ROS Production via P2Y₁-PKC-NOX2 Is Triggered by Extracellular ATP after Electrical Stimulation of Skeletal Muscle Cells

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Abstract

During exercise, skeletal muscle produces reactive oxygen species (ROS) via NADPH oxidase (NOX2) while inducing cellular adaptations associated with contractile activity. The signals involved in this mechanism are still a matter of study. ATP is released from skeletal muscle during electrical stimulation and can autocrinely signal through purinergic receptors; we searched for an influence of this signal in ROS production. The aim of this work was to characterize ROS production induced by electrical stimulation and extracellular ATP. ROS production was measured using two alternative probes; chloromethyl-2,7- dichlorodihydrofluorescein diacetate or electroporation to express the hydrogen peroxide-sensitive protein Hyper. Electrical stimulation (ES) triggered a transient ROS increase in muscle fibers which was mimicked by extracellular ATP and was prevented by both carbenoxolone and suramin; antagonists of pannexin channel and purinergic receptors respectively. In addition, transient ROS increase was prevented by apyrase, an ecto-nucleotidase. MRS2365, a P2Y₁ receptor agonist, induced a large signal while UTPyS (P2Y₂ agonist) elicited a much smaller signal, similar to the one seen when using ATP plus MRS2179, an antagonist of P2Y₂. Protein kinase C (PKC) inhibitors also blocked ES-induced ROS production. Our results indicate that physiological levels of electrical stimulation induce ROS production in skeletal muscle cells through release of extracellular ATP and activation of P2Y₁ receptors. Use of selective NOX2 and PKC inhibitors suggests that ROS production induced by ES or extracellular ATP is mediated by NOX2 activated by PKC.

Introduction

During exercise, several pathways are activated in skeletal muscle in order to maintain cellular homeostasis [1]. Skeletal muscle responds to exercise or electrical stimuli with an increased
generation of reactive oxygen species (ROS) [2]. ROS are produced during cell metabolism from different sources, among them xanthine oxidase, mitochondria and NADPH oxidase (NOX) [3]. NADPH oxidases are proteins that transfer electrons across biological membranes. In general, the electron acceptor is oxygen and the product of the electron transfer reaction is superoxide \( \text{O}_2^- \) which is then converted to hydrogen peroxide \( \text{H}_2\text{O}_2 \) by the enzyme superoxide dismutase (SOD) [4]. NOX family members are transmembrane proteins. The phagocyte NADPH oxidase 2 (NOX2) was the first identified and is the best studied member of the NOX family. Depending of the type of cell, in resting conditions \( \text{gp91}^{\text{phox}} \) and \( \text{p22}^{\text{phox}} \) are found primarily in the plasma membrane. Upon activation, the movement of cytoplasmic subunits, \( \text{p67}^{\text{phox}}, \text{p47}^{\text{phox}}, \text{p40}^{\text{phox}} \) and Rac GTPase from the cytoplasm to the membrane form the active NOX2 enzyme complex [4]. NOX2 can be activated by several mechanisms like \( \text{p47}^{\text{phox}} \) phosphorylation by PKC or by PI3K [5]. Skeletal muscle cells express NOX2 [6] and several authors suggest that this is one of the main sources of ROS during muscle contraction or electrical stimuli [7, 8], in addition, NOX protein subunits were detected in transverse tubules and triads isolated from rabbit skeletal muscle but not in sarcoplasmic reticulum vesicles [9], moreover, electrical stimulation induces NOX2 activation in skeletal muscle cells [7]; the mechanism of activation, however, is not fully understood.

ROS can modulate several pathways such as mitochondrial biogenesis, cell proliferation, muscle plasticity, phosphatase and kinase activities and antioxidant expression to maintain cellular homeostasis [5, 10–14]. For example, in myotubes, ROS stimulate ERK, CREB, early genes and glucose uptake induced by insulin [7, 15].

We have previously studied depolarization-induced calcium signals in skeletal muscle cells, describing a fast calcium transient involved in excitation-contraction coupling, and a slow, nuclei-associated calcium transient unrelated to contraction [16, 17]. The slow calcium signal is related with IP$_3$R activation [18, 19] and depends on ATP released from the stimulated muscle cells [20]. ATP signals in skeletal muscle through P2Y purinergic receptors [20]. P2Y are G-protein–coupled receptors that typically signal through the \( \beta\gamma \) subunits to activate phosphatidylinositol 3-kinase-\( \gamma \) (PI3K\( \gamma \)) and PKC [19, 21]. We hypothesized that ATP extruded from the muscle fiber increases ROS production via PKC-NOX2. Our results indicate that both electrical stimulation and extracellular ATP induced ROS production in skeletal muscle cells, at least partly through NOX2 activation via P2Y$_1$-PKC.

**Materials and Methods**

**Isolation of adult fibers**

We used C57/BL6j mice (6–8 weeks old) obtained from the Animal Facility at the Faculty of Medicine, University of Chile. Mice were sacrificed by exposure to isofluorane (5%) followed by cervical dislocation. Fibers were isolated from *flexor digitorum brevis* (FDB) muscle after enzyme digestion with type 2 collagenase (90min with 400U/ml; Worthington Biochemicals Corp., Lakewood, NJ, USA), and mechanical dissociation with fire-polished Pasteur pipettes, as described previously [17]. All the procedures performed in this work were approved by the Bioethics Committee of the Faculty of Medicine, University of Chile.

**ROS production**

ROS generation in skeletal muscle cells was evaluated using chloromethyl-2,7- dichlorodihydrofluorescein diacetate (DCF) probe (Eugene, OR). Muscle fibers were cultured on glass coverslips and incubated with 5 \( \mu \text{M} \) DCF during 15 min at 37°C. The cells were washed with PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$). Cultures on coverslip dishes were transferred to the confocal microscope (Carl Zeiss Pascal 5, LSM). DCF
fluorescence was detected using excitation-emission at $\lambda_{488}/\lambda_{510-540}$ nm. In all measurements, a control with laser excitation only was performed. The laser illumination was kept at a minimum (stand by position, 0.1–0.3% potency). All experiments were conducted in Krebs buffer with or without calcium (Krebs with calcium: 145 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.4; Krebs without calcium: 145 mM NaCl, 5mM KCl, 3.6 mM MgCl$_2$, 10 mM HEPES, 2mM EGTA, 5.6 mM glucose). The results were expressed as ($\Delta F/F_0$)$\times 100$ and fluorescence signals were processed as previously described [22], the fluorescence profiles were fit by linear regression. The DCF fluorescence change was normalized to the control to allow comparison of rates after the stimulus. NOX-specific inhibitor gp91dsTAT (5μM) (YGRKRRQRRRSTRRQRL–NH$_2$) [23] or apocynin (50μM) were used to verify the source of ROS. PnX1 inhibitor carbenoxolone (CBX) (5μM), purinergic antagonist suramin (10μM) were used to verify ATP release and purinergic receptor in NOX2 activation, PKC general inhibitor BIM (0.5μM) was used to determine the signal pathway. 25μM N-benzyl-p-toluene sulphonamide (BTS, from Sigma-Aldrich Co., St Louis, MO, USA) was used in all experiments as a fiber contraction inhibitor.

Fibers Transfection and H$_2$O$_2$ Measurement

H$_2$O$_2$ production was evaluated using a plasmid that encodes for HyPer protein. Muscle fibers were cultured on glass coverslips and transfected using Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) for 2 h (1 μg DNA/3 μL) during collagenase digestion of FDB muscle. H$_2$O$_2$ was determined 24h post transfection, as described previously [24]. HyPer imaging was achieved using an inverted Olympus IX81 microscope with a 40x objective (numerical aperture, N.A. 1.3). HyPer fluorescence was detected using an excitation/emission wavelength $\lambda_{\text{exc1}}-\lambda_{\text{exc2}/\lambda_{\text{em}}}$ = 420–490/520 nm. The ratio between the signals excited with 490 and 420 nm was used to determine the presence of H$_2$O$_2$. HyPer has a 420 nm excitation peak that decreases in proportion to the increase at 490 nm [25]. Fluorescence emitted at 520 nm was shown. Each experiment was performed alongside the effect of laser excitation alone. Noise in the images was removed using Image J Filters (http://rsbweb.nih.gov/ij/). All experiment were conducted in Krebs buffer (145 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.4). We used MRS2179 (1μM), MRS2365 (20μM) and UTP$\gamma$S (20μM) to verify purinergic receptor participation in the process. All reagents were obtained from Sigma-Aldrich Co., St Louis, MO, USA.

Muscle fibers stimulation

Adult muscle fibers were stimulated with either, electrical stimulation (ES) or extracellular ATP. Electrical stimulation was a single sequence of 270 square pulses of 0.3 ms duration at 20 Hz or 90 Hz (lasting 13.5 or 3s) using electrodes that consist of a row of six platinum wires intercalated 0.5 cm apart with alternate polarity across a circular plastic holder that fits in the dish, as described previously [26].

Data analysis

All values are expressed as means ± standard error of the mean (S.E.M.) from at least three different experiment. The significance of differences was evaluated using Student’s t-test for paired data and one-way ANOVA followed by Dunnett’s post-test for multiple comparisons or Bonferroni’s post-test for multiple paired comparisons. P<0.05 was considered to be statistically significant. GraphPad PRISM 5.03 software (GraphPad Software Inc, LA, USA) was used for data fitting.
Results

Electrical stimulation and extracellular ATP increase ROS production in skeletal muscle fibers

ES induces an increase in extracellular ATP [26] and we studied ROS production using the DCF probe. The probe fluorescence is clearly higher minutes after electrical stimulation compared with the unstimulated control (Fig 1A). Because autoxidation of the probe when exposed to light scan occurs, generating artifactual signals, the slopes of the fluorescence increase after stimulation were quantified and normalized against the control condition, as described previously by other group [27, 28]. Thus, the ES induced a sustained fluorescence increase with a slope of 1.89 fold over control condition (Fig 1B and 1C). In addition, the exogenous administration of ATP (10μM) increased the fluorescence of DCF similarly, in magnitude, timing and speed, compared to ES (2.01 fold over control) (Fig 1B and 1C). Finally, we have previously reported that ATP release from muscle cells is frequency-dependent, ATP is released from muscle fibers at low frequency but not at high frequency [26]; in agreement with those results, we observed an increase of ROS production at 20Hz but not at 90Hz, suggesting that ROS increase during muscle activity is dependent on release of extracellular ATP (Fig 1D).

doi:10.1371/journal.pone.0129882.g001
ROS production induced by electrical stimulus of adult skeletal muscle fibers is dependent on ATP release and purinergic receptor P2Y₁

We evaluated whether ATP extrusion to the extracellular medium after electrical stimulation is capable to increase ROS production in adult fibers. Accordingly, the increase in ROS production induced by ES was prevented when fibers were pre-incubated with CBX, an inhibitor of PnX channel (Fig 2A and 2B). Furthermore, pretreatment with the purinergic receptor antagonist suramin inhibited electrical stimulus-dependent ROS production (Fig 2C and 2D).

To confirm that extracellular ATP increases the specific production of H₂O₂, muscle fibers were transfected with the plasmid encoding the cytoplasmic fluorescent protein HyPer [25]; successful transfection of a fiber with the GFP containing probe can be seen in Fig 3A (left panel). ATP caused a transient increase in HyPer fluorescence of about 20% above basal, which reached a maximum after 3min and returned to resting condition 7 min post stimulation (Fig 3A, right panel and 3B). Similarly, electrical stimulation at 20 Hz induced HyPer fluorescence increase, no increment in H₂O₂ production was detected after electrical stimulation in fibers pre-incubated for 30 min with 2 U/ml apyrase, a nucleotidase that metabolizes extracellular ATP to AMP (Fig 3C). Considering that P2Y receptors appear to be involved in this H₂O₂ release, we measured the impact of suramin on these fibers and found a significant decrease in the ROS production (Fig 3D).
increase, we tried to identify the particular isoform that participate in this process. Recently our laboratory showed that skeletal muscle mainly expresses P2Y1 and P2Y2 purinergic receptors [29]. So we used agonists and antagonist of these receptors [30]. As the maximal fluorescence was detected 3 min post electrical stimulation, we used this time for all measurements.

We observed a similar H2O2 increase when using exogenous ATP or Mrs2365 (P2Y1 agonist) (18 ± 2% and 23 ± 4% respectively), while UTPγS (P2Y2 and P2Y4 agonist) and Mrs2179 (P2Y1 antagonist) plus ATP did not cause any H2O2 increase (Fig 3D). Finally, when the experiments were performed in the absence of external calcium (data not shown), similar results for electrical stimulation and ATP addition were obtained, suggesting that calcium entry, either through P2X purinergic receptors or through other pathways is not playing a relevant role in this effect. These results suggest that ATP released from the muscle fibers during ES increase H2O2 production in adult muscle; this effect appears to be mediated by the P2Y1 purinergic receptor.

Fig 3. Exogenous ATP increases H2O2 production via P2Y1 purinergic receptor. Muscle fibers were isolated and transfected with HyPer plasmid, 24h post transfection the cells were stimulated. A, H2O2 generation was measured before and after ATP (10μM) addition. Left panel shows a representative image of HyPer transfection, right panel image fluorescence in pseudo-color. The scale bar represents 50μm. B, kinetics of extracellular ATP-induced H2O2. C, muscle fibers were transfected with with HyPer plasmid and stimulated with ES in absence or presence of apyrase (2U/ml). Maximal fluorescence was plotted D, C. Effect of Mrs2365, Mrs2179, UTPγS or exogenous ATP, maximal fluorescence was plotted (n = 4), *p<0.05, **p<0.01.

doi:10.1371/journal.pone.0129882.g003
ROS production induced by extracellular ATP is dependent on NADPH oxidase 2 enzyme (NOX2).

NOX2 is one of the main sources of ROS in skeletal muscle [8] but its activation by ATP has not been shown in adult skeletal muscle fibers. In order to study the ATP-dependent source of ROS in adult muscle, ES-dependent ROS production was studied in the presence of apocynin, a NOX2 inhibitor (Fig 4A and 4B). Apocynin completely blocked H2O2 production, but as it is a nonspecific NOX2 inhibitor [31], we used gp91 ds-TAT peptide which blocks the recognition site between the NOX2 subunits [23]. ES-dependent ROS increase was inhibited by ds-tat peptide gp91 (Fig 4C and 4D) and not by the scrambled peptide used as control. In addition,

Fig 4. Electrical stimulation and exogenous ATP increase ROS production via NOX2. Muscle fibers were isolated, loaded with DCF (30min) and stimulated with electrical stimulation (ES) or exogenous ATP A, representative traces of DCF fluorescence under control conditions or stimulated with ES in the absence or presence of apocynin. B, Muscle cells were stimulated with ES and the slope of fluorescence was analyzed (see Materials and Methods). C, Representative traces of DCF fluorescence under control conditions or stimulated with ES in the absence or presence of gp91 ds-TAT or scrambled peptide. D, muscle cells were stimulated with ES and the slope of fluorescence was analyzed (see material and method). E, representative traces of DCF fluorescence under control or stimulated with exogenous ATP in the absence or presence of gp91 ds-TAT or scrambled peptide. F, muscle cells were stimulated with ES and the slope of fluorescence was analyzed (see material and method) (n = 5, *p<0.05, **p<0.01, ***p<0.001).

doi:10.1371/journal.pone.0129882.g004
exogenous ATP increased ROS production and this effect was blocked with gp91 ds-tat peptide (Fig 4E and 4F). These results strongly suggest that ROS production induced by either ES or exogenous ATP is mediated by NOX2.

ATP-dependent ROS production via NOX2 requires PKC activation

To study the possible role of PKC in ATP-dependent ROS generation we used the DCF probe. Incubation with BIM, a non selective general PKC inhibitor, strongly decreased the effect of ATP stimulation on ROS production (Fig 5A and 5B). In addition, in muscle fibers transfected with HyPer, BIM decreased significantly the ATP-dependent ROS increase. Similar effect was
observed when using rottlerin, a PKCδ inhibitor (Fig 5C). Finally, we observed that the phorbol 12-myristate 13-acetate (PMA) mimicked the effect of exogenous ATP on ROS production. These results suggest that diacylglycerol-dependent PKCs mediate ROS production induced by extracellular ATP (Fig 5C).

Discussion

In this work, we describe a novel mechanism for ROS production via PKC-NOX2 activation induced by extracellular ATP released from adult cultured muscle cells after electrical stimulation which mimics the physiological activation of skeletal muscle during exercise (Fig 6). Electrical stimulation is able to modulate several cascades triggered by ATP release and P2Y purinergic receptors such as glucose uptake, calcium signaling, gene expression and muscle plasticity [20, 26, 29, 32]. Interstitial ATP concentration increases during muscle contraction and this increase is intensity-dependent, possibly reaching micromolar concentrations in the T-tubule lumen [33, 34]. 10 μM was used to stimulate the muscle cells, a concentration previously reported as sufficient to induce effects on skeletal muscle cells [29]. Considering that purinergic receptors are located in the transverse tubule membrane and the presence of active ectonucleotidases in that region, it is not possible to calculate the actual ATP concentration that reaches the receptor after diffusion and degradation.

Different cell types increase ROS production upon membrane depolarization. For example, endothelial cells from human umbilical vein (HUVEC) increase ROS production after incubation with a high K⁺ concentration [35]. Previously our laboratory reported that electrical
stimulation induces ROS production from NOX2 in muscle cells and this effect is Cav1.1 dependent [7]. During depolarization, muscle cells release ATP through PnX1 channel; this process is prevented by nifedipine, a Cav1.1 blocker [26]. In addition, these proteins are located in the T-tubule and we recently showed that both proteins appear to be part of the same signaling complex [36]. We show here that electrical stimulation increases ROS production and that this increase was blocked by CBX, an inhibitor of PnX1 channel; this effect was mimicked by exogenous ATP, suggesting that ATP released via PnX1 during ES is necessary to increase ROS production during depolarization of skeletal muscle fibers.

There are several sources of ROS in skeletal muscle cells, such as mitochondria metabolism, xanthine oxidase, NOX2 and NOX4 [3]. However, the main ROS source during muscle contraction appears to be NOX2 [8]. Extracellular ATP can induce NOX2 activation in different cell models as macrophages and lung epithelial cells [37, 38]. Our study shows that ROS production induced by ES or exogenous ATP was prevented by both apocynin and gp91dsTAT, known NOX2 inhibitors. Our results suggest that ROS production induced by ES is dependent on ATP released from muscle cells via PnX1, which in turn induces NOX2 activation. These results are consistent with previous reports; for example, in myoblasts, extracellular ATP increases ROS production via NADPH oxidase, promoting cell proliferation [14].

DCF probe is sensitive to multiple types of ROS, including several reactive oxygen and nitrogen species [39]. Probably ES (via extracellular ATP) can induce oxidation of the probe by different oxidative species. For example, superoxide anion produced by NOX2 can dismutate to H2O2 in the cytoplasm via superoxide dismutase (CuZnSOD). However, superoxide can react with nitric oxide to form peroxynitrite (ONOO-). The rate of reaction to produce ONOO- is larger than the speed of enzymatic dismutation (7x10⁹ vs 2x10⁸ M⁻¹s⁻¹ respectively) [10]. To determine the reactive species induced by extracellular ATP, we used the protein Hyper which has high sensitivity and specificity toward hydrogen peroxide and is based on the properties of OxyR, an E. coli protein especially devoted to sensing H2O2 [25]. Extracellular ATP induced a transient H2O2 increase which was similar to that already reported by us in skeletal muscle cells depolarized with high K⁺ [24]. In addition, in fibers treated with apyrase to completely hydrolyze ATP, ES did not increase ROS production. These results suggest that extracellular ATP plays a key role on ROS production induced by ES.

Skeletal muscle cell express several purinergic receptors of the P2X and P2Y families [21]. We previously showed that the most abundant P2Y receptor subtypes in adult skeletal muscle are P2Y₁, P2Y₂, P2Y₄ and P2Y₆. Moreover, P2Y₁ and P2Y₂, two of the ATP/ADP-responsive receptors, are highly expressed in relation to the other subtypes [29]. In this work we provided evidence pointing to P2Y₁ as the target for extracellular ATP to induce ROS increase. The P2Y₁ receptor has an important role in skeletal muscle function. For example, extracellular ATP acts via P2Y₁ receptors to stimulate acetylcholinesterase and acetylcholine receptor expression [40]. Extracellular ATP appears to inhibit chloride channels in mature mammalian skeletal muscle by activating P2Y₁ receptors [41] and participates in up-regulation of Na/K-ATPase activity during muscle activity [42]. This receptor is G-protein (Gq) coupled and downstream increases IP₃ production and PKC activation via PLC [43]. NOX2 can be activated by different mechanisms, including p47phox subunit phosphorylation by PI3K or PKC [4]. Skeletal muscle expresses several PKC isoforms. We previously demonstrated that myotubes depolarization activate NOX2 via PKCs and that in muscle fibers insulin leads to activation of NOX2 by PKCδ [15, 44]. In this study, we showed that both BIM and rottlerin prevented ROS production in response to extracellular ATP. In addition, the increase ROS production was mimicked by PMA, an analogous of DAG. These results suggest that extracellular ATP induces NOX2 activation via P2Y₁/PKC and that this PKC is DAG dependent, however the specific isoform of PKC involved is yet to be identified. It is important to note that all inhibitors used in this work...
were tested first in the absence of ATP stimulation and show no effect on basal H₂O₂ production, suggesting lack of non-specific effects on NOX2. We probed the effect of suramin, CBX, BIM and rottlerin, in the fluorescence increase induced by exogenous H₂O₂ addition (data not shown), only suramin has an antioxidant effect in itself; however the role of ATP receptor is validated using exogenous ATP and P2Y1 agonist.

Intracellular ROS may produce different effects such as mitochondria biogenesis, antioxidant enzymes expression and calcium signals [12]. Physiological regulation of cell signaling events by ROS occurs primarily via selective modification of cysteine residues within proteins [45]. Since cells possess specific systems to reverse these protein redox modifications, transient modifications of cysteine residues are likely to be central to the mechanisms underlying redox regulation of normal cell function [45]. ROS can also induce changes in intracellular calcium levels, which are the result of oxidative modification of calcium channels or other proteins involved in calcium signaling [2]. In cardiomyocytes, myotubes and isolated triads from skeletal muscle, ROS originated from NOX2 induce calcium release viaryanodine receptor (RyR) [7, 9]. It has been suggested that this ROS production may be necessary for the excitation-contraction (E-C) coupling process [9]. For example, NOX2 is located in the membrane of the t-tubules and in isolated triads, NOX2 increase calcium release through RyR oxidation [9, 46]. The RyR1 isoform [46] is essential for skeletal muscle excitation-contraction (E-C) coupling, a process that requires close physical interactions between RyR1 present in junctional SR and Cav1.1, which acts as the voltage sensor protein in T-tubules responsible for RyR1 activation during E-C coupling. Despite that Sakellariou already described that NOX2 is involved in ROS generation evoked by contraction [8], our work shows new relevant data about the mechanism involved, evocating a new pathway associated to E-C coupling that depends of ATP receptor, panexins and NOX2, in heart muscle, NOX2 can induce RyR oxidation increasing calcium release from sarcoendoplasmic reticulum [27]. Calcium release during E-C is essential for muscle contraction. The privileged location of the skeletal muscle NOX2 at the T-tubules and its activation during contraction opens the possibility that conditions of increased muscle activity, such as exercise, could potentiate calcium release through NOX-induced RyR1 redox activation. Recently, Riquelme et al. [48] showed that ATP released from muscle fibers via PnX1 is required for potentiating skeletal muscle contraction and that this effect is mediated by P2Y, purinergic receptors. We suggest that ES induces ATP release from muscle fibers via PnX1; extracellular ATP can activate P2Y receptors, followed by downstream PKC activation. PKC induces NOX2 activation, increasing ROS production; ROS could induce RyR1 redox activation, enhancing the channel activity (Fig 6). At least one important function influenced by ROS-dependent RyR1 activation has been described in skeletal muscle cells; insulin elicited GLUT4 translocation to the membrane depends on calcium release after H₂O₂ oxidation of RyR1 in L-6 cells [49]. More research is needed to ascertain whether other important functions of the muscle cell may be regulated in the same way.

Acknowledgments
We thank Mónica Silva for skillful technical assistance. We also thank Yildy Utreras for transfection of fibers.

Author Contributions
Conceived and designed the experiments: MC AD SB AE EJ. Performed the experiments: AC CC AD AE. Analyzed the data: AD CC. Contributed reagents/materials/analysis tools: EJ. Wrote the paper: EJ AD AE.
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