A novel mitochondrial peptide causes recovery of skeletal muscle after burn trauma as assessed with P31 NMR and Electron Paramagnetic Resonance in vivo

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Introduction: Severe burn injury causes a major systemic catabolic response characterized by mitochondrial dysfunction in skeletal muscle, heart, and liver (1). Burn-induced mitochondrial dysfunction is associated with increased mitochondrial reactive oxygen species (ROS) and decreased oxidative phosphorylation (2,3). Here, for the first time, we show that a novel cell-permeable, mitochondria-targeted peptide (Szeto-Schiller, SS-31) (4), can reduce burn-induced mitochondrial oxidative stress and improve oxidative phosphorylation. Specifically, we report the effects of peptide SS-31 in skeletal muscle in a mouse burn model using measurements with nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR). Often, EPR is complementary to NMR (5). The significance of our findings includes the non-invasive nature of the NMR and EPR measurements, which can serve to monitor the effectiveness of mitochondrial protective agents in burn injury.

Material and Methods: Male 6-week-old CD1 mice (20-25 g) were anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital sodium and the left hind limb of all mice was shaved. Burn injury was inflicted by a nonlethal scald injury of 3-5% total body surface area by immersing the left hind limb in 90°C water for 3 sec (6). **NMR**: Mice were randomized into burn (B), burn+SS-31 (B+P), control (C) and control+SS-31 (C+P) groups. SS-31 (3 mg/kg) was injected intaperitoneally at 30 min before burn and immediately after burn. NMR experiments were performed in a horizontal bore magnet (proton frequency 400 MHz, 21 cm diameter, Magnex Scientific) using a Bruker Avance console. A 90° pulse was optimized for detection of phosphorus spectra (repetition time 2 s, 400 averages, 4K data points). Saturation 90°-selective pulse trains (duration 36.534 ms, bandwidth 75 Hz) followed by crushing gradients were used to saturate the γ-ATP peak. The same saturation pulse train was also applied downfield of the inorganic phosphate (Pi) resonance, symmetrically to the γ-ATP resonance. T_1 relaxation times of Pi and phosphocreatine (PCr) were measured using an inversion recovery pulse sequence in the presence of γ-ATP saturation. An adiabatic pulse (400 scans, sweep with 10 KHz, 4K data) was used to invert Pi and PCr, with an inversion time between 152 ms and 7651 ms. **EPR:** Mice were randomized into burn, burn + SS-31 and control groups. SS31 (3 mg/kg) was injected intraperitoneally at 0, 3, 6, 24, and 48 hours post-burn. EPR measurements were carried out with a 1.2 GHz EPR spectrometer equipped with a microwave bridge and external loop resonator specially designed for *in vivo* experiments. Typical spectrometer parameters were: incident microwave power, 10 mW; magnetic field center, 400 gauss; modulation frequency, 27 kHz. The decay kinetics of the intravenously-injected 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxyl nitroxide (CPA, 150 mg/kg) over time was used to assess mitochondrial redox status of the muscle.

Results: As shown in Table 1 and illustrated in Fig. 1: ATP synthesis rate ($P_i \rightarrow \gamma ATP$) at 6 hours after burn was significantly reduced in burned (B) mice; and SS-31 treatment resulted in significantly increased ATP synthesis rate in both control (C+P) and burned (B+P) mice. Importantly, ATP synthesis rate was significantly increased in burned mice injected with the SS-31 (B+P), as compared to burned alone mice (B) (P=0.0001). Moreover, when the ATP synthesis rate (reaction PCr → γATP) was compared in burned mice and mice injected with SS-31 the increase was statistically significant (P=0.006). According to EPR, a significant decrease in the redox status of burn and burn + SS-31 groups as compared to control was detected ($p<0.05$); also, a significant increase (recovery) in the redox status of burn + peptide group as compared to burn alone was observed ($p<0.05$), (Fig.2).

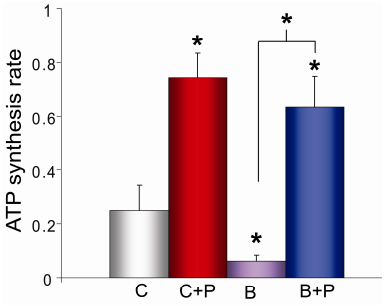

Figure 1. ATP synthesis rate (μmol/g/s) in control (C), control+SS-31 peptide (C+P), burned (B), and burned+SS-31 peptide (B+P) by ^{31}P NMR at 6 hours after burn (* $P<0.05$).

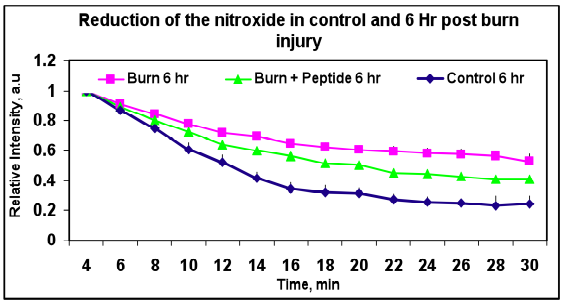

Figure 2. Mitochondrial redox status. Decay kinetics of the nitroxide (CPA) of the gastrocnemius muscle in control (n=6), burn (n=6), and burn+peptide SS-31 (n=9) mice.

Discussion: Our *in vivo* NMR findings were cross-validated by EPR measurements. Our results suggest that SS-31 enhances ATP synthesis rate possibly via recovery of the mitochondrial redox status and/or down-regulation of the expression of mitochondrial uncoupling protein 3 and its upstream key metabolic regulator peroxisome proliferator activated receptor-gamma coactivator-1beta, both shown to be dysregulated as early as 6 hours after burn (2). Thus, our results suggest that the mitochondrial dysfunction caused by burn injury is significantly reduced with the administration of SS-31, a novel mitoprotective and antioxidant peptide. Administration of SS-31 increased ATP synthesis rate substantially even in control healthy mice (Fig 1, C vs C+P). The direct measurement of tissue parameters such as redox status by EPR may be used to complement measurements by NMR *in vivo* in order to assess tissue dysfunction and the therapeutic effectiveness of mitochondrial protective agents in severe burn trauma. Our multi-modality NMR/EPR approach advances the development of novel non-destructive therapeutic approaches in murine models of mitochondrial pathologies, provides biomarkers for investigation of mitochondrial paradigms, and thus may contribute to novel therapeutic development.

Table 1. Results of *in vivo* ^{31}P-NMR saturation transfer experiments. ATP synthesis rate (reaction $P_i \rightarrow \gamma ATP$)

	Healthy Controls (n =5)	Controls + Peptide SS-31 (n =5)	Burn (n =8)	Burn + Peptide SS-31 (n = 8)
$\Delta M/M_0$	0.24 ± 0.05	0.15 ± 0.02 (P=0.097)	0.23 ± 0.05 (P=0.902)	0.31 ± 0.06 (P=0.488)
T1obs (s)	1.16 ± 0.14	1.16 ± 0.14	1.33 ± 0.27	1.33 ± 0.27
Pi (μmol/g)	1.01 ± 0.28	5.49 ± 0.28 (P=0.0008)	0.34 ± 0.25 (P=0.006)	2.93± 0.56 (P=0.035)
ATP synthesis rate (μmol/g/s)	0.25 ± 0.09	0.74 ± 0.09 (P=0.008)	0.06 ± 0.02 (P=0.026)	0.63 ± 0.11 (P= 0.046)

Values are means ± SE; $\Delta M/M_0$ is the fractional change in P_i magnetization as a result of saturation transfer; T1obs is the observed spin lattice relaxation time of P_i during γATP saturation in seconds; ATP synthesis is calculated as [Pi] × k; [Pi] is the concentration of Pi extrapolated from the baseline NMR spectrum, comparing Pi and ©ATP peaks and ATP concentration measured with bioluminescence assay; k is calculated as (1/ T1obs) x ($\Delta M/M_0$); P-values (Student's t-test).

References

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