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The Antioxidant Requirement for Plasma Membrane Repair in Skeletal Muscle

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Abstract

Vitamin E (VE) deficiency results in pronounced muscle weakness and atrophy but the cell biological mechanism of pathology is unknown. We previously showed that VE supplementation promotes membrane repair in cultured cells and that oxidants potently inhibit repair. Here we provide three independent lines of evidence that VE is required for skeletal muscle myocyte plasma membrane repair *in vivo*. We also show that when another lipid-directed antioxidant, glutathione peroxidase 4 (Gpx4), is genetically deleted in mouse embryonic fibroblasts, repair fails catastrophically, unless cells are supplemented with VE. We conclude that lipid-directed antioxidant activity provided by VE, and possibly also Gpx4, is an essential component of the membrane repair mechanism in skeletal muscle. This work explains why VE is essential to muscle health and identifies VE as a requisite component of the plasma membrane repair mechanism *in vivo*.

Graphical Abstract

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Introduction

VE is a lipid soluble nutrient with potent phospholipid-directed antioxidant activity [1]. It partitions ubiquitously into cell phospholipid bilayers and into other hydrophobic environments, such as the lipid stored in fat. Among the eight forms found in foods, α -tocopherol is the most common. All tocopherols share the basic structure of a saturated isoprenoid side chain and a 6-chromonal ring (2 position). The isoprenoid side chain interpolates into phospholipid bilayers, and may alter thereby alter the biophysical/functional properties of membranes. The chromonal ring is positioned by the interpolated side chain for peroxy radical scavenging amongst membrane phospholipid head-groups.

Given its ubiquitous location in cells and in tissues, and its general antioxidant function, the biological roles of VE are predicted to be multifarious. However, skeletal muscle, in particular, has a well-demonstrated requirement for this vitamin; many studies have indicated that deficiency results in profound skeletal muscle pathology [2]. The mechanism by which VE promotes muscle health is unknown.

We have been investigating the hypothesis that one function of VE is in cell plasma membrane repair [3]. In support of this, we found that supplementation of cultured cell models with α -tocopherol or Trolox (a water soluble analogue) promotes plasma membrane repair, whereas challenge with oxidants strikingly inhibits repair, unless cells are supplemented beforehand with VE. We showed additionally that oxidants potently inhibit repair and that this can be prevented by prior supplementation with VE. Importantly, repair inhibition after oxidant exposure and its prevention by prior exposure to VE could also be demonstrated in intact skeletal muscle using the laser assay employed here [3]. This study clearly implicated VE as an antioxidant in cell plasma membrane repair. However, these studies did not answer the question whether native, pre-existent levels of cellular VE are required for repair *in vivo*, or whether this repair role of innate VE applies specifically to skeletal muscle.

Plasma membrane repair is a fundamental cellular activity of the skeletal muscle myocyte (muscle fiber). Disruption injuries are common in skeletal muscle, especially when high-force, eccentric contractions are applied in exercise [4]. Failure to repair a plasma membrane disruption rapidly (seconds) results in cell death (by necrosis). Both increased fragility,

resulting in an enhanced rate of muscle cell plasma membrane disruption injury, and repair failure, are associated with congenital human muscular dystrophies [5].

We here directly test the hypothesis that VE is required for plasma membrane repair in mammalian skeletal muscle using three independent, *in vivo* and *in situ* assays of repair capacity. Striking evidence of repair failure in the skeletal muscle of VE-deprived rats is presented. We demonstrate further that cell depletion of the phospholipid hydroperoxidase, Gpx4, also results in repair failure, and that this failure can be prevented by VE supplementation, providing further evidence of the crucial role of lipid-based antioxidant activity in membrane repair. This work is the first to explain, at the cell biological level, why VE is essential for skeletal muscle homeostasis: without VE, myocyte repair of contraction-induced membrane disruptions fails, myocytes consequently die, and, over time, the muscle atrophies.

Materials and Methods

VE deprivation

Male Sprague Dawley rats (Harlan Laboratories) were introduced to a VE-deprived diet, rodent chow stripped of VE (Harlan Laboratories), at age 4 weeks. Controls were fed normal chow, or chow to which α -tocopherol had been added back. This was accomplished by dissolving ($\pm$) α -tocopherol (96% pure, Sigma-Aldrich) at 20 mg/ml in 95% ethanol and spraying it onto the VE deficient chow, followed by air-drying in the dark. Control chow for this addition received vehicle only. Rats were fed ad libitum on these various chows. The measured content of α -tocopherol in all chows, and the muscle of rats fed each is given in Supplementary Fig. 1.

Gpx4 MEF KO

Tmx-inducible Gpx4^{-/-} and respective control mouse embryonic fibroblasts with reconstituted Gpx4 expression were employed as previously described [6, 7]. Briefly, Gpx4 KO was initiated by incubating cells in 1 μ M tmx (Sigma-Aldrich). Controls included inducible Gpx4 knockout cells not exposed to tmx and inducible Gpx4 knockout cells stably expressing Gpx4 that did receive tmx. For the latter, a cell line transduced with empty virus served as respective control.

Treadmill Running

Rats were run on a downhill (15°) treadmill (Exer 3/6 treadmill, Columbus Instruments) once a week 6 weeks for training and preliminary observation of running ability before the final run. At the 11 month interval, a final run was made until exhaustion (sitting on the electrical grid for more than 10 shocks) and the number of visits to the back of the treadmill and shocks received during the run were recorded for each pair member.

Evans Blue Repair Analysis

After (15 min) the final down-hill run, rats were injected with Evans blue dye (50 mg/kg weight). Rats were sacrificed 16–20 hours later, and the quadriceps processed without fixation for frozen sectioning. Frozen sections were mounted in an anti-fade agent (Prolong

Gold, Life Technologies) and a montage of images encompassing 80–100% of the muscle recorded using an Olympus IX71 Deltavision microscope. The fluorescence fibers, indicative of repair failure, were counted using Image J (NIH) which was programmed to identify fibers based on fluorescence intensity, size and circularity.

Laser Analysis Repair

The front paw was excised at animal sacrifice after down-hill run for use in laser analysis of membrane repair. The palmar surface skin was incised, and peeled to either side to reveal underlying muscles. One of these, the flexor digitorum brevis muscle was imaged for repair assay. This paw preparation was found to be far superior to the previous one we have employed, the soleus muscle [8]: less damage was done during muscle exposure and most importantly the muscle retained its normal skeletal support. This minimized the movement effect of injury-induced contractions and resultant focusing problems. The paw was stuck to the bottom of a 35 mm dish using New Skin Liquid Bandage (CVS Drugstore) and then immersed Tyrode's buffer and immediately used in repair analysis or left on ice no more than 2 hs before use. FM 1-43 dye was added (2.5 μ M) to the saline maintained at 37°C and the muscle imaged confocally (Argon-2, 488 nm) on a Zeiss LSM510 Meta two-photon microscope. Any fibers damaged by the dissection could be detected based on intracellular labeling with FM 1-43 and were excluded from analysis. Disruptions were created irradiating the plasma membrane of myofibers at 80% laser power (Coherent, Mira 900F Ti:Sapphire, 820nm), 1 iteration over a circular area of 3.12 μ m diameter placed centrally over the fluorescent plasma membrane boundary to created plasma membrane disruptions. Images were acquired confocally at 1 s intervals thereafter for a period of 5–6 min. The integrated fluorescence intensity within a rectangular shaped measurement area (360 μ m²) placed in cytoplasm immediately beneath the disruption site was measured as a function of time using Zeiss Physiology software. Plasma membrane disruption and subsequent repair analysis of MEFs grown in 35 mm dishes were as previously described [8].

Western Analysis of Gpx4 Levels

Cells were plated in the presence of 1 μ M α -tocopherol. Tmx was added to the cell culture medium to induce the knockout (1 μ M), and cells were lysed at the varies time points after tmx addition. On each lane, 15 μ g total protein were loaded and blotted against Gpx4 (Abcam # ab125066 - 1:1000) and β -actin as loading control (Sigma # A5441 - 1:10000 dilution), as described previously [9].

Scraping Analysis Repair

Scraping analysis of repair was performed as previously described^[8].

VE Analysis

Samples of gastrocnemius muscle or rat chow were analyzed for their tocopherol profile as previously described, but with slight modifications [10]. A 1 g sample was weighed into an extracting tube to which 10 ml of 6% (w/v) pyrogallol in 95% ethanol were added. After flushing the tube with N₂ and sonicating the sample for 5 min, 3 ml of a 60% (w/v) KOH solution were pipetted in, and a Snyder column was attached to the extracting tube. The

samples were saponified in a 70°C orbital-shaking water bath for 45 min, after which they were cooled in an ice bath. Then, 20 ml of a 2% (w/v) NaCl solution were added followed by extraction of the tocopherols 3× with 10 ml portions of 10% (v/v) ethyl acetate in hexane containing 0.01% BHT [11]. A 20 µl aliquot of the resuspended extract was injected into a Shimadzu HPLC system consisting of a LC-10AT controller/pump, CBM-20A Prominence Communications Bus Module, DG-14A degasser, RF-10AXL fluorescence detector and a PC with EZStart Chromatography software to control the instrument. A normal-phase LiChrosorb® Si 60 column (4 mm × 250 mm, 5 µm particle size; Hibar® Fertigsäule RT, Merck, Darmstadt, Germany) connected to a LiChroCART® 4-4 guard column packed with LiChrospher® Si 60 (5 µm), and an isocratic mobile phase comprising 0.85% (v/v) isopropanol in hexanes at a flow rate of 1 ml/min were used. The excitation and emission wavelengths for the fluorescent determination of tocopherol isomers were 290 and 330 nm, respectively. Quantification was achieved using a set of high-purity tocopherol standards (Calbiochem) and extinction coefficients.

Statistical analysis

P-values were calculated using Student's t-test to compare groups in all bar graphs, or ANOVA with Tukey's post analyses for all temporal laser data. All graphs display the data as the mean plus and minus standard error.

Results

Rats had previously been shown [12] to display a pronounced muscle phenotype when deprived of VE and were thus selected as a model system. To induce deprivation, rats were fed a chow stripped of VE. Control rats were fed either, a) standard chow, or b) VE deficient chow to which α-tocopherol had been added back. These two controls yielded identical results. At the 11 month interval on these diets, the α-tocopherol content of deprived rat muscle was significantly lower than that of both controls (Fig. S1).

Rats were run downhill on a treadmill after the initiation of VE deprivation, as a non-invasive index of myocyte repair function. At 11 months, a highly significant deficit in downhill running ability was measured in VE deprived rats: both the number of visits to the rear platform of the treadmill and the number of shocks received increase, relative to controls, in the VE deprived cohort (Fig. 1a). This running deficit is consistent with a repair defect since downhill running induces plasma membrane disruptions that must be repaired for continuing muscle functioning [13].

Histological comparison of control (Fig. 1b) and deprived muscle (Fig. 1c) clearly indicated that VE deprivation resulted in an increase in: a) central nucleation of fibers, b) inflammatory infiltrate in connective tissue; c) fibers of reduced diameter; d) the presence of necrotic fibers. Thus, VE deprivation resulted in overt muscle pathology in rats, as expected based on previous studies [12].

The key question of whether membrane repair is defective in VE-deprived muscle was further addressed using two additional approaches. First, cohorts were injected i.p. with Evans blue dye 15 min after the final downhill run, in which pairs of deprived and control

animals were exercised until one member was exhausted (sat on the grid while receiving more than 10 shocks). Downhill running induces plasma membrane disruptions in leg muscle undergoing eccentric contractions^[13]. Evans blue dye can only enter into muscle fibers with a compromised plasma membrane boundary, e.g. a plasma membrane disruption [14]. Since the dye was injected 15 min after the disruption-inducing run, and repair of disruptions occurs on a second time scale, fibers stained are those that suffered but failed to repair run-induced plasma membrane disruptions. Downhill running of VE-deprived rats resulted in a significant increase in the number of Evans blue positive fibers compared to controls (Fig. 2). Thus an *in vivo* assay, based on the imposition of disruption-inducing physiological stress, indicates that repair fails in VE deprived skeletal muscle myocytes.

Second, as a method for assessing repair capacity, we applied a laser assay to whole muscle taken immediately after the run. In this assay, the flexor digitorum brevis is immersed in saline containing the dye, FM 1-43 [8]. This dye can partition rapidly between aqueous (saline) and lipidic (phospholipid bilayer) environments but cannot cross intact membrane bilayers. It is fluorescent only in the lipidic environment. Thus, prior to a membrane disruption, FM 1-43 labels the plasma membrane only of muscle fibers imaged by two-photon microscopy (Fig. 3a, 0 s; see also Supplementary Movies 1 & 2). When a plasma membrane disruption is created, by abruptly and temporarily increasing infrared laser power, dye can enter the fiber and begin to stain internal membrane (Fig. 3a, 110 s, arrowhead): a ‘hot-spot’ of fluorescence caused by initial rapid dye entry marks the injury site. The initial (7 sec time-point) intensity of the fluorescence ‘hot-spot’ created, and the rate of dye entry, did not differ between control and VE-deprived muscle (Fig. S2), indicating that equivalent injuries were initially induced by the laser irradiation as documented in previous studies [3, 8]. If repair of this laser-induced disruption succeeds, as in controls, further entry of dye is blocked, and consequently the fluorescence signal imaged surrounding the disruption site stabilizes: the ‘hot-spot’ remains but does not increase in size (Fig. 3a, 445 s). If repair of the laser disruption fails, as in VE deprived muscle, dye entry continues and consequently the fluorescence signal imaged increases: there is a visible spreading of staining into the fiber (Fig. 3a, 445 s). The integrated fluorescence accumulating surrounding the disruption site as a function time post-disruption (Time = 0s) is plotted in Fig. 3b, c: significantly more dye enters into VE deprived muscle and the fluorescence recorded over time does not plateau. Thus, in a direct measurement of membrane repair capacity, VE deprived muscle is strikingly repair defective.

Glutathione peroxidase 4 (Gpx4) is a phospholipid hydroperoxidase that cells deploy in concert with VE to prevent unwanted lipid peroxidation [15]. Hence, we asked whether Gpx4, which shares only this functional activity with VE, is required for membrane repair using mouse embryonic fibroblasts (MEFs) derived from conditional Gpx4 knockout embryos, stably expressing tmx-inducible Cre recombinase^[6]. These inducible Gpx4 KO cells display nearly undetectable levels of Gpx4 protein 48 h after exposure to tmx (Fig. S3), confirming previous findings [6]. Inducible MEFs were exposed to tmx for 48 hours to initiate KO of Gpx4 and then repair assayed either after creating plasma membrane disruptions with a laser, or mechanically, by scraping the cells from their culturing substratum [16]. Controls included both inducible KO cells that did not receive tmx, and phenotypically ‘wild type’ cells (with a tmx-independent Gpx4 wild-type sequence added

back secondarily by lentivirus-mediated cell transduction and empty-virus transduced cells as the respective control) that did receive tmx [7]. Laser injury revealed a striking failure of repair after Gpx4 KO using both controls (Fig. 4ab). Scraping injury also revealed a repair defect in the Gpx4 KO MEFs (Fig. 4c). We next attempted to prevent by a VE supplementation the repair failure phenotype that developed in Gpx4 KO MEFs. Incubation of MEFs Gpx4 KOs in either α -tocopherol or Trolox (a water-soluble analogue of VE) completely eliminated the repair defect (Fig. 4d). These results strongly suggest that VE's role in membrane repair is as an antioxidant that maintains phospholipids in a native, reduced state, given that this is antioxidant activity is the only feature VE shares in common with Gpx4. Moreover, these results suggest that Gpx4 also may be important in for cell plasma membrane repair.

Discussion

'After nearly one century of research and thousands of publications, the physiological function(s) of VE remain unclear' [17]. Thus begins a recent review of the field. However, one tissue clearly affected by deprivation is skeletal muscle. Dietary deficiency results in severe (lethal) muscular dystrophy in rabbits [18], ducklings [19] and fish [20]. Weaker muscle phenotypes have been reported in rodents. For example, treadmill exercise dramatically increases muscle damage in rats deprived of VE: post-exercise serum creatine kinase levels were increased and histological assessment revealed pathological structure [12]. The same finding has been made in mice, and has been attributed to the levels of this vitamin present in muscle, rather than in blood [21, 22]. In humans, one of the problems associated with VE deficiency is skeletal myopathy, and low VE levels in the elderly are correlated with 'frailty' syndrome, characterized primarily by loss of muscle strength [23].

We report here that the skeletal muscle of the VE-deprived rat has a phenotype highly consistent with myocyte repair failure: 1) deficient downhill running ability; 2) elevated uptake by myocytes *in vivo* of an indicator for repair failure; 3) failure of myocytes *in situ* to prevent dye ingress after laser-induced plasma membrane disruption.

These results, from three independent assays, clearly indicate a novel and specific biological function for VE *in vivo*: it is required for plasma membrane repair by skeletal muscle fibers. This repair function explains why VE is required for muscle health and is consistent with additional muscle disease paradigms. Skeletal muscle exercise and VE deprivation both generate reactive oxygen species and consequent lipid and protein oxidation, and a large body of literature ranging from birds to man clearly shows these oxidative events can be reduced by VE supplementation [24–31]. In the absence of VE, our work now suggests, repair failure elevates the incidence of fiber deaths following membrane disruption injury, and in consequence, over the long-term, muscle pathology develops. At least one congenital muscular dystrophy, limb girdle type IIB/Myoshi's myopathy, is thought to have a similar etiology. In this disease, myofibers fail, due to a genetic defect, to produced functional dysferlin, a muscle-associated protein that binds membranes when activated by calcium. Skeletal muscle fibers from a mouse model of this disease (KO of dysferlin) were shown, using the laser assay employed here, to be repair defective [5]. Skeletal muscle membrane

repair is also defective in diabetes, one complication of which is muscle weakness and, in severe cases, muscle wasting [8].

Our earlier finding that VE supplementation can enhance repair in cultured cells, and protect skeletal muscle cells from repair failure induced by oxidants [3], may explain why VE supplementation has been found to be protective in eccentric exercise, which strongly induces fiber plasma membrane disruptions, but not in other forms of exercise, such as aerobic, where injury may be primarily metabolically based [32]. VE or some more potent analogue may be useful as a therapy for muscular dystrophies, such as those just described above involving myocyte plasma membrane fragility or an inadequate repair response.

The selenoenzyme Gpx4 is the only member of its family (8 in total) whose genetic deletion has been shown to be embryonic lethal [33]. Three distinctive forms, a cytosolic (short), a mitochondrial (long) and a nuclear one, are expressed by the *Gpx4* gene; the latter two being almost exclusively localized to testis. The short form, by contrast, is essential for embryonic development [34] and is widely expressed in embryonic and adult mouse, including in skeletal muscle, where it is found in the cytosol and also associates with certain organelles such as mitochondria and the nucleus [35]. Various biological functions have been ascribed to it, including the generalized role of reducing (phospho)lipid hydroperoxides. It has also been hypothesized more specifically to inhibit caspase-independent, non-apoptotic cell death, initiate eicosanoid signaling and mediate chromatin condensation [36]. Whether these latter, more specific roles, are dependent on its antioxidant capacity is not known.

Importantly, selenium, in form of selenocysteine, is an essential structural component of active Gpx4 [7]. Selenium deficiency results in muscle pathology, specifically degenerative muscle disease, affecting both skeletal and cardiac muscle [37–39]. Often known in the livestock trade as white muscle disease, this muscle degeneration develops in sheep and goats grazing on selenium deficient soils. Administration of either supplemental selenium or VE alleviates the skeletal muscle problems [37]. Of particular relevance, a recent report shows that selenium deprivation results in a striking reduction in Gpx4 protein in skeletal muscle, based on western blotting and immunostaining analysis [40].

We here have tested whether Gpx4 KO in cultured cells results in repair failure, and found that it does, unless the KO cells are supplemented with VE. Gpx4 KO has previously been shown in this model system to result in profound phospholipid peroxidation [6]. The only common biological activity these two molecules share is in preventing peroxidation. Moreover, we have previously shown that oxidants potently inhibit repair, and that this inhibition is preventable by prior supplementation with VE [3]. We conclude that prevention of phospholipid peroxidation is the key function of VE in membrane repair. This raises the question, not yet answered, of how exactly antioxidant activity contributes to plasma membrane repair, and whether Gpx4 is required, in addition to VE, for membrane repair in skeletal muscle and hence for muscle health.

The mechanism of membrane repair is an active area of current investigation. Repair is initiated by calcium influx through a disruption into cytosol. This triggers homotypic/heterotypic membrane fusion events that produce an enlarging membrane-bound

compartment, the 'patch', in cytosol beneath the disruption site [41, 42]. Exocytotic fusion then anneals this patch to the plasma membrane, completing repair [43, 44]. Numerous proteins have been identified as participants in this process, including SNAREs [43], dysferlin [45], annexins [46–48], calpains [49], synaptotagmin [50] and MG53 [51, 52]. None of these protein components of the repair machinery is likely to be the direct target of antioxidants such as VE or Gpx4, though at least two of them, annexins and calpains possess redox sensitive cysteine moieties. Are effects on protein transcription/translation involved? This an important unanswered question, but we do know inhibition of repair by oxidants is a rapid process (minute timescale), and that cell exposure to Trolox results in rapid protection oxidant-induced repair failure [3]. These *in vitro* results do not support a transcriptional/translational mechanism of repair inhibition.

The fusion events of repair necessarily involve not only membrane proteins but also membrane phospholipids. Membrane phospholipid composition can influence membrane deformation-requiring events such as endocytosis and exocytosis [53]. In neurons, oxidants inhibit SNARE-mediated exocytotic fusion at the synapse [54, 55], though whether this is an effect on the SNAREs or membrane phospholipids is unknown. Membrane lipid oxidation alters many membrane biophysical properties and cell functions, and ultimately causes outright loss of plasma membrane barrier function [56]. An untested hypothesis arising from this work is that the membrane fusion events of repair, homotypic and exocytotic, require maintenance of phospholipids in the fusing membranes in an unoxidized state.

An alternative explanation of repair failure, that cannot be excluded, is that, in the VE-deficient lipid bilayer, uncontrolled extension of an initial disruption occurs. In support of this, VE has long been described as a membrane stabilizing molecule and phospholipid oxidation can lead to bilayer leakiness [57]. On the other hand, there is no direct experimental evidence to support this concept, and, moreover, in a previous study, we found that the rate of dye entry was equivalent plus or minus VE in the absence of extracellular calcium, which prevents activation of the vesicle/protein-based repair apparatus [3].

Clearly, determining the molecular mechanism of repair failure is a key area for future research. Meanwhile, promising recent work suggests that small molecule inhibitors might be useful in preventing pathology arising from a failure of the glutathione/Gpx4 axis [9].

Conclusion

VE, acting as an antioxidant, is required for plasma membrane repair in skeletal muscle myocytes. This explains why it is essential for muscle homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

VE	Vitamin E
Gpx4	glutathione peroxidase 4
FM 1-43	<i>N</i> -(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide
MEFs	mouse embryonic fibroblasts
tmx	4-hydroxytamoxifen
SNARE	soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptor
MG53	mitsugumin 53

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Figure 1. Muscle pathology in vitamin E deprived rats

- (a) A cohort of 6 rats was subject to downhill running on a treadmill, and the number of visits (NOV) made to the electrified grid, and the number of shocks (NOS) received while there, were recorded (an automated function on this treadmill). The deprived rats (VED) were clearly deficient in running ability relative to controls as indicated by their failure to keep pace: hence the striking elevation in visits to and shocks received at the electrified grid.
- (b) H&E stained section of the quadriceps muscle taken from a rat fed chow containing VE added experimentally to the VE stripped chow. Muscle architecture is normal and identical to that of rats fed standard chow. Scale bar 50 μ m.
- (c) Muscle taken from a rat fed VE stripped chow. VE-deprived muscle is characterized by central nucleation of myofibers, reduced fiber size, necrotic myofibers (arrow) and extensive inflammatory infiltrate (high degree of cellularity between myofibers). Scale bar 50 μ m.

Figure 2. Uptake of Evans blue dye indicates that VE-deprived skeletal muscle myocytes fail to repair membrane disruptions created during eccentric contraction exercise

(a) Fluorescence micrographs of the entire quadriceps muscle from a control and VE-deprived rat. Rats ran down-hill until exhaustion and then, 15 min later, were injected with the vital dye, Evans blue, which labels fibers (red) that failed to repair the disruptions created by this type of exercise. Uptake of dye by myofibers is strikingly elevated in the VE-deprived muscle, indicating that repair failure is induced by deprivation. *Inset*: One region of the whole-muscle montage from the VE-deprived muscle is shown at higher magnification and clearly reveals myofibers labeled with Evans blue. Scale bar 1 mm.

(b) This difference was assessed quantitatively by automated, blinded counting of labeled fibers. A significant increase in labeled fibers (per muscle) was measured in the VE-deprived rat muscles (VED) compared to normal chow diet (Control).

Figure 3. Direct assay of dye uptake indicates that VE-deprived skeletal muscle myocytes fail to repair membrane disruptions created by a laser

(a) A laser disruption is made at the site of the arrowhead (0 second time-point). Consequent entry of FM1-43 dye is recorded here at 110 and 445 seconds after this injury. Repair limits dye entry into muscle cell derived from control rats fed a normal chow diet, but fails allowing unrestricted dye entry in the VE deficient muscle derived from a rat fed chow lacking VE. Scale bar 10 μ m.

(b) The fluorescence intensity measured in myofiber cytoplasm as a function of time after disruption of the plasma membrane with a laser. Dye uptake by the fibers of VE-deprived rats is significantly more rapid and extensive than in control rats fed standard chow, indicating repair failure in the deprived myofibers (n = number of myofibers measured, a minimum of 6 from each of 5 rats).

(c) An equally striking failure was recorded when rats fed VE stripped chow were compared with controls fed the stripped chow to which VE had been added back (n = number of myofibers measured, a minimum of 6 from each of 6 rats).

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Figure 4. Repair fails in a mouse embryonic fibroblast KO of Gpx4 and failure is preventable by supplementation with VE

(a) Conditional Gpx4 KO MEFs were exposed to tmx for 48 h (Gpx4^{-/-}) or vehicle alone (Gpx4^{+/+}) and repair assessed by the laser assay. A striking increase in dye uptake was observed in the Gpx4 KO relative to controls indicating that Gpx4 is required for repair (n = number of cells).

(b) An alternative cell model system gives an equivalent result. Laser analysis of MEFs used in 'A' but transfected with empty vector (Gpx4^{-/-}) and those same source MEFs transfected with a second, tmx-independent Gpx4 sequence (Gpx4^{+/+}) after a 48 h exposure to tmx. Use of this alternate model system demonstrates that repair failure of the Gpx4 KO cannot be explained as an artifact of exposure to tmx or as an unintended consequence of the genetic manipulations made.

(c) MEFs were exposed to tmx (+ tmx) or vehicle and then repair assayed by scraping, which mechanically induces plasma membrane disruptions (n = 5, number of cultures assayed). The Gpx4 KO repair defect is not confined to laser injuries only.

(d) MEFs were exposed to tmx for 48 h and then membrane repair was measured in the laser assay. Fibroblasts receiving unmodified medium (No Supplement) displayed a prominent repair defect. Those supplemented with α-tocopherol (200 μM) or Trolox (200 μM) for 24 h before displayed a strikingly improved repair response.