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FORUM REVIEW ARTICLE

Mechanical Stretch-Induced Activation of ROS/RNS Signaling in Striated Muscle

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Abstract

Significance: Mechanical activation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) occurs in striated muscle and affects Ca^{2+} signaling and contractile function. ROS/RNS signaling is tightly controlled, spatially compartmentalized, and source specific. ***Recent Advances:*** Here, we review the evidence that within the contracting myocyte, the trans-membrane protein NADPH oxidase 2 (Nox2) is the primary source of ROS generated during contraction. We also review a newly characterized signaling cascade in cardiac and skeletal muscle in which the microtubule network acts as a mechanotransduction element that activates Nox2-dependent ROS generation during mechanical stretch, a pathway termed X-ROS signaling. ***Critical Issues:*** In the heart, X-ROS acts locally and affects the sarcoplasmic reticulum (SR) Ca^{2+} release channels (ryanodine receptors) and tunes Ca^{2+} signaling during physiological behavior, but excessive X-ROS can promote Ca^{2+}-dependent arrhythmias in pathology. In skeletal muscle, X-ROS sensitizes Ca^{2+}-permeable sarcolemmal "transient receptor potential" channels, a pathway that is critical for sustaining SR load during repetitive contractions, but when in excess, it is maladaptive in diseases such as Duchenne Musclar dystrophy. ***Future Directions:*** New advances in ROS/RNS detection as well as molecular manipulation of signaling pathways will provide critical new mechanistic insights into the details of X-ROS signaling. These efforts will undoubtedly reveal new avenues for therapeutic intervention in the numerous diseases of striated muscle in which altered mechanoactivation of ROS/RNS production has been identified. *Antioxid. Redox Signal*. 20, 929–936.

Introduction

Reactive oxygen species (ROS) or reactive nitrogen species (RNS) modulate numerous biochemical processes through the targeted modification of specific protein residues. In striated muscle, contractile activity and/or stretch increases ROS/RNS signaling and modulates a host of biochemical processes, including glucose uptake, gene expression, calcium signaling, and contractility. In pathological conditions, ROS/RNS signaling excess or dysfunction contributes to contractile dysfunction and arrythmogensesis in the failing heart as well as contractile dysfunction and myopathy in skeletal muscle.

Much progress has been made in unraveling the sources and molecular targets of ROS/RNS in the cardiovascular and skeletal muscle systems (*i.e.,* myocyte, endothelium, and vascular smooth muscle). It is obvious that extra-myocyte-generated ROS and RNS demonstrate paracrine signaling to myocytes, and several excellent reviews have been published that consider these pathways (12, 15, 40, 41, 55). Much less is known, however, about the mechanisms by which ROS/RNS is generated within the myocyte during contraction or stretch (34, 54). Here, we provide a brief review of the mechano-signaling during contractile activity or *via* stretch, the subsequent ROS/RNS production, and oxidative/nitrosative signaling within the striated muscle myocyte. These pathways are now being revealed with single-cell studies that are enabled by new methods and reagents, enhancing our understanding of how mechanosensing *via* ROS/RNS contributes to physiological and pathophysiological processes within striated muscle.

ROS/RNS Signaling in Contracting Muscle Cells

Striated muscle generates superoxide as the primary ROS species and NO as a parental species from which RNS are

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generated. Each arises from several sources, and the content of each is increased with contractile activity. Once generated, each leads to the formation of secondary ROS or RNS, each with specific signaling roles. A basic schematic is presented in Figure 1 with greater detail provided in the later text.

Superoxide

Superoxide is free-radical oxygen that is generated by the addition of a single electron to ground-state oxygen ($O_2^{\bullet -}$) (73). Superoxide is a highly reactive, unstable species that is rapidly dismuted by superoxide dismutase (Cu/ZnSOD) to hydrogen peroxide (H_2O_2), a weaker but more stable reactive oxidant which is not a "free radical," as it does not contain an unpaired electron. H_2O_2 is highly diffusible within and between cells, activates multiple signaling pathways, and is decomposed by either catalase or glutathione peroxidase to water and oxygen (52, 71). H_2O_2 is also the substrate for the extremely reactive hydroxyl radical (OH) that is generated *via* the Fenton Reaction with divalent metal cations, mainly ferrous iron (Fe^{2+}) (35).

Within proteins, sulfhydryl groups on cysteine molecules are preferential targets for oxidation or the formation of disulfide bonds. Here, the sulfhydryl (–SH) group may initially be reversibly oxidized to a sulfenic acid (protein–SOH). This group can then be further oxidized to a sulfinic acid (protein-SOH + ($O^{\bullet}$) → protein SO_2H) or irreversibly oxidized to sulfonic acid (protein-SO_2H + ($O^{\bullet}$) → protein SO_3H). The $OH^{\bullet}$ can then form irreversibly oxidized protein sulfhydryl groups that are indicative of protein damage.

Mitochondria

The production of superoxide occurs in many locations within the cell. The most studied is the mitochondria where

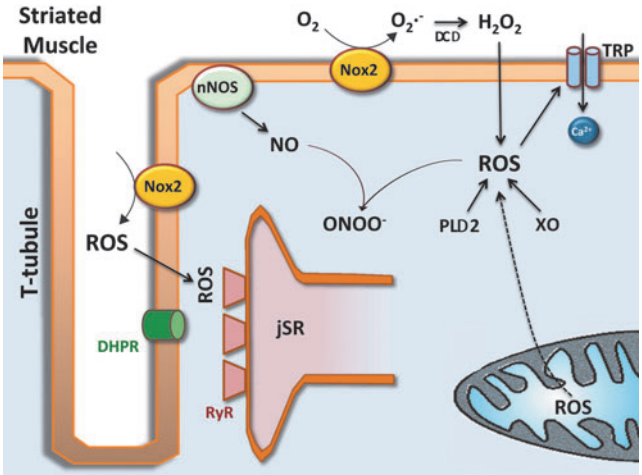

FIG. 1. Schematic representation of RNS and ROS production on the striated myocyte. See main text for details. ROS, reactive oxygen species; RNS, reactive nitrogen species; PLA2, phospholipase A2; TRP, transient receptor potential; RyR, ryanodine receptor; DHPR, dihydropyridine receptor; SOD, superoxide dismutase; XO, xanthine oxidase; jSR, junctional sarcoplasmic reticulum; t-tubule, transverse tubule; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; $ONOO^-$, peroxynitrite; Nox2, NADPH oxidase 2. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

superoxide is produced within the electron transport chain (ETC) (3). Earlier studies suggested that 2%–5% of the total oxygen consumed underwent one electron reduction in the ETC (43); therefore, as mitochondrial activity increases >50-fold with aerobicaly sustained contractile activity, a >50-fold increase in activity-dependent ROS generation could theoretically be accounted for by mitochondrial sources (75). Recent data, however, have led to a downward revision in both the magnitude of ROS produced during steady-state contraction (approximately two to fourfold increase) and the percentage of the ROS generated by electron flow through the ETC (<1%) (66). It is now thought that little superoxide is contributed by the mitochiondria during steady-state contractions [see Powers and Jackson (52) for review]. This estimate is in line with our recent finding that the redox state of the mitochondria (a surrogate measure of ROS generation) is unchanged during steady-state contractions (47) as well as with recent direct measures of ROS within the mitochondria of contractile muscle fibers (61).

Xanthine oxidase

The enzyme xanthine oxidase (XO) has also been shown to produce superoxide in response to contractile activity in rodent muscle (69, 73). Available evidence, however, supports either a vascular source of XO-generated superoxide due to contractile shear stress or an increase in XO activity secondary to anaerobic metabolism that increases the availability of XO substrates (20, 21, 69). In either case, neither can account for steady-state ROS production during aerobic metabolism, and the latter supports only a mechanism that increases superoxide after exhaustive or fatiguing contractions.

Phospholipase A2

Superoxide anion is produced by phospholipase A2 (PLA2)-dependent processes (53). A Ca^{2+}-independent PLA2 mechanism may act under resting conditions (22), whereas a Ca^{2+}-dependent PLA2 may contribute to ROS production during contraction when cytosolic [Ca^{2+}] is elevated (19, 33, 60). In both cases, PLA2 is believed to act by arachidonic acid-dependent activation of NADPH oxidase (24, 70).

NADPH oxidase

Superoxide is generated by NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase; Nox), a multimeric enzyme complex that generates superoxide by transferring electrons from NADPH to oxygen. Several Nox isoforms are expressed in striated muscle and located within the sarcoplasmic reticulum (SR), the sarcolemma, and the transverse tubules (t-tubules) (63, 65).

The Nox family of oxidases comprises seven members, each of which is based on a distinct core catalytic subunit. Striated muscle cells express NADPH oxidase 2 (Nox2) and 4, each of which bind $p22^{phox}$, a small subunit that is essential for enzyme activity (65). Nox4 is constitutively active and does not require association with regulatory subunits, with regulation considered as occurring mainly by changes in expression level. Therefore, Nox4 likely contributes to the basal rate of ROS production in the myocyte. In contrast, Nox2 (also known as $gp91^{phox}$) is activated by specific agonists (*e.g.*, G-protein-coupled receptor agonists such as angiotensin II, growth

factors, and cytokines) and mechanical/contractile stress, which induce the association of regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac1) and activation of the enzyme (1, 63).

Recent work in striated muscle by our group and others has implicated the Nox pathway as the major source of superoxide ROS during repetitive contraction in both skeletal and cardiac muscle cells (47, 61). ROS production with contraction has been shown to arise from the sarcolemma and t-tubules by activation of transmembrane Nox2 (14, 38, 57, 61). It was further suggested that Nox2-generated superoxide then promoted Nox4 activation within the mitochondria and generated ROS that was released into the cytosol (80, 81); however, experiments which directly address this hypothesis have not supported that idea (61).

Nitric oxide

In striated mucle cells, nitric oxide (NO) is generated by the nitric oxide synthases (NOSs). These enzymes are the products of three different genes: neuronal nitric oxide synthase (nNOS, or NOS1), endothelial nitric oxide synthase (eNOS, or NOS3), and inducible nitric oxide synthase (iNOS, or NOS2) (45, 68). All three enzymes catalyze the production of NO from the precursor L-arginine (15, 68). nNOS and eNOS contain a calmodulin (CaM)-binding domain, making them Ca^{2+} sensitive and therefore responsive to contractile activity (68, 71). iNOS contains constutively bound CaM at resting levels of Ca^{2+}, and its activity is inhibited by association with kalirin or NAP110 (68). All NOSs generate NO and *S*-nitrosothiols (SNO) (68). NO primarily functions to stimulate soluble guanylate cyclase to produce cyclic guanosine monophosphate (cGMP), which has numerous downstream targets through cGMP-dependent kinases and phosphodiesterases (9). NO also readily reacts with superoxide to form perioxynitrite ($NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$), which, in turn, reacts potently with thiol groups to S-nitrosylate-specific protein targets. The formation of peroxynitrite also decreases the bioavailability of NO and superoxide, thus modifying the redox balance in the myocyte (36, 37).

X-ROS Signaling: Mechanotransduction-Activated Nox2-Dependent ROS Production

The concept of cellular "tensegrity" proposes that the stabilized microtubule (MT) network resists mechanical perturbations in cells and in doing so acts as a mechanotransducer (26, 67, 77, 78). Experiments and methods used to mechanically manipulate enzymatically isolated heart cells have been pioneered by several groups (7, 17, 42) and have provided a means to assess the "tensegrity" concepts in muscle cells. Our initial hypothesis that the MT network was a mechanosignaling element in the heart arose from elegant work in the literature (7, 8) and from experiments between our group and that of G. Iribe and P. Kohl (29). As a demonstration of our initial findings, Figure 2 shows a single isolated cardiomyocyte loaded with a calcium indicator dye to monitor Ca^{2+} sparks (local Ca^{2+} release from the ryanodine receptor; RyR2) and attached with carbon filaments that allowed cell stretch to be applied. Having demonstrated that an acute stretch of the myocyte caused a rapid increase of spark occurrence only in the stretched portion of the cell, we reasoned that the signal for stretch-dependent activation of the RyR should be rapid, reversible, and highly localized.

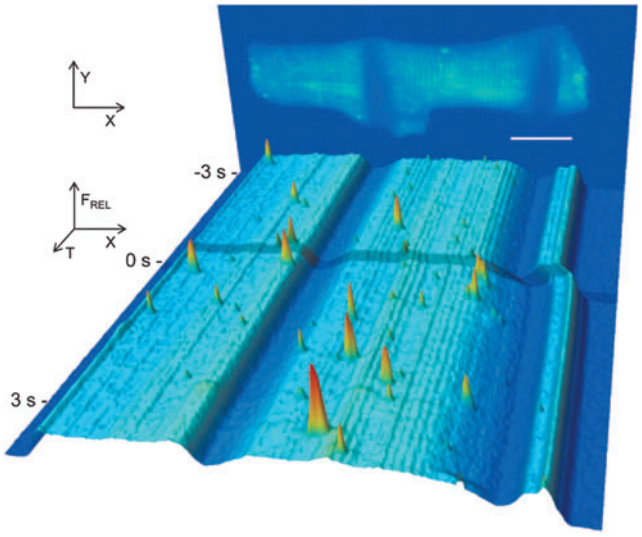

FIG. 2. Time course of Fluo-4 signal intensity in a ventricular cardiomyocyte subjected to a half-cell stretch. The *back panel* shows an image of a cell, averaged from 10 confocal XY scans before the application of stretch. The low intensity areas indicate points of CF attachment at resting length (scale bar = 20 μm). The *front panel* represents a pseudo color, pseudo 3D rendering of XT images temporally from back to front. Note that the shading across the image and the CF's fluorescence troughs indicate the stretch of one-half of the cell (right half) and that Ca^{2+} spark signals arise only in the stretched portion of the cell. This figure was used with permission from Iribe *et al.* (30). CF, carbon filament. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Since we had evidence from electron microscopy that MT filaments interdigitate with t-tubule/SR junctions (29), our initial hypothesis speculated on an interaction of MTs with clusters of calcium channels that somehow altered channel sensitivity in a stretch-dependent fashion. To support this hypothesis, we demonstrated that MT destabilization with colchicine was solely sufficient to abrogate the stretch-dependent activation of local calcium signals (*i.e.*, Ca^{2+} sparks) in cardiomyocytes. Further experimentation, however, led us to refine our hypothesis.

Working with the knowledge that Nox2 is activated by contraction (*i.e,* contraction/compression followed by relaxation/stretch), osmotic stress (stretch or compression (31, 46, 64, 74)), or stretch (49), we focused our attention on a hypothesis by which an MT-dependent mechanotransduction pathway could translate a mechanical signal to Nox2 to activate ROS. To address this question within our own laboratories, we developed new tools and methods (38, 57), enabling us to establish a model of stretch-activated mechanotransduction in striated muscle that avoids potential confounders of contraction, membrane-damaging force (eccentric injury), or non-physiologic stress such as osmotic shock.

Using these new methods coupled with high-speed confocal fluorescence imaging approaches, we revealed that in cardiomyocytes loaded with the ROS indicator dichlorofluorescein (DCF), a brief, acute physiologic stretch elicited a burst of ROS production (Fig. 3). Since DCF is a non-specific ROS indicator, we used selective inhibitors and genetic

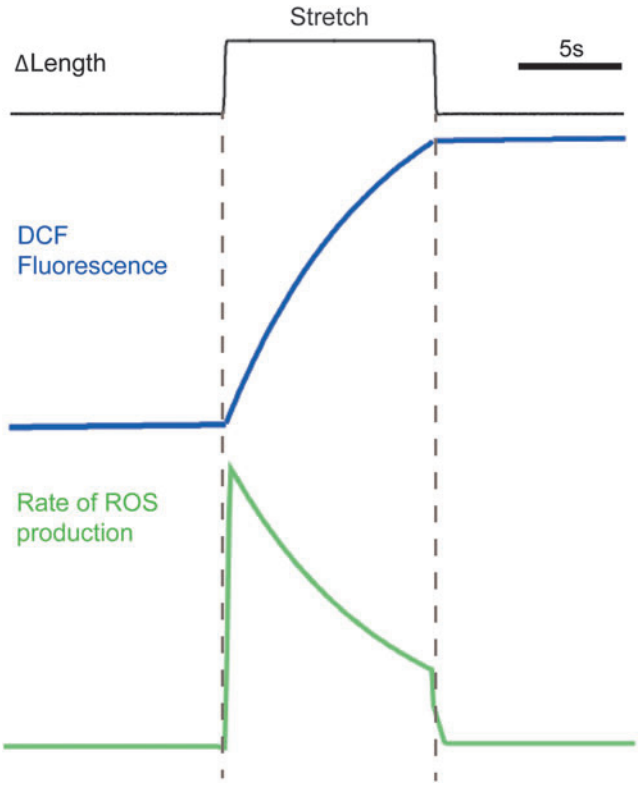

FIG. 3. Kinetic response of the ROS signal generated during a brief cardiomyocyte stretch. Data are stylized based on experiments of a single cardiomyocyte loaded with the ROS indicator DCF and subjected to an 8% sarcomere length excursion (black trace) (57). DCF fluorescence intensity (blue trace) rises rapidly on the initiation of stretch and terminates rapidly with the relaxation of the stretch. The new level of DCF fluorescence after stretch reflects DCF as a nonreversible ROS indicator. The rate of ROS production estimated by the first derivative of the DCF fluorescence profile (green trace) indicates a burst of ROS production at the initiation of stretch that decays monotonically until relaxation. DCF, dichlorofluorescein. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

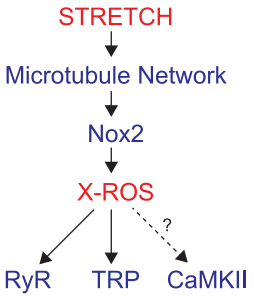

FIG. 4. Graphical representation of the X-ROS activation in striated muscle. The stress of mechanical stretch is transmitted through the filamentous microtubule network to Nox2, which generates superoxide and secondary ROS species (see review). We have demonstrated that the downstream targets of Nox2-derived ROS (*i.e.*, X-ROS) result in sensitization of RyR's in heart and TRP channels in skeletal muscle. Other potential targets include CaMKII (76). CaMKII, calmodulin kinase II. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

approaches to reveal that the MT network acted as a mechanotransducer to activate Nox2-dependent ROS generation, a pathway termed X-ROS signaling (38, 57). In heart cells, X-ROS leads to post-translational modification of RyR2s either directly (56, 57) or, possibly, indirectly through a second messenger such as Ca^{2+}-CaM kinase II (76). Regardless of the mechanism, X-ROS increases the sensitivity of RyR2s to $[Ca^{2+}]_i$ and, thus, promotes the fidelity of excitation-contraction coupling (Fig. 4).

Many of the features of X-ROS signaling in the heart are also found in skeletal muscle (38, 51), but the signaling involves additional molecular components (38). One prominent new component in skeletal muscle is a calcium-conducting sarcolemmal "transient receptor potential" (TRP) channels (2, 6, 18, 48, 82) whose opening is enhanced by Nox2-derived ROS (Fig. 4). This signaling system is an important pathological component in Duchenne muscular dystrophy, where an increase in MT network density and Nox2 expression leads to a detrimental enhancement of X-ROS signaling (38). In addition, whether X-ROS may affect cardiac TRP channels or skeletal RyR1 under dystrophic conditions or with dynamic stretching paradigms (58) remains to be determined.

One striking finding was the speed at which X-ROS production was enhanced with mechanical stretch in muscle (Fig. 3), a finding that likely was due to specialization within the signaling pathway. It is well established that Nox2 and its regulatory subunits are localized to the t-tubule membranes in the heart (57, 62) and skeletal muscle (25). MTs bisect this junctional space (29) and may interact with the Nox2 subunit Rac (4). Since ROS are short-lived species that are confined to interaction within their immediate vicinity, this tight spatial organization is likely critical for the rapid and controlled signaling reported here (38, 57); signaling fidelity is necessary for mechanoactivation of X-ROS during the contraction cycle.

Nox2 activation also typically requires phosphorylation and recruitment of multiple cytosolic regulatory subunits to its transmembrane catalytic subunit ($gp91^{phox}$). The rapid nature of X-ROS signaling likely necessitates pre-assembly of the Nox2 complex to minimize steps that activate ROS production. In support of this, Nox2 subunits may tether to sarcolemmal membranes in hetero-multimeric complexes primed for rapid activation (50). With pre-assembly, mechanoactivation of this complex would then require an MT-dependent "switch" for activation. Rac is an MT-associated protein that associates with $gp91^{phox}$ separately from other regulatory subunits (50). Rac1 is a required subunit for Nox2 activation, and we have demonstrated that inhibition of Rac1 blocks stretch-activated X-ROS signaling (57).

Based on our results, we propose a minimal model in which MT-associated Rac1 is a critical activator of a pre-assembled Nox2 complex that generates superoxide during mechanical stress of contraction. While being exciting, this model requires validation and expansion in many key areas. Experimental demonstration of stretch-dependent Rac1 recruitment to $gp91^{phox}$ in an MT-dependent fashion is needed. In fact, no mechanistic detail is available that explains how the MT network acts as a dynamic mechanotransduction element in the muscle. Advances in other areas of MT biology will need to be exploited and adapted to address these key issues in striated muscle.

Future Directions

Most of these insights on contraction-generated ROS/RNS have been made possible by the real-time detection of these species in single, intact living cells that were either electrically activated to elicit contraction or mechanically stretched to assay passive mechanical responses. The combination of mechanical control of length and the measure of contractility in single isolated myocytes was pioneered by several groups (8, 10, 16, 27, 28, 29, 42, 79) and recently advanced in our laboratories (38, 57). While the approaches are still being refined, the availability of new commercial systems (38, 57) will undoubtedly increase the pace of research in this area.

Our laboratory and many other laboratories have used DAF-FM to monitor RNS/NO, and DCF (or CMDCF) to monitor ROS (*i.e.,* superoxide) in single-cell preparations (33, 55, 59). While DAF is fairly species specific, DCF is known to be sensitive to a number of RNS and ROS species, including H_2O_2, superoxide, and peroxynitrite, and, thus, requires careful experimentation and controls (56). Each of these probes, however, also suffers from time-dependent mitochondrial compartmentalization (72), light-induced oxidation of the dye (5), and limited signal:noise, all of which complicates the interpretation of results. This represents a limitation in the field, and there is a great need for more selective and rapidly responsive indicators.

New advances in ROS/RNS detection are now available with probes to permit the monitoring of ROS in specific cell compartments. For example, redox-sensitive green fluorescent protein (ro-GFP) (13) and glutaredoxin-tagged ro-GFP (23) allow the monitoring of local subcellular redox potentials. In addition, genetically encoded probes which are specific for H_2O_2 (Hy-Per) that can be targeted to cytosol, mitochondria, or nuclei are now available (44). However, it remains to be determined whether these genetically encoded sensors are suitable for experiments that demand the highest temporal resolution.

An important goal for this research is the identification of specific targets for therapeutic intervention within the ROS/RNS pathway. In diseases of both the heart (*i.e.,* cardiomyopathy, arrythmia) and skeletal muscle (*i.e.,* muscular dystrophy), pre-clinical studies targeting either the MT cytoskeketon (*i.e.,* MT network destabilization) (11, 32, 38, 39) or Nox-dependent ROS production (*i.e.,* non-specific Nox inhibition) (38, 83, 84) have shown benefit and, thus, provide proof of concept for these approaches. The use of new techniques, reagents, and models will undoubtedly reveal new mechanistic details on the mechanoactivation of ROS/RNS production and oxidative/nitrosative signaling that will translate to new more specific targets for therapeutic intervention. For example, can ROS production be reduced by specifically targeting Nox2? Can the mechanisms that are responsible for the increase in MT density be targeted, thus reducing the mechanoactivation of Nox2? One thing is clear, the efforts to understand the mechanoactivation of ROS/RNS production have yielded, and will continue to yield, exciting and important rewards.

Acknowledgments

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Abbreviations Used

Ca^{2+} = calcium
CaM = calmodulin
CF = carbon filament
cGMP = cyclic guanosine monophosphate
DCF = dichlorofluorescein
DHPR = dihydropyridine receptor
eNOS = endothelial nitric oxide synthase
ETC = electron transport chain
H_2O_2 = hydrogen peroxide
iNOS = inducible nitric oxide synthase
jSR = junctional sarcoplasmic reticulum
MT = microtubule
nNOS = neuronal nitric oxide synthase
NO = nitric oxide
Nox2 = NADPH oxidase 2
$ONOO^-$ = peroxynitrite
PLA2 = phospholipase A2
RNS = reactive nitrogen species
ro-GFP = redox-sensitive green fluorescent protein
ROS = reactive oxygen species
RyR = ryanodine receptor
SOD = superoxide dismutase
SR = sarcoplasmic reticulum
TRP = transient receptor potential
t-tubule = transverse tubule
XO = xanthine oxidase
X-ROS signaling = Nox2 dependent ROS generation during mechanical stretch; a pathway termed X-ROS signaling