± 0.8	3.5 ± 0.2	1.7 ± 1.7	2.5 ± 0.5

^aMean ±SD of two experiments.

Fig. 1

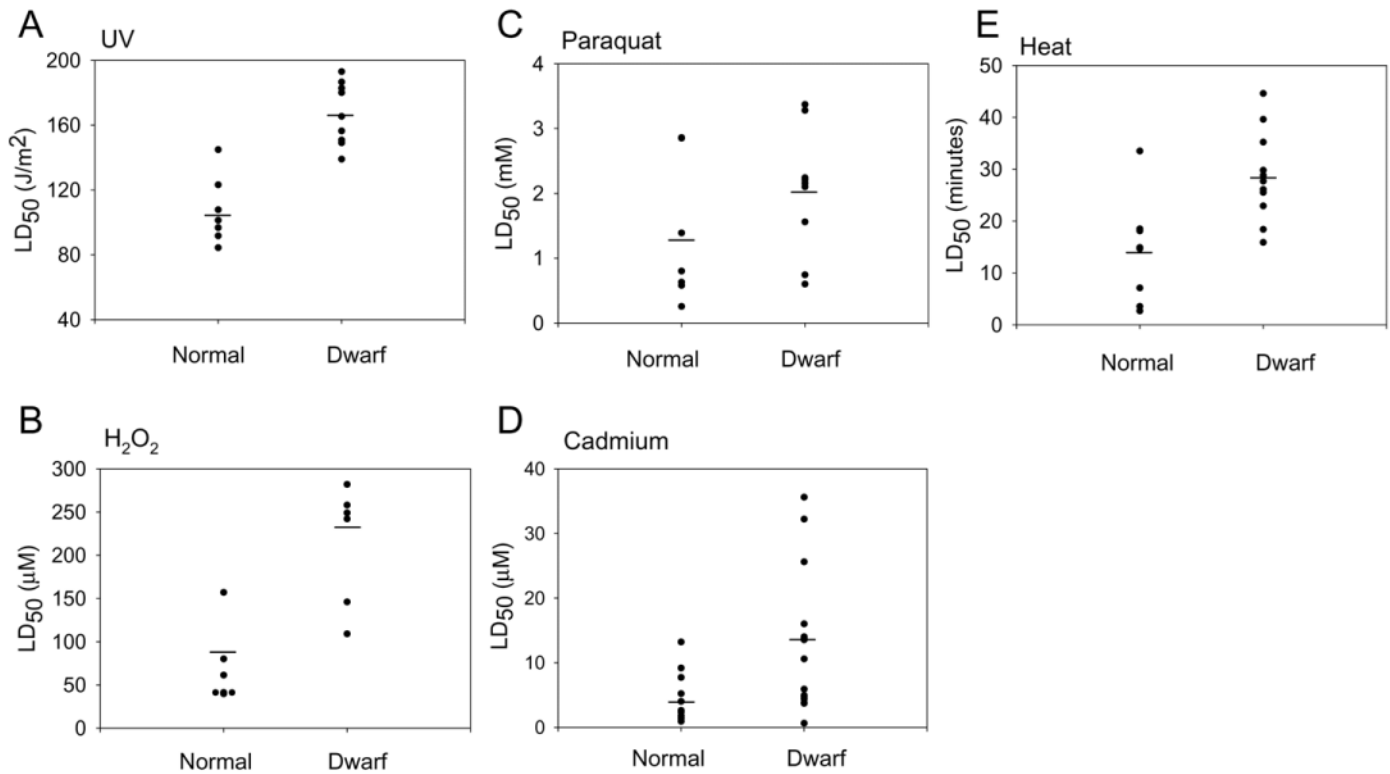


Figure 1. Skin-derived fibroblasts from dwarf mice are resistant to multiple forms of stress. Each symbol in the figure represents an individual mouse of the indicated genotype; the horizontal dash indicates the mean value in each case. The results for each stress are compiled from three to five separate experiments, each containing a balanced number of samples from dwarf and control mice. Percent increases in LD₅₀ and *P* values were as follows: UV light (45%; *P*<0.0001), hydrogen peroxide (147%; *P*=0.002), paraquat (53%; *P*=0.01), cadmium (180%; *P*=0.007), and heat (102%; *P*=0.006).

Fig. 2

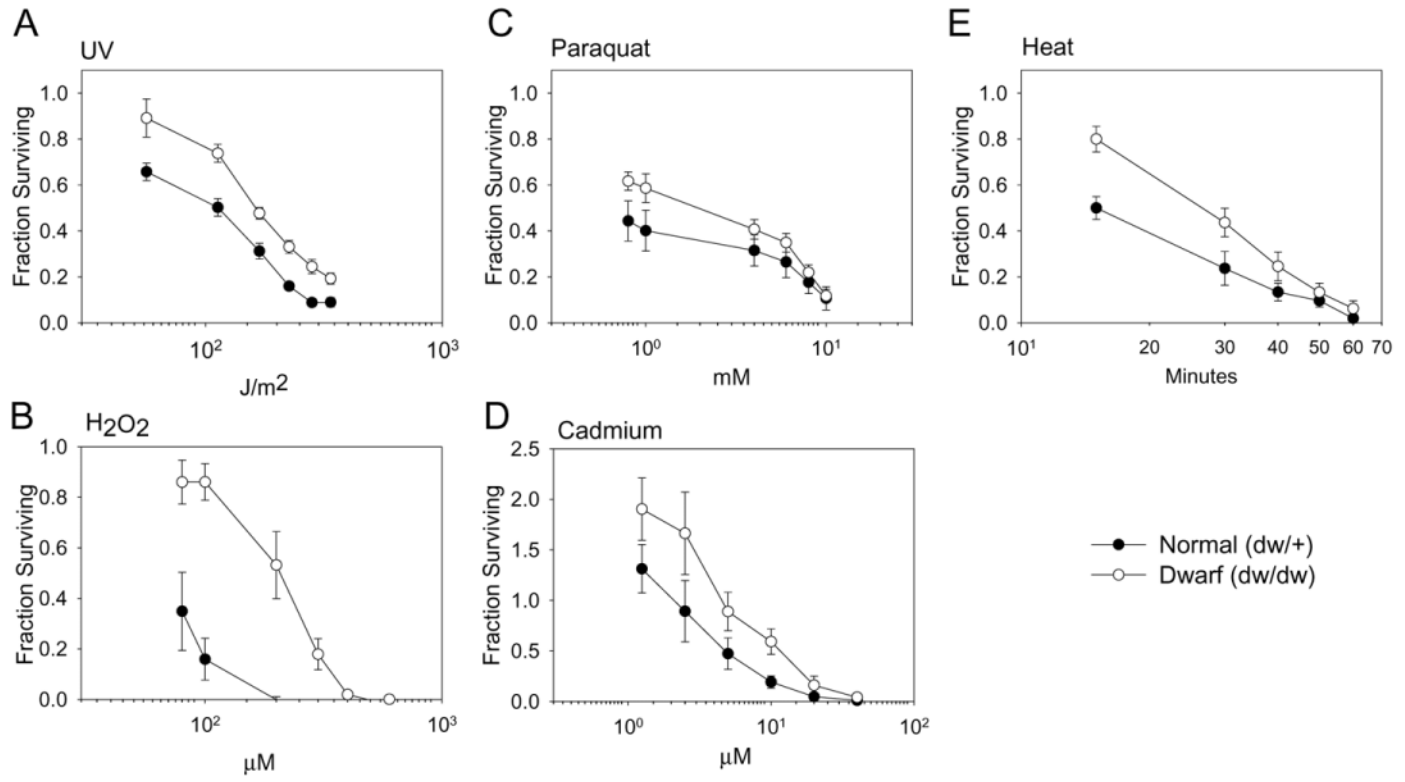


Figure 2. Dose-response curves of the fibroblasts shown in Figure 1. Each symbol shows the mean and SD at the indicated dose across the set of experiments performed.

Fig. 3

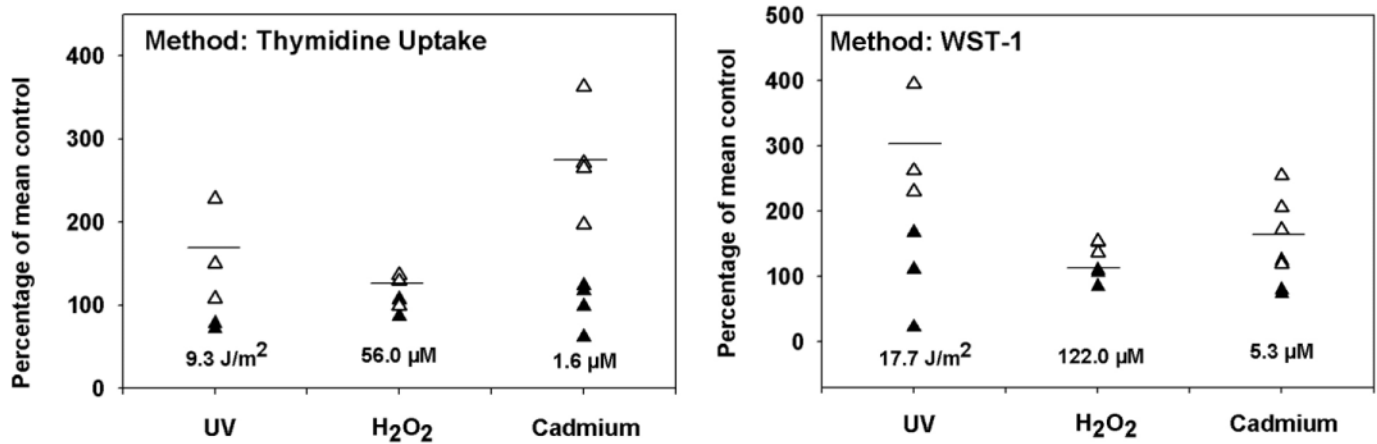


Figure 3. Comparison of WST-1 method (right panel) to thymidine uptake method (left panel) as a measure of cell viability after stress exposure. Three or four dwarf (open symbols) and three or four normal mice (filled symbols) were tested in parallel; each symbol represents LD₅₀ from one mouse, expressed as the percentage of the mean response of the control mice tested. The mean LD₅₀ level for the control mice is shown in each column. The short horizontal lines show the mean values for dwarf mice for each of the stress agents.