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ZG, FNG, SV conceived the idea; TG, GDV, ZG performed the experiments; ZS prepared the draft of the manuscript; all authors contributed to data analysis and writing.

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Impaired Ca^{2+} signaling indicates disturbed mitochondrial function in fibroblasts from patients with sporadic and familial ALS

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Summary

Amyotrophic lateral sclerosis (ALS) is a progressive, devastating, neurodegenerative disorder affecting upper and lower motor neurons. Common mechanisms of ALS pathogenesis disturb cellular calcium homeostasis and cause mitochondrial dysfunction. Both influence mutually each other. As a result, chronic mitochondrial energy stress impairs cellular signaling and transport processes, leading to degeneration of motor neurons. We measured cytosolic Ca^{2+} in healthy and ALS fibroblasts. Mitochondrial calcium retention capacity in fibroblasts obtained from patients with sporadic (sALS) and familial (fALS) ALS differed between two subtypes and from healthy individuals. Changes of $[Ca^{2+}]_{cyt}$ dynamics in ALS fibroblasts was partially rescued by treatment with antioxidants (Trolox and CoQ_{10}). These results confirm a causative role of oxidative stress in mitochondrial dysfunction linked to ALS.

1. Introduction

Calcium toxicity is a key factor of degeneration of motor neurons in Amyotrophic Lateral Sclerosis (ALS) (Grosskreutz et al 2010). Sustained neuronal hyperactivation leads to elevated cytosolic Ca^{2+} and its abnormal intracellular re-distribution causing depletion in the endoplasmic reticulum (ER) and overload in mitochondria (Marambaud et al 2009). Among the first Ca^{2+} toxicity consequences are mitochondrial injury, impaired autophagy, protein misfolding, and oxidative stress (Rotunno, Bosco 2013; Edens et al 2016; Obrador et al 2021, Prell et al 2013). The mitochondrial Ca^{2+} overload-associated energy deficit affects the activity of key Ca^{2+} transporting mechanisms: plasma membrane Ca^{2+}-ATPase (PMCAs) (Brini et al 2011), endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Walker, Atkin 2011; Lautenschlaeger et al 2012), Na^{+}/Ca^{2+} exchanger (Castaldo et al 2009), and mitochondrial Ca^{2+}-uniporter (Kawamata, Manfredi 2010). The crosstalk between these transport systems is tightly controlled in healthy cells to maintain the physiological level of cytosolic Ca^{2+} but is misaligned in the ALS pathology (Kawamata, Manfredi 2010).

Most of the known ALS incidents are sporadic (sALS), and only 5-10 % are inherited familial (fALS), although, 10 % of sALS patients have a genetic component (Renton et al 2014; Volk et al 2018). Bioenergetic characteristics of neuronal mitochondria from sALS and fALS patients — including membrane depolarization, impaired oxidative phosphorylation and protein transport, and elevated oxidative stress — are similar (Singh et al 2021; Kaus, Sareen 2015). However, in non-neuronal tissues, i.e. fibroblasts, data are diverse and contradictory (Konrad et al 2017; Volk et al 2018; Walczak et al 2019; Debska-Vielhaber et al 2021).

In fALS, the major mutations are known to be associated with SOD1 (Bidhendi 2016) and TDP43 (Ludolph, Brettschneider 2015; Wang et al 2017) proteins that accumulate into insoluble aggregates. However, it is unclear whether sporadic and familial ALS share common mechanisms or have distinct mechanisms of pathogenesis.

The focus of this study is to differentiate the nuances of cytosolic Ca^{2+} homeostasis in fibroblasts from sporadic and familial ALS and explore the effect of antioxidants on the crosstalk between cytosolic Ca^{2+} and mitochondria-driven damage.

2. Methods

2.1. Subjects

We used fibroblasts from skin biopsies of three sALS, four fALS patients, and three healthy volunteers. The demographic and clinical data of the cohort are shown in Table 1. All ALS patients were diagnosed in accordance with the revised El Escorial criteria (Brooks et al 2000). Written informed consent was obtained from all participants prior to taking the skin biopsy. The study was approved by the Ethical Committee of the University of Magdeburg (No. 101-02/06-09; No. EK45022009).

Table 1. Clinical characteristics of healthy and ALS patients.

Patient groups	Healthy (*N* = 3)	sALS (*N* = 3)	fALS (*N* = 4)
Age at onset (years)			
Mean (SD)	58.0 ± 8.8	64.7 ± 4.0	51.0 ± 7.9
Median	59.5	65.8	51.5
Range	46-67	59-68	42-59
Disease duration (months)			
Mean (SD)	-	17.7 ± 12.5	41.8 ± 32.1
Median		12	39
Range		9-32	7-65
ALSFRS-R*			
Mean (SD)	-	37.3 ± 4.9	33.3 ± 7.8
Median		35	33
Range		34-43	24-43
Gender			
M:F ratio	1:2	2:1	1:1

*ALSFRS-R, the ALS Functional Rating Scale (Revised), an established rating mechanism to monitor disease progression in patients with ALS (Cedarbaum et al 1999).

2.2. Cell culture

Primary fibroblasts from ALS and healthy participants were derived from 5·5 mm punch skin biopsies. Cells were grown in DMEM medium supplemented with 10 % FCS, 2 mM glutamine, 100 U·mL^{-1} penicillin, 100 U·mL^{-1} streptomycin, 4 µg·mL^{-1} Ciprolaxin and 10 µg·mL^{-1} Tylosin, at a 5 % CO_2 in air and 37 °C (Kunz et al 1995). All cultures were used between passages 6 and 10.

In the experiments with antioxidants, ALS cells in the growth medium were exposed to 300 µM Trolox for 5 days or to 5 µM CoQ_{10} for 3 weeks. Every second day the growth medium was changed, and fresh solutions of antioxidants were added.

2.3. Cytoplasmic Ca^{2+}

Cytoplasmic free Ca^{2+} in fibroblasts was measured using a Carry Eclipse fluorescence multichannel spectrophotometer (Varian, Darmstadt, Germany). Cells were loaded with 2 µM Fura-2AM for 30 min at 37 °C in the dark (Grynkiewicz et al 1985). After incubation, cells were washed twice with HBSS (without Ca^{2+}, Mg^{2+}) and resuspended in the same buffer. For each experiment, a cell concentration of 10^6 x·mL^{-1} was added into a quartz cuvette and placed in the thermostatically controlled (37 °C) holder of the spectrophotometer with continuous stirring. Cells were first exposed to 2 mM Ca^{2+} and then sequentially challenged by the stressors 100 µM histamine and 10 µM FCCP. Excitation and emission wavelengths were 340/380 nm and 510 nm, respectively. At the end of each experiment, cells were disrupted by addition of 0.5 % Triton X-100 to assess the maximal Ca^{2+} signal. The 340/380 nm fluorescence excitation ratio (R) and the 380 nm fluorescence emission (F_s) were obtained at a saturating Ca^{2+} concentration and at <1 nM Ca^{2+}. The latter was reached in the presence of 1 mM EGTA in the buffer. $[Ca^{2+}]_{cyt}$ was calculated using the following equation (Grynkiewicz et al 1985):

$$[Ca^{2+}]_{cyt} = K_d \cdot F_0/F_s \cdot (R - R_{min})/(R_{max} - R)$$

where F_0 and R_{min} are the Fura-2AM signal at 380 nm and 340/380 nm fluorescence excitation ratio, respectively, in a medium lacking Ca^{2+}. The F_s and R_{max} are the

corresponding values obtained at saturating Ca^{2+} concentration. The K_d for the Ca^{2+}-Fura-2AM complex was 224 nM.

2.4. Statistical analysis

Experimental runs were performed at least in triplicates. Data were analyzed using the paired, two-sample Student's t test assuming unequal variances. Data are presented as mean ± SD. Linear relationships between measured variables were assessed via Pearson product moment correlation (CP) using the Sigma Plot 11. Statistical significance was accepted at $p \leq 0.05$.

3. Results

3.1. Cytosolic Ca^{2+} in control vs ALS fibroblasts

Cytosolic $[Ca^{2+}]_{cyt}$ was monitored as a function of signal changes of endogenously accumulated Fura-2AM before and upon subsequent addition of histamine and FCCP to the experimental medium. To mimic a physiological interstitial environment, 2 mM Ca^{2+} was added to the medium before challenging the cells with the modulators. Figure 1 illustrates representative Ca^{2+} responses to the stressors of human control and ALS fibroblasts. $[Ca^{2+}]_{cyt}$ in sALS was 28 % higher (Figure 1A,B,D) and in fALS 20 % lower (Figure 1A,C,D) compared to control cells.

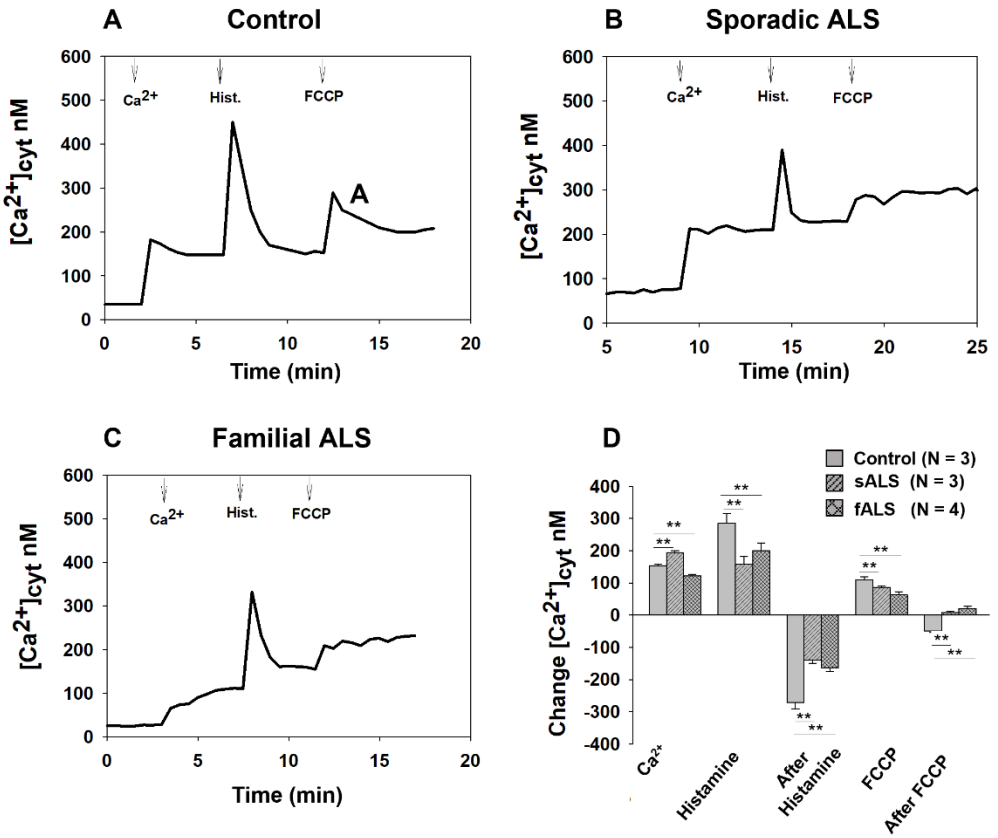

Figure 1. Cytosolic $[Ca^{2+}]_{cyt}$ in fibroblasts from healthy volunteers and ALS patients. (A) Healthy control. **(B)** ALS sporadic. **(C)** ALS familial (SOD1 mutation). **(D)** Summary of changes of fluorescence signal upon addition of modulators. Additions: fibroblast cells 10^6 x·mL^{-1}, Ca^{2+} 2 mM, histamine 100 µM, FCCP 10 µM. Data are means ± SD (N = individuals). ** $p < 0.01$.

Next, cells were exposed to histamine, the Ca^{2+} mobilizing agonist that acts via H- and G-protein-coupled receptors (Kirischuk et al 1996; Hofstra et al 2003). This compound was used to modulate elevated cytosolic Ca^{2+} and evaluate the vulnerability and/or functional activity of Ca^{2+} signaling mechanisms of ALS affected cells. Addition of 100 μM histamine increased the cytosolic Ca^{2+} signal, presumably by stimulating the ion influx from extracellular environment (Li et al 2012). The Ca^{2+} peaks were lower in ALS fibroblasts than in controls, by 45 % in sALS (Figure 1B) and 30 % in fALS (Figure 1C). However, 3-5 min after histamine stimulation, cytosolic Ca^{2+} recovered back to pre-histamine levels by -84 % in sALS and -78 % in fALS versus -98 % in controls (Figure 1D). This cytosolic signal decrease occurs due to a removal of free Ca^{2+} ions from cytosol by redistribution between intracellular compartments (mitochondria and ER), and extrusion of Ca^{2+} from cells via PMCAs.

To address mitochondrial functionality, FCCP was added to histamine-stimulated cells. Uncoupling facilitated Ca^{2+} release from mitochondria. The FCCP-induced Ca^{2+} peak was indicative of the amount of free Ca^{2+} in the mitochondria and in this experimental setting referred to the mitochondrial calcium retention capacity. As seen in Figure 1D (FCCP columns), the Ca^{2+} signal in sALS cells was 68 % and in fALS 57 % of control cells. This suggests that the calcium retention capacity of ALS mitochondria is lower than in controls, and they are not capable of retaining Ca^{2+} in the matrix.

The "After FCCP" columns demonstrate the changes of $[Ca^{2+}]_{cyt}$ after mitochondrial depolarization with FCCP. As seen in Figure 1A, in the control sample, the FCCP-induced increase of $[Ca^{2+}]_{cyt}$ was transient because cells tended to eliminate the excess of free Ca^{2+}; the level of basal $[Ca^{2+}]_{cyt}$ quickly restored to 60 %, by virtue of available cellular ATP (Figure 1D, After FCCP). However, the elevated signals in both ALS samples sustained, indicating a compromised ability of ALS cells to maintain $[Ca^{2+}]_{cyt}$.

3.2. Modulation of cytosolic Ca^{2+} by antioxidants

The altered mitochondrial functions are prerequisites for oxidative stress (Cunha-Oliveira et al 2020). To reverse the effects of oxidative damage, ALS-derived fibroblasts were incubated with the antioxidants Trolox (Babahajian et al 2019) and CoQ_{10} (Chaturvedi, Beal 2008), which have neuroprotective potential. The same experimental run was carried out as described in the previous section but with additional pretreatment of cells with the antioxidants.

As seen in Figure 2A, in the presence of 2 mM Ca^{2+}, the sALS+Trolox and sALS+CoQ_{10} cells had slightly elevated $[Ca^{2+}]_{cyt}$ compared to healthy controls. However, the signals were quite similar to the untreated sALS. The responses of sALS+Trolox and sALS+CoQ_{10} fibroblasts to histamine and FCCP differed from the untreated ALS cells. Moreover, both ALS-antioxidants signals were approximately equal to those of healthy fibroblasts (Figure 2A).

Figure 2B shows the results of $[Ca^{2+}]_{cyt}$ alterations in fALS fibroblasts pretreated with antioxidants. In the presence of 2 mM Ca^{2+}, both fALS+Trolox and fALS+CoQ_{10} cells had approximately a 20 % higher $[Ca^{2+}]_{cyt}$ than the untreated fALS, nearly identical to healthy fibroblasts. The stimulation of Ca^{2+} transport by histamine increased $[Ca^{2+}]_{cyt}$ in both ALS groups bringing close to healthy fibroblasts. The dynamics of $[Ca^{2+}]_{cyt}$ changes after addition of FCCP demonstrated an improvement of mitochondrial metabolism by antioxidative agents, particularly an increase of mitochondrial calcium retention capacity

(Figure 2B). Overall, our data indicate that Trolox and CoQ_{10} are capable to stabilize Ca^{2+} homeostasis of ALS-affected fibroblasts close to the level of healthy cells.

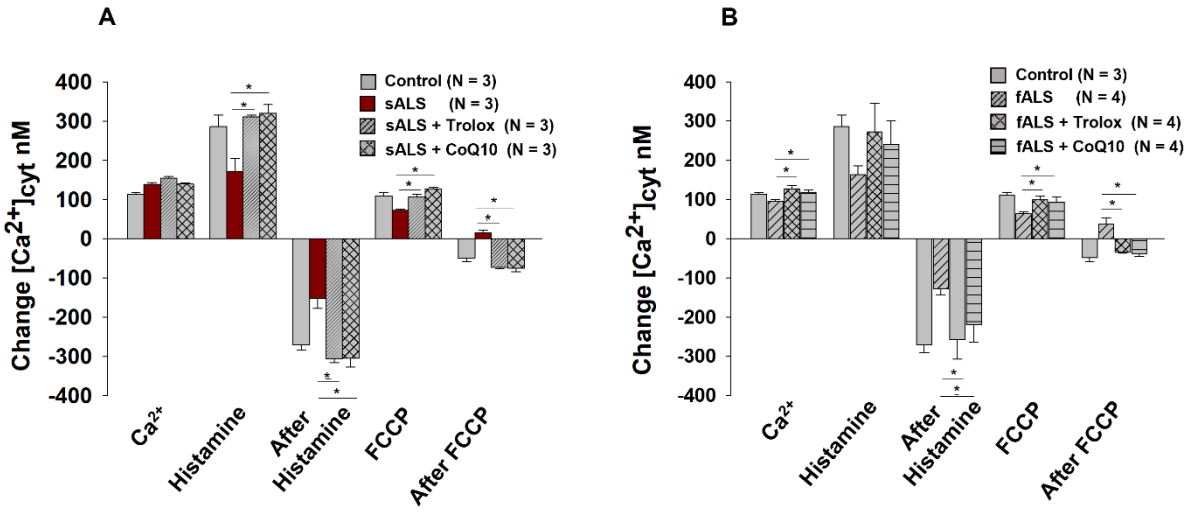

Figure 2. Modulation of cytosolic $[Ca^{2+}]_{cyt}$ in ALS fibroblasts by pretreatment with antioxidants. A. Healthy controls; untreated and treated with Trolox and CoQ10 sALS fibroblasts. **B**. Healthy controls; untreated and treated with Trolox and CoQ10 fALS fibroblasts. Data are means ± SD (*N*= individuals). * $p < 0.02$.

4. Discussion

To explore the nuances of the mechanisms of Ca^{2+} regulation associated with sporadic and familial ALS we performed the stress analysis of skin fibroblasts obtained from the ALS patients. The rationale behind the evaluation of cellular mechanisms under the induced Ca^{2+} stress lies in our earlier studies of skeletal muscle mitochondria isolated from the wild type and transgenic mice with Huntington's disease (Gizatullina et al 2006). In that study, only the condition of an elevated cytosolic Ca^{2+} enabled us to distinguish otherwise hidden metabolic differences between the pathologic and healthy samples. Using the same approach, in the current study, the dynamics of $[Ca^{2+}]_{cyt}$ changes were evaluated fluorimetrically using the stimulation of cells with histamine to provoke high cytosolic Ca^{2+} and then uncoupling of mitochondria.

To mimic the physiological extracellular concentration of Ca^{2+}, suspensions of fibroblasts were preincubated with 2 mM Ca^{2+} (Peacock 2010; Carafoli, Krebs 2016) prior to challenging with the stressors. The mechanisms involved in elevation of cytosolic Ca^{2+} consist of influx through the plasma membrane transporters and voltage-dependent ion channels, and of Ca^{2+} release from intracellular stores, with the endoplasmic reticulum being the major Ca^{2+} harbor. $[Ca^{2+}]_{cyt}$ is controlled by the buffering activity of cytosolic Ca^{2+} binding proteins and by its uptake/release from mitochondria. The sequestration of Ca^{2+} by mitochondria stimulates the citric acid cycle enhancing ATP production for cellular needs, including the ATP-driven Ca^{2+} pumps of endoplasmic reticulum and plasma membrane for removal of excess Ca^{2+} from the cytosol. Retaining the accumulated Ca^{2+} in mitochondria is as important as its sequestration and depends on the mitochondrial membrane potential and ΔpH, i.e., on the protonmotive force. Under heavy depolarization of mitochondria, the mtPTP is activated facilitating leakage of Ca^{2+} from the matrix to the cytosol. In our experimental setting this was the case of Ca^{2+} overloaded ALS fibroblasts

with a resultant sustained mitochondrial energetic depression and inability to hold Ca^{2+} in the matrix.

Ca^{2+} related parameters in ALS fibroblasts were different from those in healthy cells. Histamine-induced transport of Ca^{2+} into the ALS cells was 30-45 % weaker than in healthy cells. The post-stimulation decrease of $[Ca^{2+}]_{cyt}$ in both ALS subtypes was not efficient either, but nevertheless, sALS cells eliminated $[Ca^{2+}]_{cyt}$ somehow better than fALS cells (Figure 1D, After Histamine). These data indicate mis-regulated mechanisms of Ca^{2+} homeostasis in ALS pathogenesis, one of which is the perturbed metabolism of mitochondria.

The experimental setup used in this work — uncoupling of mitochondria under elevated cytosolic Ca^{2+} — is an approach for indirect qualitative assessment of the ability of mitochondria to accumulate and retain calcium in the matrix. A reduced calcium retention capacity was observed in ALS fibroblasts which upon addition of FCCP release lower amounts of Ca^{2+} than healthy cells (Figure 1D, FCCP). Moreover, the mitochondria of both ALS subtypes were not able to sequestrate back the elevated free Ca^{2+} which kept spontaneously releasing during the course of measurement. Our observations agree with a study that revealed a reduced mitochondrial polarization in fALS and sALS fibroblasts (Walczak et al 2019).

To eliminate the oxidative stress mediated by malfunctional mitochondria, the ALS fibroblasts were treated with Trolox and CoQ_{10} before evaluating the Ca^{2+} signaling. Both compounds were equally effective against the sporadic and familial ALS cells, almost completely reverting the dynamics of $[Ca^{2+}]_{cyt}$ modulations to the healthy control background. These data agree with our previous finding of the recovery of mitochondrial functions in sALS and fALS skin fibroblasts by antioxidants (Debska-Vielhaber et al 2021).

Conclusion

We demonstrated the dysregulated mechanisms of cytosolic Ca^{2+} control and mitochondrial calcium retention capacity in ALS skin fibroblasts. The observed impairments were rescued by antioxidants that could be considered a promising therapeutic approach to treat the ALS disorder (Chaturvedi, Beal 2008; Babahajian et al 2019; Debska-Vielhaber et al 2021).

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