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Research Paper

Ablation of the mitochondrial complex IV assembly protein Surf1 leads to increased expression of the UPR^{MT} and increased resistance to oxidative stress in primary cultures of fibroblasts

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ABSTRACT

Mice deficient in the electron transport chain (ETC) complex IV assembly protein SURF1 have reduced assembly and activity of cytochrome *c* oxidase that is associated with an upregulation of components of the mitochondrial unfolded protein response (UPR^{MT}) and increased mitochondrial number. We hypothesized that the upregulation of proteins associated with the UPR^{MT} in response to reduced cytochrome *c* oxidase activity in *Surf1*$^{-/-}$ mice might contribute to increased stress resistance. To test this hypothesis we asked whether primary cultures of fibroblasts from *Surf1*$^{-/-}$ mice exhibit enhanced resistance to stressors compared to wild-type fibroblasts. Here we show that primary dermal fibroblasts isolated from *Surf1*$^{-/-}$ mice have increased expression of UPR^{MT} components ClpP and Hsp60, and increased expression of Lon protease. Fibroblasts from *Surf1*$^{-/-}$ mice are significantly more resistant to cell death caused by oxidative stress induced by paraquat or tert-Butyl hydroperoxide compared to cells from wild-type mice. In contrast, *Surf1*$^{-/-}$ fibroblasts show no difference in sensitivity to hydrogen peroxide stress. The enhanced cell survival in response to paraquat or tert-Butyl hydroperoxide in *Surf1*$^{-/-}$ fibroblasts compared to wild-type fibroblasts is associated with induced expression of Lon, ClpP, and Hsp60, increased maximal respiration, and increased reserve capacity as measured using the Seahorse Extracellular Flux Analyzer. Overall these data support a protective role for the activation of the UPR^{MT} in cell survival.

1. Introduction

Mitochondrial electron transport chain complex IV (cytochrome *c* oxidase) is composed of 13 protein subunits that are assembled in the mitochondrial inner membrane into the holoenzyme by a regulated series of assembly proteins. Previous studies have shown that mice with a complex IV assembly factor *SURF1* null mutation (*Surf1*$^{-/-}$ mice) have impaired assembly of complex IV resulting in a 50–75% reduction in cytochrome *c* oxidase content and activity [8,35,39]. Surprisingly, despite the significant reduction in cytochrome *c* oxidase activity, the *Surf1*$^{-/-}$ mice exhibit a number of beneficial phenotypic changes including increased insulin sensitivity [7], increased mitochondrial number and activation of the UPR^{MT} in several tissues [35], increased blood flow and memory function in brain [23], increased resistance to kainic acid toxicity, and increased lifespan [8].

Cellular stress resistance is considered an important component of longevity in mammalian organisms [16,27]. For example, primary fibroblasts isolated from long-lived mouse models, such as the Ames dwarf mouse, have been shown to have a significantly elevated LD_{50} compared to wild-type controls in response to exogenous oxidative stressors such as paraquat or hydrogen peroxide [22,38]. Additionally, fibroblasts isolated from species with divergent lifespans indicate that greater survival following various stressors corresponds to the lifespan of the animal species [38]. The increased kainic acid resistance in adult *Surf1*$^{-/-}$ mice and increased longevity in *Surf1*$^{-/-}$ mice led us to hypothesize that cells from the *Surf1*$^{-/-}$ mice would exhibit increased resistance to

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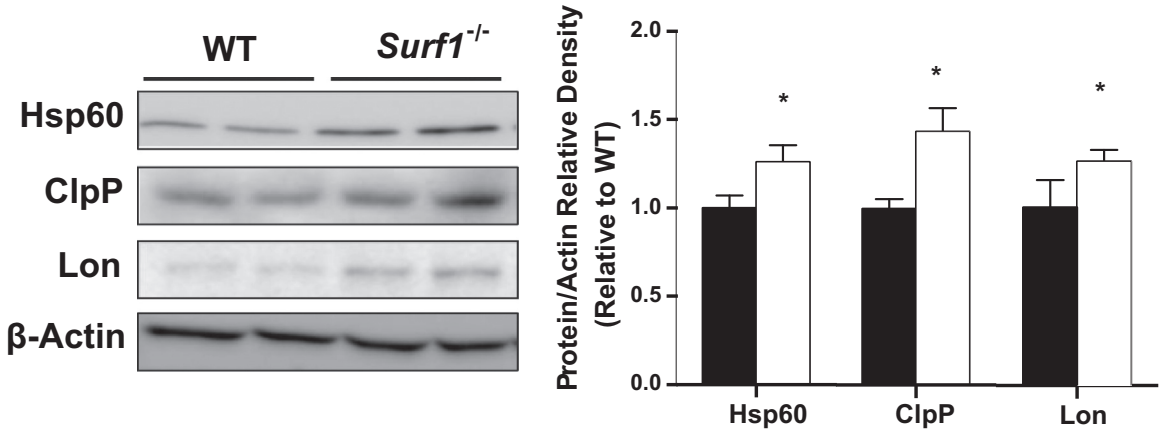

Fig. 1. The UPR^{MT} is elevated in *Surf1*$^{-/-}$ dermal fibroblasts. Primary dermal fibroblast cultures were isolated from 3 to 6 month old wild-type and *Surf1*$^{-/-}$ tails. Following an overnight incubation, cell lysates were prepared and immunoblotted with antibodies to Hsp60, ClpP, and Lon as surrogate for UPR^{MT} activation. Right panel indicates the histogram of the immunoblot quantification. Bars depict the mean ± SEM from 3 independent experiments. Statistical significance was determined by two-tailed Students *t*-test; $^{*}p < 0.05$.

oxidative stress [8]. Consistent with this, we report that primary dermal fibroblasts isolated from *Surf1*$^{-/-}$ mice are more resistant to cell death caused by paraquat (PQ) or tert-Butyl hydroperoxide (*t*-BuOOH), but not to hydrogen peroxide (H_2O_2), compared to cells from wild-type mice. Interestingly, PQ and *t*-BuOOH also result in induction of the UPR^{MT} related proteins Hsp60 and ClpP protease as well as Lon protease. These data suggest that reduced assembly of complex IV holoenzyme associated with reduced cytochrome *c* oxidase activity leads to enhanced resistance to stress that may be associated with enhanced upregulation of the UPR^{MT}.

2. Methods

2.1. Animals

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Health Science Center at San Antonio (UTHSCSA) and the Oklahoma Medical Research Foundation (OMRF). The *Surf1*$^{-/-}$ mice were generated as previously described [8]. The *Surf1*$^{-/-}$ colony was maintained by breeding male and female heterozygous *Surf1*$^{+/-}$ mice on a B6D2F1/J (C57/Bl6JxDBA2) mixed background. All wild-type mice were littermate controls of the *Surf1*$^{-/-}$ animals. The mice were maintained under specific pathogen-free barrier conditions with access to water and food ad libitum.

2.2. Primary fibroblast isolation

Primary fibroblasts were isolated from tail snips collected from *Surf1*$^{-/-}$ and wild-type mice. Tail snips taken from young, 3- to 6-month old mice were washed in 70% ethanol and rinsed in DMEM (Gibco, 4.5 g/l glucose, Glutamax) supplemented with 1% Penicillin/Streptomycin (P/S) antimicrobial solution (Gibco). The tail snip was added to fresh DMEM (4.5 g/l glucose with 1%P/S) and minced using a sterile scalpel. 1 mg of Liberase DL (Roche) was added to the minced tail and samples were incubated overnight in a cell culture incubator at 37 °C, 5% CO_2, and 21% O_2. The next day, the tail fragments were pipetted 7–10 times in complete DMEM (DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% P/S) and centrifuged at 200 × *g* for 5 min. The supernatant was aspirated and the cell pellet resuspended in complete DMEM and transferred to a 25 ml cell culture flask for subsequent culturing and expansion.

2.3. Cell survival assay

Primary fibroblasts were seeded 2.5×10^4 per well in a 96-well plate in complete DMEM. Following an overnight incubation, complete DMEM was replaced with DMEM+2% Bovine Serum Albumin (BSA) for 18 h prior to addition of the stressors in serum-free DMEM. Fibroblasts were treated with increasing concentrations of PQ, *t*-BuOOH, or H_2O_2 in triplicate for 6 h. Following stress, the media was aspirated and the cells were washed with dPBS and cultured in DMEM+2% BSA for 18 h. Cell viability was then measured after a three-hour incubation with the extracellular tetrazolium dye WST-1. The LD_{50} was calculated using non-linear regression (curve fit) analysis for wild-type and *Surf1*$^{-/-}$ fibroblasts.

2.4. XF24 Seahorse Flux Analyzer

Cells were seeded into Seahorse Flux Analyzer plate (30,000 cells/well) and incubated overnight at 37 °C, 5% CO_2 in complete DMEM. Following overnight incubation, cells were treated with vehicle (MEM), 1 mM PQ, 100 μM *t*-BuOOH, or 200 μM H_2O_2 for 30 min. These concentrations were chosen based on the LD_{50} measurements for each stress. We used a concentration that would give a stress that did not result in significant loss of cell viability. Following stress, the culture medium was changed to DMEM, pH 7.4 supplemented with 4 mM glutamine and placed in a dry incubator (non-CO_2) for 1 h at 37 °C. Mitochondrial function was then assessed by monitoring changes in oxygen consumption rate (OCR) as previously described ([30] (3T3 fibroblast); [31] (C2C12 cells); [6] (C2C12 cells)). Briefly, at the time of the assay run, three measurements of the basal level of oxygen consumption were recorded. Subsequently, oligomycin (1 μM), a complex V inhibitor, was injected, mixed, and two measurements were recorded to determine ATP-linked oxygen consumption and proton leak. Following oligomycin, FCCP (1 μM), a proton uncoupler, was injected, mixed, and another two measurements were recorded to determine maximal respiration capacity. Finally, antimycin A (1 μM), a complex III inhibitor, was injected, mixed, and two measurements were recorded to determine non-mitochondrial oxygen consumption. The oxygen consumption rate of each well was then normalized to total protein.

2.5. Cell culture, treatment and preparation of extracts

Primary fibroblasts from *Surf1*$^{-/-}$ and wild-type mice were seeded at 1.5×10^5 in a 6-well plate with 3 ml complete DMEM