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Calcium dependence of damage to mouse motor nerve terminals following oxygen/glucose deprivation

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Abstract

Motor nerve terminals are especially sensitive to an ischemia/reperfusion stress. We applied an in vitro model of this stress, oxygen/glucose deprivation (OGD), to mouse neuromuscular preparations to investigate how Ca^{2+} contributes to stress-induced motor terminal damage. Measurements using an ionophoretically-injected fluorescent $[\text{Ca}^{2+}]$ indicator demonstrated an increase in intra-terminal $[\text{Ca}^{2+}]$ following OGD onset. When OGD was terminated within 20–30 min of the increase in resting $[\text{Ca}^{2+}]$, these changes were sometimes reversible; in other cases $[\text{Ca}^{2+}]$ remained high and the terminal degenerated. Endplate innervation was assessed morphometrically following 22 min OGD and 120 min reoxygenation (32.5 °C). Stress-induced motor terminal degeneration was Ca^{2+} -dependent. Median post-stress endplate occupancy was only 26% when the bath contained the normal 1.8 mM Ca^{2+} , but increased to 81% when Ca^{2+} was absent. Removal of Ca^{2+} only during OGD was more protective than removal of Ca^{2+} only during reoxygenation. Post-stress endplate occupancy was partially preserved by pharmacological inhibition of various routes of Ca^{2+} entry into motor terminals, including voltage-dependent Ca^{2+} channels (ω -agatoxin-IVA, nimodipine) and the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (KB-R7943). Inhibition of a Ca^{2+} -dependent protease with calpain inhibitor VI was also protective. These results suggest that most of the OGD-induced motor terminal damage is Ca^{2+} -dependent, and that inhibition of Ca^{2+} entry or Ca^{2+} -dependent proteolysis can reduce this damage. There was no significant difference between the response of wild-type and presymptomatic superoxide dismutase 1 G93A mutant terminals to OGD, or in their response to the protective effect of the tested drugs.

Keywords

Motor nerve terminal; Oxygen/glucose deprivation; Hypoxia; Calcium; Muscle endplate; Calpain; $\text{Na}^+/\text{Ca}^{2+}$ exchanger; Superoxide dismutase 1; SOD1-G93A; Mouse

Introduction

Motor axons and motor nerve terminals can be subjected to transient partial ischemia during intense (anaerobic) muscle activity (Sadamoto et al., 1983). They can also experience an

ischemia/reperfusion (I/R) stress following temporary interruption of limb blood supply due to tourniquets applied to produce a bloodless field for hand or foot surgery (reviewed by Horlocker et al., 2006; Wakai et al., 2001). Studies in rodent hind limbs demonstrate that motor nerve terminals are especially vulnerable to I/R stress. For example, in rat hind limbs subjected to 2 h of tourniquet ischemia and then examined 1–28 days later, electron microscopic analysis detected no changes in axons, Schwann cells or muscle fibers, but did detect multiple signs of degeneration in motor nerve terminals, including disruption of the presynaptic membrane, the appearance of vacuoles, and degeneration of mitochondria (Makitie and Teravainen, 1977; Tombol et al., 2002). Functional measurements also indicate that the neuromuscular junction is a major site of I/R injury (Eastlack et al., 2004). Baxter et al. (2008) found that mouse motor nerve terminals are also selectively vulnerable to hypoxia/reoxygenation, an in vitro model of I/R stress.

Motor terminal degeneration following these stresses occurs rapidly, within 2 h following both the tourniquet-induced I/R stress studied by David et al. (2007) and the hypoxia/reoxygenation stress studied by Baxter et al. (2008). Studies in the central nervous system and myelinated axons indicate that rapid stress-induced cell damage usually occurs via necrotic/excitotoxic mechanisms, mediated at least in part by elevations of cytosolic $[Ca^{2+}]$ (reviewed by Arundine and Tymianski, 2003; Kristián and Siesjö, 1998; Stys, 2004). This mechanism might help explain why motor terminals are especially vulnerable to I/R stress, since they are small structures with a high density of depolarization-activated Ca^{2+} channels. The present study tested whether motor terminal damage produced by oxygen/glucose deprivation (OGD), an in vitro model of ischemia, is indeed Ca^{2+} -dependent. We measured elevations in $[Ca^{2+}]$ produced within motor terminals by OGD, and present evidence that during OGD the more damaging condition is lack of oxygen (rather than lack of glucose).

If elevation of cytosolic $[Ca^{2+}]$ contributes importantly to OGD-induced motor terminal degeneration, then treatments that reduce Ca^{2+} influx into the terminal would be predicted to reduce this degeneration. Consistent with this prediction, we report that OGD-induced denervation of motor endplates is reduced by removing bath Ca^{2+} , by inhibiting P/Q-type Ca^{2+} channels, and by inhibiting the plasma membrane Na^+/Ca^{2+} exchanger, which can mediate Ca^{2+} influx in depolarized cells (Kiedrowski, 2007; Petrescu et al., 2007). Endplate denervation was also reduced by an inhibitor of the $Na^+, K^+, 2Cl^-$ co-transporter (NKCC1) and by an inhibitor of calpains, a family of Ca^{2+} -dependent proteases. These results suggest that Ca^{2+} influx and Ca^{2+} -activated proteolysis are major contributors to OGD-induced damage in motor terminals.

Another goal of the present study was to test whether OGD-induced endplate denervation would be greater in mice that express a mutation of human superoxide dismutase 1 (SOD1-G93A) than in wild-type (wt) mice. SOD1-G93A mice are an animal model of a familial form of the motor neuron degenerative disease amyotrophic lateral sclerosis (Chiu et al., 1995; Gurney et al., 1994). David et al. (2007) found that motor terminals innervating fast muscles in presymptomatic SOD1-G93A mice were more sensitive to in vivo I/R injury than wt terminals. However, for the in vitro OGD/reoxygenation stress studied here, presymptomatic SOD1-G93A terminals did not differ significantly from wt terminals in either the stress-induced degeneration or its mitigation by the tested drugs.

Materials and methods

Preparations

Experiments used mice maintained in the C57BL/6 background for at least 10 generations. Some of these mice expressed human SOD1-G93A (bred from founders purchased from Jackson Labs, Bar Harbor, ME; B6.Cg-TgN(SOD1-G93A)1Gur/J, stock #4435). These

SOD1-G93A mice developed disease ~60 days later than the founder mice, suggesting a reduced copy number of the mutant SOD1 gene (Acevedo-Arozena et al., 2011) and/or protective genes in this mouse background (Heiman-Patterson et al., 2011). Wt mice ranged from 45–235 days old; mutant SOD1 mice were used at presymptomatic ages (43–132 days) at which non-stressed muscle endplates were still fully innervated. To facilitate identification of motor terminals in experiments involving morphological assays of endplate occupancy, both mutant SOD1 and wt mice were crossed with mice that express yellow fluorescent protein (YFP) in many neurons (including motor neurons), but not in muscle or Schwann cells (B6.Cg-Tg(Thy1-YFP)16Jrs/J, Jackson Labs, Bar Harbor ME, stock #3709). Tail clips obtained between postnatal days 18–20 were used to detect YFP expression (in sensory axons) by fluorescence microscopy, and expression of human SOD1 by PCR (as described in Vila et al., 2003).

Mice were euthanized with 100% CO₂. Dissected muscle preparations were bathed in physiological saline containing (in mM) NaCl 137, NaHCO₃ 15, KCl 4, CaCl₂ 1.8, MgCl₂ 1.1, glucose 11.2 and NaH₂PO₄ 0.33, aerated with 95% O₂/5% CO₂. pH was maintained at ~7.4.

Measurements of intra-terminal [Ca²⁺] and mitochondrial membrane potential (Ψ_m)

These measurements used levator auris longus (LAL) neuromuscular preparations (rostral band). This muscle (which adducts the ears) is very thin, permitting good visualization of motor axons and terminals (Angaut-Petit et al., 1987). LAL is composed primarily of fast muscle fibers (Erzen et al., 2000), whose innervation tends to be more susceptible to stresses than that of slow muscle fibers (Gordon et al., 2004; Hegedus et al., 2007). Muscles were pinned flat in a chamber with silicon walls built atop a microscope slide, and perfused with physiological saline maintained at 35–37 °C, monitored with a thermistor. As described in David and Barrett (2000), the membrane-impermeable hexapotassium salt form of a fluorescent Ca²⁺ indicator, Oregon green 488 BAPTA1 (OG-1), was injected ionophoretically into the internodal region of a motor axon, and allowed to diffuse into motor terminals. This high-affinity indicator (K_d 0.2–0.8 μ M) is well suited to detect small elevations of cytosolic [Ca²⁺] above the resting level.

Action potentials were evoked in the motor axon (usually 50 Hz for 20 s) by passing brief, suprathreshold depolarizing pulses via a suction electrode. Muscle contractions were blocked using d-tubocurarine (15 μ M), an inhibitor of muscle nicotinic acetylcholine receptors.

Following measurements of fluorescence at rest and during stimulation in physiological saline, an OGD stress was initiated by bathing the preparation with saline lacking glucose, equilibrated with a gas mixture in which N₂ replaced O₂. This gas mixture was also blown through an open dome above the experimental chamber. In some cases fluorescence (background-subtracted) was normalized to that measured in the resting terminal prior to application of the OGD stress (F/F_{control}). Following measurements of OGD-induced changes in resting and stimulated fluorescence, the stress was terminated by restoring O₂ and glucose. In Fig. 3 glucose and O₂ deprivation were applied singly instead of together.

Measurements of stress-induced changes in Ψ_m used rhodamine (Rh)-123, a potentiometric fluorescent indicator that is readily sequestered by viable mitochondria, using techniques described in Nguyen et al. (2011). Ψ_m depolarization causes Rh-123 to leak out of the mitochondrial matrix into the cytosol and become unquenched, producing an increase in fluorescence (reviewed by Nicholls and Ward, 2000).

Imaging used a Nikon TE2000E microscope (Nikon, Melville, NY) and a Retiga EXi camera (Q Imaging, Surrey, British Columbia, Canada). Both OG-1 and Rh-123 were excited at 488 nm, and emitted light was filtered with a 535 nm band pass filter (40 nm bandwidth, Chroma, Rockingham, VT). Excitation light was delivered using a monochromator (Delta Ram V, Photon Technology International, Birmingham, NJ). Photobleaching was minimized by using minimal intensities of excitation light and exposing the preparation to light for only 50 s, with a rest period of ~10 min between subsequent measurements. Net fluorescence (F_{net}) was calculated by subtracting background fluorescence from the total fluorescence of a region of interest encompassing a motor nerve terminal ($F_{\text{net}} = F_{\text{total}} - F_{\text{background}}$). Images were acquired using IPLAB (v. 3.61, Scanalytics Inc, Fairfax, VA) and analyzed using ImageJ software (Abramoff et al., 2004).

Measurement of OGD-induced decrease in endplate innervation

These experiments used extensor digitorum longus (EDL) muscles from mice whose motor neurons expressed YFP. This predominantly fast muscle (Luedeke et al., 2004) is one of the first to become denervated in SOD1-G93A mice (David et al., 2007; Schaefer et al., 2005). Even at the early, presymptomatic ages studied here, EDL motor terminals in SOD1-G93A mice are more vulnerable to a tourniquet-induced I/R stress than those in wt EDL or in slow muscle fibers (soleus) from SOD1-G93A mice (David et al., 2007). EDLs were dissected from the right and left hind limbs such that the proximal regions of the 4 heads of EDL remained attached, whereas the distal tendons were separate and free-floating. Right and left EDLs were placed in separate adjoining chambers containing the physiological saline solution described above, bubbled with 95% O₂/5% CO₂. The temperature of the water bath surrounding these chambers was maintained at 32.5±1 °C, the highest temperature at which endplate innervation was reliably maintained for >3 h under optimal conditions (physiological saline and O₂). After 30 min muscles were transferred to chambers containing the experimental solutions. OGD was achieved using glucose-free saline bubbled with 95% N₂/5% CO₂; bath pO₂ in muscle-containing chambers fell to <1 torr in <2 min. The OGD stress lasted 22 min, a duration that consistently reduced endplate occupancy (normally >98%) by at least 40% (see Results). One EDL of each pair experienced this standard OGD stress (stressed control); the other EDL experienced the OGD stress in saline lacking Ca²⁺ or in saline containing a test drug (stressed test). In experiments involving removal of bath Ca²⁺, the Ca buffer BAPTA (2 mM) was added to saline containing no added Ca²⁺. Muscles were preincubated for 5–10 min in glucose-containing low [Ca²⁺] saline (oxygenated) prior to initiating the OGD stress. In experiments testing drugs the muscle was pre-incubated in the test drug for 25 min prior to the onset of OGD. Following the OGD stress, glucose and O₂ were restored, and muscles were incubated for a further 120 min prior to fixation in 4% paraformaldehyde for 60 min.

Endplate acetylcholine receptors were labeled with 25 µg/ml α-bungarotoxin (α-BgTx, Alexa Fluor 594 conjugate, Invitrogen, Carlsbad, CA) for 60 min, then rinsed with physiological saline for 40 min. Each EDL muscle was divided into 4–5 longitudinal segments and mounted between #1 glass coverslips (Thomas Scientific, Swedesboro, NJ) with hard mounting medium (Vectashield, Vector Laboratories, Burlingame, CA). Images were acquired with a confocal system using a 60× water immersion lens (NA 1.2, Olympus, Melville, NY) and a 4× lens (NA 0.2, Nikon). YFP and α-BgTx Alexa Fluor 594 conjugate were excited at 488 or 561 nm, respectively (argon 488 laser, Laser Physics, West Jordan, UT; diode-pulsed 561 laser, Melles-Griot, Albuquerque, NM), with emissions monitored at 535±40 nm for YFP and at >590 nm for α-BgTx. The investigator set the upper and lower z-axis limits using the red (α-BgTx) fluorescence, and then collected image pairs (α-BgTx, YFP) between these limits with a z-separation of 1 µm, using IPLAB scripts. Data were analyzed using a macro (written in ImageJ, available upon request) that for each field

displayed side-by-side image stacks of α -BgTx fluorescence (red), YFP fluorescence (green) and the merged image (in which innervated endplates appeared yellow/orange, see Fig. 4). Using a slider at the bottom of this composite stack, the investigator then advanced through the depth of the composite stack, scoring the occupancy of each endplate encountered. An endplate was considered to be occupied (innervated) if the YFP-labeled motor terminal (1) occupied $\geq 80\%$ of the corresponding α -BgTx-labeled endplate and (2) remained connected with its pre-terminal axon. A minimum of 200 α -BgTx-labeled endplates were imaged for each muscle, including at least 5 muscle fields ($4\times$ magnification) for each of the 4 heads of EDL. Endplate occupancy measurements from stressed control and stressed test EDLs were compared using the Wilcoxon signed rank, one-tailed test. Experiments were excluded from analysis in those rare instances in which (a) the stress denervated all endplates (i.e. neither of the paired EDL muscles had an endplate occupancy of $\geq 10\%$), or (b) the stress produced little damage (i.e. endplate occupancy $>70\%$ in normal $[Ca^{2+}]$ with no protective drug).

Reagents

Reagents and their sources include: α -BgTx Alexa Fluor 594 conjugate and OG-1 (Invitrogen, Carlsbad, CA); nimodipine and calpain inhibitor VI [N-(4-fluorophenylsulphonyl)-L-valyl-L-leucinal] (Calbiochem/EMD Chemicals, Gibbstown, NJ); KB-R7943 mesylate [(2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourethane methane sulfonate)] (Tocris Bioscience, Ellisville, MO); ω -agatoxin IVA (Alamone Labs, Jerusalem, Israel). All other reagents came from Sigma-Aldrich (St. Louis, MO).

Results

Intra-terminal $[Ca^{2+}]$ increases during OGD

Fig. 1A illustrates results of an experiment investigating the time course of OGD-induced increases in intra-terminal $[Ca^{2+}]$, monitored as increases in the fluorescence of intra-axonally injected OG-1. The fluorescence micrographs show the branches of a single injected axon connecting to 5 motor terminals (labeled a–e). The fluorescence of these terminals was measured under resting (control) conditions and then following 45 min of an OGD stress, which increased $[Ca^{2+}]$ throughout the axon and its terminals. Upon reoxygenation the axonal branches and terminals developed a beaded appearance and then disintegrated. Traces in Fig. 1B show the time course of OGD-induced fluorescence changes for these 5 terminals (normalized to control fluorescence, F/F_{control}). In each terminal intra-terminal $[Ca^{2+}]$ began to rise rapidly after an interval ranging from ~ 30 – 40 min (range 25–55 min in the population of sampled terminals). At least part of this interval was likely due to a delay in reducing bath pO_2 (see Fig. 1 legend). The solid line in Fig. 1C plots the time course of the OGD-induced fluorescence increase averaged over 12 terminals from 4 wt mice. The dashed line shows that OGD-induced fluorescence increases averaged from 4 terminals from presymptomatic SOD1-G93A mice had a similar time course. The variable onset, delayed increase in intra-terminal $[Ca^{2+}]$ during OGD is consistent with the variable delay of the hypoxia-induced increase in miniature endplate potential frequency in mouse neuromuscular junctions (Nishimura, 1986).

The OGD stress was terminated 20–40 min following the elevation of resting $[Ca^{2+}]$. If the duration of the high-cytosolic- $[Ca^{2+}]$ phase did not exceed 20–30 min, OGD-induced changes were sometimes at least partially reversible upon reoxygenation. Fig. 2A illustrates a wt terminal in which resting $[Ca^{2+}]$ (monitored as F/F_{control}) increased during OGD but recovered following oxygenation. Fig. 2B shows the increases in $[Ca^{2+}]$ produced in this terminal by 50 Hz stimulation administered to the motor nerve, superimposing responses recorded at the times indicated by the arrows in A. These responses are plotted as F/F_{rest} .

where F_{rest} is the fluorescence recorded just prior to the onset of stimulation. Stimulation produced a clear increase in F/F_{rest} prior to stress onset (control). No stimulation-induced increase was detected during the OGD-induced increase in resting cytosolic $[Ca^{2+}]$, but the response returned following reoxygenation. The failure of stimulation to produce a detectable increase in fluorescence when resting $[Ca^{2+}]$ was increased during OGD has at least two likely causes: hypoxia-induced failure of nerve conduction (as documented by Hubbard and Loynning, 1966), and saturation of the high-affinity indicator OG-1.

In other cases terminals exposed to OGD disintegrated following restoration of the normal, oxygenated control solution, as illustrated for wt terminals in Fig. 1A, and for an SOD1-G93A terminal in Figs. 2C, D. In the terminal of Fig. 2C resting $[Ca^{2+}]$ continued to rise during reoxygenation, and the stimulation-induced $[Ca^{2+}]$ increase did not reappear following reoxygenation (Fig. 2D). All terminals innervated by a particular axon shared the same fate: either all recovered, or all disintegrated following reoxygenation.

These results indicate that some motor axons and terminals can survive a 20–30 min episode of OGD-induced elevation of intracellular $[Ca^{2+}]$. Using this assay we detected no difference in survival between wt and mutant SOD1 terminals, but our sample size of OG-1-injected axons was small due to the difficulty of the dye-ionophoresis technique. Interestingly, terminals that disintegrated did so during reoxygenation rather than during OGD, consistent with reports that oxidative stress contributes importantly to neurodegeneration following an I/R stress (Jenkins et al., 1981; reviewed by Saito et al., 2005).

OGD is an in vitro model of ischemia, involving withdrawal of both glucose and O_2 . Figs. 3A, B show results of an experiment in which these deprivations were applied separately and sequentially, to test which deprivation is the more important determinant of cytosolic $[Ca^{2+}]$ dysregulation. Glucose deprivation lasting 2 h had no significant effect on either resting $[Ca^{2+}]$ or the stimulation-induced increase in $[Ca^{2+}]$. In contrast, O_2 deprivation reproduced both effects of OGD: elevation of resting $[Ca^{2+}]$ and abolition of the stimulation-induced increase in $[Ca^{2+}]$. Fig. 3C shows results of a similar experiment testing the effects of separate glucose and O_2 deprivation on Ψ_m in a motor terminal, monitored as changes in fluorescence of Rh-123. Glucose deprivation had no effect, but O_2 deprivation increased Rh-123 fluorescence, signaling Ψ_m depolarization. A separate experiment in which O_2 deprivation preceded glucose deprivation (not shown) confirmed the finding that (at least under these experimental conditions) the effects produced by O_2 deprivation were more severe than those produced by glucose deprivation. This finding is consistent with reports that synaptosomes derive their energy primarily via oxidative metabolism (and therefore would be severely affected by O_2 deprivation, Ereci ska et al., 1996), and that intracellular glycogen stores are an important source of ATP in peripheral nerves (permitting temporary survival during glucose deprivation, Stewart et al., 1965).

Removal of bath Ca^{2+} protects motor terminals from OGD-induced degeneration

Since a prolonged increase in cytosolic $[Ca^{2+}]$ can damage cells, we tested whether omission of Ca^{2+} from the bathing medium would preserve motor terminals during an OGD stress. Fig. 4 shows pseudocolor micrographs of EDL muscles in which endplates were labeled with α -BgTx (red, left column) and motor axons and terminals were labeled by transgenic expression of YFP (green, middle column). In the overlay images (right column), innervated endplates appear yellow-orange and denervated endplates are red. The upper row illustrates a field from an EDL muscle maintained for 2.5 h in physiological saline at 32.5 °C (no stress), showing maintenance of intact axons and innervated endplates. The middle and lower rows show fields from a mouse in which one EDL was subjected to an OGD stress (22 min OGD followed by 120 min reoxygenation) in the normal 1.8 mM bath Ca^{2+} (middle

row), and the contralateral EDL was subjected to the same OGD stress in saline with no added Ca^{2+} (lower row). In both stressed EDLs the α -BgTx-labeled endplates appeared normal. In OGD with normal $[\text{Ca}^{2+}]$ there was extensive damage, not only to motor terminals but also to motor axons, many of which appeared discontinuous and beaded. There was much less damage following OGD in low $[\text{Ca}^{2+}]$ medium; most axons remained intact and most endplates remained innervated. Results summarized in Fig. 5A show that removing Ca^{2+} from the bathing solution (along with addition of 2 mM BAPTA and 1.8 mM Mg^{2+}) during OGD and reoxygenation yielded a median endplate occupancy of 81%, compared to only 24% for EDLs exposed to Ca^{2+} throughout ($n=10$ pairs, $p<0.001$, Wilcoxon signed rank one-tailed test).

To test whether the Ca^{2+} -dependent damage occurred mainly during OGD or during the following reoxygenation, additional experiments were performed in which one EDL received the standard OGD stress in normal bath $[\text{Ca}^{2+}]$, and the contralateral EDL had Ca^{2+} removed either only during the OGD stress or only during the post- OGD reoxygenation. As summarized in Fig. 5B, removal of Ca^{2+} only during OGD was protective, resulting in a median endplate occupancy of 80%. Removal of Ca^{2+} only during the reoxygenation stage yielded a median endplate occupancy of only 26% (Fig. 5C). These findings suggest that the vulnerability of synapses to Ca^{2+} overload in vitro occurs mainly during the OGD stress itself. However, since removing Ca^{2+} from the bathing solution requires time, we cannot rule out the possibility that significant Ca^{2+} -induced damage also occurred during a brief interval at the onset of reoxygenation.

An alternative explanation for the results in Fig. 5 is that OGD irreparably damages most terminals whether or not Ca^{2+} is present, and the absence of Ca^{2+} just delays their inevitable degeneration. This possibility was tested in an experiment in which paired EDLs both underwent the standard OGD/reoxygenation protocol in low $[\text{Ca}^{2+}]$, after which one EDL remained in low $[\text{Ca}^{2+}]$ for an additional 2 h, while the other EDL spent the additional 2 h in 1.8 mM Ca^{2+} . If most OGD-exposed terminals are fated to degenerate once exposed to bath Ca^{2+} , then the terminals exposed to Ca^{2+} for the final 2 h interval would be expected to exhibit more degeneration. However, endplate occupancies in these two conditions were not significantly different (97% for EDLs kept throughout in low $[\text{Ca}^{2+}]$, 89% for EDLs exposed to Ca^{2+} during the final 2 h, $n=2$ experiments), suggesting that lack of Ca^{2+} during the stress does indeed preserve motor terminals rather than simply postpone their stress-induced degeneration.

The paired data of Figs. 5A–C include results from both wt (YFP-only, solid lines) and presymptomatic SOD1-G93A mice (dashed lines). Visual inspection suggests substantial overlap between results from these two groups. Separate analysis of these two groups, summing together data from Fig. 5A and no-drug data from Fig. 6 (see below), revealed no significant difference between wt and mutant SOD1 mice in the degree of motor terminal damage produced by the OGD stress (Ca^{2+} present throughout; median post-stress endplate occupancies 26% for wt, $n=31$; 25% for G93A, $n=21$). Thus data from comparably-treated wt and SOD1-G93A EDLs were averaged together.

Inhibition of Ca^{2+} entry and Ca^{2+} -activated protease reduce OGD-induced motor terminal degeneration

Since the magnitude of OGD-induced terminal degeneration is Ca^{2+} -dependent, one would predict that agents that reduce Ca^{2+} entry into the terminal would increase post-OGD endplate occupancy. In the following experiments paired EDLs each experienced the standard OGD/reoxygenation protocol in normal $[\text{Ca}^{2+}]$, with one member of each pair exposed throughout to the test drug. The diagram in Fig. 6A indicates the likely mechanism of action of each tested drug.

The major voltage-dependent Ca^{2+} channel type in mouse motor terminals is P/Q (Cav2.1, Westenbroek et al., 1998). L-type Ca^{2+} channels (Cav1.2) can also contribute to Ca^{2+} entry into mouse motor terminals (Urbano et al., 2002) and into optic nerve axons during anoxia (Brown et al., 2001). Inhibition of P/Q-type and L-type channels reduced depolarization-induced death of cultured embryonic mouse motor neurons (Gou-Fabregas et al., 2009). Figs. 6B, C show that both ω -agatoxin IVA (1 μM , a P/Q-type channel inhibitor, Teramoto et al., 1993), and nimodipine (10 μM , an L-type channel inhibitor) increased post-OGD endplate occupancy.

Under stress conditions, Ca^{2+} entry into neurons can also be mediated by reverse operation of the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Reverse operation of this exchanger is a major contributor to Ca^{2+} overload and damage in central axon tracts during ischemic/anoxic events (Li et al., 2000; Stys et al., 1992). Fig. 6D shows that KB-R7943 (10 μM), which blocks operation of this $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Elias et al., 2001; Iwamoto et al., 2007) increased post-stress endplate occupancy.

Ca^{2+} entry via reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger would be favored by any mechanism that increases intracellular $[\text{Na}^+]$. Spinal cord motor neurons express an $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC1, Chabwine et al., 2009; Gonzalez-Islas et al., 2009; Price et al., 2006), which can contribute to excessive Na^+ influx under pathophysiological conditions (Beck et al., 2003; Lenart et al., 2004), resulting in cellular damage via several mechanisms (Kintner et al., 2007; Xie et al., 1994). Blocking this transporter with bumetanide reduced brain edema and infarction following an I/R stress in rats (Chen and Sun, 2005; Yan et al., 2001). Bumetanide (10 μM) produced a modest increase in post-stress endplate occupancy (Fig. 6E).

Calpains, a family of Ca^{2+} -dependent cysteine proteases, are rapidly activated in injured motor neurons (Momeni and Kanje, 2005; Momeni et al., 2007), and contribute to anoxia-induced injury in central axons (Li et al., 2000). Fig. 6F shows that calpain inhibitor VI (100 μM , Momeni and Kanje, 2005), a membrane-permeable inhibitor of μ - and m-calpains, increased endplate occupancy following the OGD/reoxygenation stress.

The finding that inhibition of various routes of Ca^{2+} entry and of a Ca^{2+} -activated protease partially protects motor terminals reinforces the evidence that accumulation of intracellular $[\text{Ca}^{2+}]$ contributes importantly to the rapid degeneration of motor terminals following an OGD/reoxygenation stress.

Discussion

We have presented evidence that application of an OGD/reoxygenation stress to motor terminals innervating fast muscle fibers increases cytosolic $[\text{Ca}^{2+}]$, due mainly to O_2 deprivation rather than to glucose deprivation. We also show that most OGD-induced degeneration of motor terminals is Ca^{2+} -dependent, and that reducing Ca^{2+} entry (via various strategies) or inhibiting Ca^{2+} -activated proteases in the calpain family can protect motor terminals from OGD-induced damage. This Ca^{2+} dependence and the rapid time course of the motor terminal degeneration suggest a necrotic death mechanism.

We detected no difference between wt and presymptomatic SOD1-G93A terminals in their response to the OGD stress. Thus the in vitro stress applied here did not reproduce the increased vulnerability of SOD1-G93A EDL terminals reported for an in vivo I/R stress by David et al. (2007). This difference might be due to aspects of the in vivo stress not duplicated by the in vitro stress, and/or to disease-delaying factors in the SOD1-G93A mice used in the present study (see Materials and methods).

The motor terminal damage reported here occurred with briefer stress durations than those reported by Baxter et al. (2008). For example, we measured significant OGD-induced motor terminal degeneration in EDL after only 22 min of OGD, compared to the 2 h durations used for the hypoxia stress applied to lumbrical, transversus abdominis and triangularis sterni muscles by Baxter et al. (2008). At least part of this discrepancy likely relates to temperature: the hypoxia stress in Baxter et al. (2008) was applied at room temperature (23–24 °C), whereas the OGD stresses used here were applied at more physiological temperatures ~30 °C. Lower temperatures reduce the degree of nervous system damage produced by an ischemia/reperfusion stress (reviewed by Ginsberg et al., 1992). We applied both O₂ and glucose deprivation, whereas Baxter et al. (2008) applied only O₂ deprivation; results in Fig. 3 suggest that O₂ deprivation was the more damaging component of the combined OGD stress, but it is possible that the combination of O₂ and glucose deprivation was more damaging than hypoxia alone. The relatively rapid onset of motor terminal degeneration in our study was *not* likely due to the use of transgenic mice whose neurons expressed YFP. Comley et al. (2011) reported that neuronal expression of YFP had either no detectable effect (on Wallerian degeneration) or a protective effect (on distal axonopathy in *wasted* mice) for the stresses they tested.

The OGD stress applied here also damaged EDL motor terminals more rapidly than the tourniquet-induced ischemic stress applied by David et al. (2007; 45 min ischemia required to denervate 50% of wt endplates). Perhaps, due to absence of blood perfusion, the inner regions of the dissected EDL muscle experienced a prolonged partial OGD stress in addition to the timed OGD stress, and thus in effect were exposed to a longer OGD stress than that experienced by EDLs in the ischemic limb. Another possibility is that the large volume of bathing medium in vitro minimized tissue-protective changes in the muscle environment that normally accompany ischemia in vivo, such as reduction in extracellular [Ca²⁺], pH acidification (Tombaugh and Sapolsky, 1990), and accumulation of released adenosine and/or AMP (Bouma et al., 2010; Pang and Forrest, 1995).

Fig. 4 shows that the OGD/reoxygenation stress damaged not only motor terminals, but also motor axons, and that this axonal damage was also Ca²⁺-dependent. Axonal damage may have resulted from influx of Ca²⁺ via voltage-dependent Ca²⁺ channels in or near nodes (David, 2008) and/or via reverse operation of axonal Na⁺/Ca²⁺ exchangers (Li et al., 2000). Some axonal damage may also have resulted from diffusion of Ca²⁺, or of damaging Ca²⁺-induced or Ca²⁺-activated agents, from motor terminals.

The main reason for the reduction in endplate occupancy following the OGD/reoxygenation stress was likely motor terminal degeneration rather than axonal retraction. This statement is supported by the time-lapse imaging reported in David et al. (2007, their Fig. 5B), in which EDLs from YFP-expressing mice like those used in the present study were exposed to limb ischemia in vivo, and then rapidly dissected and observed over time in control, oxygenated solution in vitro. In terminals that degenerated rapidly the pattern seen was always disintegration (beading followed by disappearance of YFP fluorescence) rather than axonal retraction. In the present study the various axon branches in Fig. 1A also developed a beaded pattern before their motor terminals disappeared.

All five of the drugs tested here (ω-agatoxin-IVA, nimodipine, KB-R7943, bumetanide and calpain inhibitor VI) significantly preserved EDL motor terminals following an OGD/reoxygenation stress. The stress-protective effects of both Ca²⁺ removal and the tested drugs were similar for wt and mutant SOD1 terminals. KB-R4973 and calpain inhibitor VI protected motor terminals almost as well as withdrawal of bath Ca²⁺, and have the possible therapeutic advantage of not blocking neuromuscular transmission. KB-R7943 inhibition of the plasma membrane Na⁺/Ca²⁺ exchanger was likely a major factor underlying its strong

neuroprotective effect, but a variety of additional reported actions might also have contributed (inhibition of L-type Ca^{2+} channels, Ouardouz et al., 2005; inhibition of TRPC channels, Kraft, 2007; activation of Ca^{2+} -activated K^{+} channels, Liang et al., 2008; blockage of ryanodine receptors, Barrientos et al., 2009; inhibition of mitochondrial Ca^{2+} accumulation, Brustovetsky et al., 2011; Consolini and Bonazzola, 2008; Santo-Domingo et al., 2007; Storozhevyykh et al., 2010).

The strong protective effect of calpain inhibitor VI suggests an important role of the calpain family of Ca^{2+} -activated proteases in OGD-induced degeneration of axons and motor terminals. This protective effect may relate to O'Brien et al.'s (1984) finding that protease inhibitors (leupeptin, pepstatin) protected rat motor nerve terminals exposed to a different Ca^{2+} -mediated stress (nerve stimulation in high bath $[\text{Ca}^{2+}]$). Calpain activation might contribute to rapid degeneration by increasing intracellular Ca^{2+} accumulation, e.g., by increasing Ca^{2+} conductance by proteolytic cleavage of a subunit of L-type Ca^{2+} channels (Hell et al., 1996), and/or by cleavage of the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger, which occurs during brain ischemia and increases Ca^{2+} overload in neurons exposed to an excitotoxic stress (Bano et al., 2005).

It will be important in future work to determine which of the drugs found to protect motor terminal *morphology* during OGD also protect motor terminal *function*. Jiang and Stys (2000) and Stys and Jiang (2002) reported that pharmacological inhibition of calpain greatly reduced proteolysis of spectrin and neurofilament in optic nerve axons exposed to ischemia/reperfusion or anoxia/reoxygenation, but did not improve electrophysiological recovery. In contrast, removal of bath Ca^{2+} did protect function. Thus they concluded that stress-induced damage of optic axons involves activation of calpain but also of additional deleterious Ca^{2+} -activated pathways.

Conclusions

In summary, we applied an OGD/reoxygenation stress at near-physiological temperatures to fast-twitch mouse muscles, and present evidence that the rapid motor terminal degeneration produced by this stress is Ca^{2+} -dependent. Motor terminals were protected from this stress by agents that inhibit several routes of Ca^{2+} entry and by an agent that inhibits the calpain family of Ca^{2+} -activated proteases. It is possible that these agents might also help protect motor terminals exposed to an ischemic stress in vivo.

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Abbreviations

α-BgTx	alpha-bungarotoxin
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
calpain inhibitor VI	N-(4-fluorophenylsulphonyl)-L-valyl-L-leucinal
EDL	extensor digitorum longus muscle
I/R	ischemia/reperfusion
KB-R7943 mesylate	(2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourethane methane sulfonate)

LAL	levator auris longus muscle
NKCC1	$\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ co-transporter 1
OG-1	Oregon green 488 BAPTA1
OGD	oxygen-glucose deprivation
Rh-123	rhodamine 123
SOD1	superoxide dismutase 1
wt	wild-type
YFP	yellow fluorescent protein
Ψ_m	potential across inner mitochondrial membrane

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Fig. 1.

OGD increases resting cytosolic $[Ca^{2+}]$ in motor terminals. A, Fluorescence micrographs show branches of a wt LAL axon that was filled by ionophoretic injection with the Ca^{2+} indicator OG-1. The upper micrograph (labeled “control”) indicates 5 motor terminals of this axon (labeled a–e) whose fluorescence changes are plotted in B. Fluorescence increased during the OGD stress. This axon began to disintegrate within 10 min following reoxygenation, as indicated by dye loss from some terminals and beading of the axon (micrograph labeled reox 35 min). All images are displayed with the same gray scale settings. B, Fluorescence changes (normalized to pre-OGD control fluorescence, $F/F_{control}$) produced in each of the 5 motor terminals labeled in A by exposure to a 60 min OGD stress (vertical dashed lines indicate stress onset and termination). In this axon stimulation-induced changes in fluorescence became undetectable 35 min after OGD onset (not shown, but see Figs. 2, 3). Measurements after reoxygenation (not shown) were dominated by dye loss associated with disintegration of the terminals and parent axon. C, Averaged OGD-induced fluorescence increases were similar for 12 wt (solid line, 4 mice) and 4 SOD1-G93A (dashed line, 2 presymptomatic mice) terminals. An $F/F_{control}$ value of 2.5 corresponds to $[Ca^{2+}]$ of $\sim 1.1 \mu M$, at least 7 times greater than the assumed resting value of $0.1\text{--}0.15 \mu M$ (calculation assumes OG-1 $K_d=0.8 \mu M$). Part of the interval between the onset of OGD and the increase in cytosolic $[Ca^{2+}]$ was attributable to the time needed to reduce the partial pressure of O_2 in the bath. The need to image the terminal precluded rapid perfusion or bubbling the experimental chamber with N_2 , and the chamber remained open to allow axonal stimulation.

Fig. 2.

Sample recordings from OG-1-filled motor terminals that recovered (A,B) or failed to recover (C,D) following OGD. A, recovery of resting $[Ca^{2+}]$ ($F/F_{control}$) and B, recovery of the stimulation-induced elevation of $[Ca^{2+}]$ (F/F_{rest}) in a wt terminal following OGD. C, lack of recovery of resting $[Ca^{2+}]$ and D, lack of recovery of the stimulation-induced elevation of $[Ca^{2+}]$ in an SOD1-G93A terminal following OGD. In this terminal resting $[Ca^{2+}]$ continued to increase following reoxygenation, and the terminal disintegrated thereafter. In B and D, F_{rest} is the fluorescence recorded just prior to stimulation; these responses were obtained at the times indicated by the arrows in A and C, respectively. The absence of a response to stimulation when resting $[Ca^{2+}]$ was elevated could reflect OGD-induced failure of nerve conduction and/or saturation of the Ca^{2+} indicator OG-1. Wt: 50 days old; SOD1-G93A: 43 days old (presymptomatic). Stimulation 50 Hz, 20 s.

Fig. 3.

The disruption of Ca²⁺ homeostasis (A,B) and Ψ_m depolarization (C) produced by OGD are due mainly to O₂ deprivation (hypoxia). Circles in B plot changes in resting [Ca²⁺] (OG-1 fluorescence) in a wt terminal exposed sequentially to 120 min glucose deprivation and 50 min O₂ deprivation, with return to control medium following each stress. Traces in A superimpose (on a faster time scale than B) pairs of representative stimulation-induced [Ca²⁺] elevations recorded (a) prior to the stresses, (b) at the end of glucose deprivation, (c) after resting [Ca²⁺] rose during O₂ deprivation, and (d) after control conditions were restored. Stimulation was 50 Hz for 10 s, repeated at 10 min intervals. C shows fluorescence changes in Rh-123 produced in a different wt terminal subjected to the same sequence of stresses. Resting [Ca²⁺], the stimulation-induced increase in [Ca²⁺], and Ψ_m were well maintained throughout 120 min of glucose deprivation. In contrast, with O₂ deprivation mitochondria depolarized and the fluorescence increase in response to stimulation disappeared after resting [Ca²⁺] increased. Values for Ca²⁺ indicator and Rh-123 fluorescence are in arbitrary fluorescence units. 5% CO₂ was present in all gas mixtures.

Fig. 4. OGD-induced damage to motor terminals and axons is Ca^{2+} -dependent. Each row shows fluorescence micrographs of a representative field from an EDL muscle of a YFP-expressing mouse: non-stressed (top), following OGD in normal bath $[\text{Ca}^{2+}]$ (middle), and following OGD without bath Ca^{2+} (bottom). The left column shows muscle endplate acetylcholine receptors (AChR, red, labeled with α -BgTx Alexa Fluor 594 conjugate), which appear unchanged following the OGD stress. The middle column shows that motor terminals and axons (green, labeled by transgenically expressed YFP) were severely damaged following OGD with Ca^{2+} , but mostly spared following OGD in the absence of Ca^{2+} . The right column shows the merged images, in which innervated endplates appear yellow-orange, and denervated endplates appear red. Arrows indicate two endplates that were denervated following OGD in the absence of Ca^{2+} . The Ca^{2+} -free solution was prepared by adding 2 mM BAPTA to saline with no added Ca^{2+} . The two OGD-treated EDLs came from the same mouse; both EDLs received 22 min OGD followed by 2 h reoxygenation, all at 32.5 °C. All micrographs are montages prepared from fields imaged at 20 $\times$; measurements of the occupancy of individual endplates were made at higher magnification. Calibration bar: 200 μm .

Fig. 5.

Removal of bath $[Ca^{2+}]$ reduces motor terminal degeneration following OGD. A–C, each line connects the % endplate occupancy measured from paired EDLs from one mouse. Both EDLs were subjected to 22 min OGD followed by 2 h reoxygenation/glucose restoration; one EDL was bathed throughout in the normal 1.8 mM Ca^{2+} and the other EDL was bathed (A) in a zero Ca^{2+} solution throughout both OGD and reoxygenation, (B) in zero Ca^{2+} during OGD with normal $[Ca^{2+}]$ present during reoxygenation, or (C) in zero Ca^{2+} during reoxygenation with normal $[Ca^{2+}]$ present during OGD. Solid lines connect wt (YFP-only) pairs; dashed lines connect SOD1-G93A pairs. Squares indicate the median value for each group. Endplate occupancies were higher for the zero Ca^{2+} EDLs than for the normal $[Ca^{2+}]$ EDLs in A and B ($p < 0.001$, $n = 10$ muscle pairs, 6 wt and 4 SOD1-G93A in A; $p < .01$, $n = 8$, 4 wt, 4 G93A in B, Wilcoxon signed rank, one-tailed). Data in C were insufficient to assess statistical significance.

Fig. 6.

Drugs that reduce Ca^{2+} entry or Ca^{2+} -activated protease preserve endplate occupancy following OGD. A, diagram summarizes the action of each tested drug. B–F, each graph plots % endplate occupancy for paired EDJs exposed to OGD alone (stress) or OGD plus the indicated drug. Solid lines connect stress/stress+drug pairs for wt (YFP-only) mice; dashed lines connect pairs for SOD1-G93A mice. Squares indicate the median value for each group. Drug concentrations: ω -agatoxin IVA, 1 μM , $n=6$ mice [3 G93A, 3 wt]; nimodipine, 10 μM , $n=5$ [2,3]; KB-R7943, 10 μM , 6 [4,2]; bumetanide, 10 μM , 5 [1,4], calpain inhibitor VI, 100 μM , 5 [1,4]. Each of the 5 tested drugs increased endplate occupancy (Wilcoxon signed rank, one-tailed, $p<0.05$). There was substantial overlap between drug effects for wt and mutant SOD1 terminals. VGCC = voltage-gated calcium channels; NCX = sodium-calcium exchanger; NKCC = sodium potassium two chloride cotransporter.