NOX2-dependent ROS is required for HDAC5 nuclear efflux and contributes to HDAC4 nuclear efflux during intense repetitive activity of fast skeletal muscle fibers

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Submitted 27 April 2012; accepted in final form 24 May 2012


In skeletal muscle, myocyte enhancer factor 2 (MEF2) is a master regulator of fiber type-specific gene expression (4). The transcriptional activity of MEF2 is partially repressed by class IIa histone deacetylases (HDACs; HDACs 4, 5, 7, and 9) through the formation of HDAC-MEF2 complexes within the cell nucleus. Translocation of HDACs from the nucleus to the cytoplasm relieves the HDAC repression on MEF2. Skeletal muscle activity results in HDAC4 translocation from nucleus to cytoplasm by activating calcium-dependent protein kinase IIa histone deacetylases (HDACs; HDACs 4, 5, 7, and 9) in cardiac muscle. Here we use HDAC-GFP fusion proteins expressed in isolated adult mouse flexor digitorum brevis muscle fibers to study ROS mediation of HDAC localization in skeletal muscle. H2O2 causes nuclear efflux of HDAC4-GFP or HDAC5-GFP, which is blocked by the ROS scavenger N-acetyl-L-cysteine (NAC). Repetitive stimulation with 100-ms trains at 50 Hz, 2/s (“50-Hz trains”) increased ROS production and caused HDAC4-GFP or HDAC5-GFP nuclear efflux. During 50-Hz trains, HDAC5-GFP nuclear efflux was completely blocked by NAC, but HDAC4-GFP nuclear efflux was only partially blocked by NAC and partially blocked by the calcium-dependent protein kinase (CaMK) inhibitor KN-62. Thus, during intense activity both ROS and CaMK play roles in nuclear efflux of HDAC4, but only ROS mediates HDAC5 nuclear efflux. The 10-Hz continuous stimulation did not increase the rate of ROS production and did not cause HDAC5-GFP nuclear efflux but promoted HDAC4-GFP nuclear efflux that was sensitive to KN-62 but not NAC and thus mediated by CaMK but not by ROS. Fibers from NOX2 knockout mice lacked ROS production and ROS-dependent nuclear efflux of HDAC5-GFP or HDAC4-GFP during 50-Hz trains but had unmodified Ca2+ transients. Our results demonstrate that ROS generated by NOX2 could play important roles in muscle remodeling due to intense muscle activity and that the nuclear effluxes of HDAC4 and HDAC5 are differentially regulated by Ca2+ and ROS during muscle activity.

Therefore, whether or how HDAC5 is regulated during skeletal muscle activity, especially in fast fibers, requires further investigation. The issue of possible selective or common mechanisms for nuclear cytoplasmic movements of different class IIa HDACs is important in regard to both possible distinct and redundant effects of these transcriptional regulators in muscle fiber nuclei.

In addition to phosphorylation as a mechanism for regulation of nuclear cytoplasmic distribution of HDACs, a novel reactive oxygen species (ROS) signaling pathway for regulation of class IIa HDAC translocation has been recently reported (1,31). ROS generated during cardiac hypertrophy oxidize HDAC4 at Cys-667 and Cys-669 to form a disulfide bond, which exposes the nuclear export signal on HDAC4. This leads to the nuclear export of HDAC4 from cardiac cells. Alignment of HDAC4 with other class II HDACs shows that the two critical cysteines are conserved among HDAC4, 5, 7, and 9 (1). Therefore, we hypothesize that both HDAC4 and 5 should respond to ROS generation by skeletal muscle activity and translocate from nucleus to cytoplasm.

It is well established that contracting skeletal muscles produce ROS (13, 17, 35), although the amount of ROS production is still an open question (13). Growing evidence suggests that intracellular ROS production is required for the remodeling that occurs in skeletal muscle in response to repeated bouts of endurance exercise (12, 35). ROS is also reported to regulate redox-sensitive kinases, phosphatases, and transcription factors in skeletal muscle (35). ROS production is greatly increased in isolated mouse skeletal muscle stimulated by certain patterns of activity (41).

Both mitochondria and NADPH oxidases (NOXs) are considered as potential sources of ROS production (36), and NOX2 is expressed in skeletal muscle fibers (44). However, there are few studies on how the changes in redox status regulate gene expression in skeletal muscle (35) and whether...
particular subcellular sources of ROS are involved. Furthermore, no information is available on the subcellular sources and functional roles of ROS in the regulation of the nuclear/cytoplasmic distribution of HDAC4 or HDAC5 during skeletal muscle contraction.

The experiments presented in this study were designed to determine the possible roles of ROS in the regulation of HDAC4 and 5 during skeletal muscle activity. We find that during intense activity of fast muscle fibers HDAC5 is translocated out of fiber nuclei exclusively in response to NOX2-dependent ROS production. In contrast, during the same intense activity in the same type of fast fibers, HDAC4 is also translocated out of fiber nuclei, but this response is only partially dependent on NOX2 generated ROS and partly dependent on CaMK activation.

**METHODS**

**Animals.** All animals were housed in a pathogen-free area at the University of Maryland, Baltimore. The animals were killed according to authorized procedures of the Institutional Animal Care and Use Committee, University of Maryland Baltimore, by regulated delivery of compressed CO2 overdose followed by cervical dislocation. CD1 mice were purchased from Charles River. NOX2 knockout (KO; B6.129S6-Cybbtm1Din/J, stock number 002365) and wild-type littermate control (C57BL/6J) were purchased from The Jackson Laboratory.

**Infection of recombinant adenoviruses in muscle fibers.** Single muscle fibers were enzymatically dissociated from flexor digitorum brevis (FDB) muscles of female 4- to 5-wk-old CD-1 mice or from NOX2 KO and wild-type control mice and cultured as described previously (19). This age mouse is convenient due to ease of collagenase digestion of FDB muscle and for adeno viral transduction. Isolated fibers were cultured on laminin-coated glass coverslips, each glued over a 10-mm-diameter hole through the center of a plastic petri dish. Fibers were cultured in MEM containing 10% FBS and 50 μg/ml gentamicin sulfate in 5% CO2 (37°C). Virus infections were performed as previously described (19).

**Microscopy, image acquisition, and analysis.** To study the localization of HDAC4-GFP or HDAC5-GFP, FDB fibers were infected with adenovirus containing HDAC4-GFP or HDAC5-GFP cDNA. Two days after infection, culture medium was changed to Ringer’s solution (in mM; 135 NaCl, 4 KCl, 1 MgCl2, 10 HEPES, 10 glucose, and 1.8 CaCl2, pH 7.4). The culture dish was mounted on an Olympus IX70 inverted microscope equipped with an Olympus PflugView 500 laser scanning confocal imaging system. Fibers were viewed with an Olympus 60×/1.2 NA water immersion objective. Excitation light was delivered by a 150-W Xenon arc-lamp and gated by a computer-controlled shutter (Vincent Associates). Fibers were illuminated at 360 ± 10 nm, and the fluorescence emitted at 405 ± 15 and 485 ± 20 nm was detected simultaneously.

For fluorescence data from CM-H2DCFDA, the AOI was placed in cytoplasm area (see Fig. 1A for demonstration of typical AOI). The average fluorescence of pixels in each AOI was also quantified using Imaging J and normalized to the initial value in each AOI, to give a set of values of fluorescence-to-average resting fluorescence (F/F0) at successive time points in each fiber. Data from successive fluorescence images were fit by a straight line to obtain the slope of increasing fluorescence. Results are expressed as the means ± SE. Paired t-test was used for data from the same fiber before and after treatment.

**MEF2 activity reporter assay.** For MEF2 reporter assay, cultured muscle fibers were infected with adenovirus encoding MEF2-driven luciferase reporter (45) for 48 h. The cultures were then stimulated for 1 h with 50-Hz trains. The cultures were kept in the incubator for another 24 h. Cultures were then lysed in passive lysis buffer (Promega). Luciferase activity was determined with a luciferase assay kit (Promega).

**Indo-1 measurements.** Single fiber indo-1 ratiometric recordings and analysis were performed as previously described (6, 37) with minor modifications. When required, cultured FDB fibers from wild-type and NOX2 KO mice were loaded with indo-1 AM at 1 μM for 60 min at 22°C. The fibers were washed thoroughly to remove residual indo-1 AM and incubated at 22°C for another 30 min to allow dye conversion. The culture dish was mounted on an Olympus IX71 inverted microscope, and fibers were imaged with an Olympus 60×/1.20 NA water immersion objective. Excitation light was delivered by a 150-W Xenon arc-lamp and gated by a computer-controlled shutter (Vincent Associates). Fibers were illuminated at 360 ± 10 nm, and the fluorescence emitted at 405 ± 15 and 485 ± 20 nm was detected simultaneously.

Fluorescence emission from a region of interest within a fiber was selected by an image-plane pinhole and measured with an ambient temperature photomultiplier tube (Hamamatsu). Four cycles of measurements were taken for each fiber, and the results were averaged. To correct for autofluorescence and background light scatter, averages from individual unloaded fibers were subtracted from same-day experimental values. The emission signals were sampled at 2 KHz and digitized using a built-in AD/DA converter of an EPC10 amplifier and the acquisition software Patchmaster (HEKA Instruments). Field stimulation (square pulse, 14 V × 1 ms) was produced by a custom pulse generator through a pair of platinum electrodes. The electrodes were closely spaced (0.5 mm) and positioned directly above the center of the objective lens to achieve semilocal stimulation. Only fibers exhibiting reproducible and consistent responses to field stimulation of alternate polarity were used for the analysis.

**Fluo-4 measurements.** High-speed fluo-4 fluorescence confocal microscopy measurements were carried out on a Zeiss LSM 5 Live system as previously described (32, 38). Fibers were loaded with fluo-4 AM (Invitrogen) at 2 μM for 30 min at 22°C. The fibers were washed thoroughly to remove residual fluo-4 AM and incubated at 22°C for another 30 min to allow dye conversion. The culture dish was mounted on a Zeiss Axiovert 200M inverted microscope on a Zeiss LSM 5 Live confocal system and viewed with a 63×/1.2 NA water immersion objective. Excitation was provided by a 488-nm laser with emission detected using a long-pass 505-nm filter. Fibers were stimulated using a protocol consisting of a single stimulus followed by a pulse train (400 ms; 100 Hz) separated by an interval of 400 ms at a set time after the start of a confocal scan. Field stimulation (square pulse, 14 V × 1 ms) was produced by a custom pulse generator through a pair of platinum electrodes. Fibers were selected based on the above inclusion criteria as well as an additional criterion of minimum fiber movement in the line scan plane to prevent movement artifacts in signals. The confocal system was operated in line scan mode, with images collected at 100 μs/line for 1.4-s acquisition time using Zeiss LSM 5 live software. Average intensity of fluorescence within selected regions of interest (ROI) was measured with a Zeiss LSM Image Examiner (Carl Zeiss). Images were background corrected by 10.220.33.2 on June 23, 2017 http://ajpcell.physiology.org/ Downloaded from
Fig. 1. Monitoring reactive oxygen species (ROS) generation with ROS-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) in flexor digitorum brevis (FDB) fibers in resting condition or treated with H$_2$O$_2$. A and B: FDB fibers were first loaded with CM-H$_2$DCFDA. Then, 8 fibers were imaged every 10 min for 60 min without any treatment (A), or 8 other fibers were first imaged for 30 min under control conditions and then imaged for another 30 min in the presence of 40 $\mu$M H$_2$O$_2$ (B). Images were quantified and fit with a linear fit to obtain slopes for the first 30 min and second 30 min, respectively, which reflects the speed of CM-H$_2$DCF oxidation by endogenous ROS and/or H$_2$O$_2$. Addition of H$_2$O$_2$ (B) significantly increased the slope of CM-DCF fluorescence by enhancing the speed of ROS generation. Scale bar = 10 $\mu$m. White line in A demonstrates how the cytoplasmic areas of interest (AOI) is defined. C: preincubation with 10 mM N-acetyl-L-cysteine (NAC) antagonized the increase in the rate of CM-H$_2$DCF oxidation by H$_2$O$_2$ addition. Data were from 5 fibers. D: addition of 10 mM NAC did not significantly change the slope of fluorescence increase. Data were from 7 fibers. Dashed lines in A–D are extrapolation of the linear fit of the first 30-min data. In A and C, the dashed lines are nearly superimposed on the measured data. However, in B the measured data are significantly higher than the extrapolation. E: ratio of slopes of the second 30 min over the first 30 min. Columns from left to right are data from A–D respectively. F/F$_0$, fluorescence-to-average resting fluorescence. **$P < 0.01$. 

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corrected by subtracting an average value recorded outside the cell. The average resting fluorescein (F₀) value in each ROI before electrical stimulation was used to scale fluo-4 signals in the same ROI as ΔF/ΔF₀.

All experiments (CM-H₂DCFDA or HDAC4 or 5-GFP fluorescence imaging, fiber stimulation, and calcium measurement) were carried out at room temperature, 21–23°C.

Data analysis and statistics. All values are presented as means ± SE. Statistical significance was tested with ANOVA or t-test as appropriate. For all comparisons, the level of statistical significance was set at P < 0.05.

RESULTS

Monitoring ROS production in muscle fibers due to application of H₂O₂. We first examined the increase in intracellular ROS levels in response to H₂O₂ addition to cultured FDB fibers by monitoring fluorescence of the ROS-sensitive dye CM-H₂DCFDA. Fibers were loaded with CM-H₂DCFDA and imaged every 10 min. CM-H₂DCFDA is hydrolyzed to CM-DCF in the cell, and CM-DCF is oxidized to form highly fluorescent CM-DCF (33, 47) in the presence of the appropriate oxidant (15). The rate of change (slope) of the CM-DCF fluorescence signal is a measure of the rate of oxidative CM-DCF generation due to ROS present within the cell (22, 33).

Figure 1A presents images of a fiber maintained under control conditions for 60 min of observation. The CM-DCF fluorescence increases continuously with time, even in the absence of added H₂O₂, indicating a resting rate of ROS production under control conditions. To characterize the resting rate of ROS production, a group of fibers was loaded with CM-H₂DCFDA and the CM-DCF fluorescence was observed for 60 min under control conditions without any addition of H₂O₂. Straight-line segments were fit to the CM-DCF normalized fluorescence. The average slope of the normalized fluorescence for the first 30 min and second 30 min, 1.57 ± 0.02 and 1.62 ± 0.02%/min, respectively, was not significantly different (8 FDB fibers, P > 0.05), indicating a constant steady rate of ROS production throughout the entire 60 min period. This is shown graphically by the dashed line in Fig. 1A (also Figs. 3 and 6), which represents an extension of the solid straight line fit to the data from 0 to 30 min to the time interval from 30 to 60 min. In another group of fibers, after the first 30-min period, 40 μM H₂O₂ was added to the culture dishes (Fig. 1B). The slopes of the CM-DCF fluorescence increase for the first 30 min (without H₂O₂) and the second 30 min (with H₂O₂) were significantly different (0.81 ± 0.06 and 2.73 ± 0.47%/min, respectively; 8 FDB fibers; P < 0.01). The increase in fluorescence above the dashed extrapolation of the solid line fit to the first 30 min demonstrates that addition of 40 μM H₂O₂ effectively increased ROS production in the cultured fibers. In the presence of 10 mM of added ROS scavenger N-acetyl-l-cysteine (NAC), addition of H₂O₂ to the culture dishes did not change the slope of CM-DCF fluorescence (Fig. 1C; 0.84 ± 0.07 and 1.09 ± 0.13%/min for the first 30 min and second 30 min, respectively; 5 FDB fibers; P > 0.05). In a separate group of FDB fibers, we tested the effects of NAC on the slope of CM-DCF fluorescence without other treatment (Fig. 1D). After 30 min at rest, 10 mM NAC were added but the rate of CM-DCF oxidation was not significantly changed (1.49 ± 0.29 and 1.15 ± 0.12%/min, respectively, for the first and second 30 min; 7 FDB fibers; P > 0.05).

As judged from the rate of change of CM-DCF fluorescence, the normalized rate of CM-DCF production varied considerably from experiment to experiment, even under control conditions. To quantify the effects of fiber manipulation on ROS production, we normalized the slope of CM-DCF fluorescence during the test period to the slope determined in the same fiber in the control period (Fig. 1E). When no change in conditions was made (Fig. 2A), the ratio of the slope in the second to the first interval was 1.07 ± 0.03 (Fig. 1E, first bar), not significantly different than 1.00 (P > 0.05). When H₂O₂ was applied in the second interval, the slope increased to 3.33 ± 0.46 of control (Fig. 1E, second bar from left; P < 0.01), and this increase was eliminated in the presence of NAC during both the control and test intervals (Fig. 1E, third bar from left). NAC itself caused no significant change in the rate of CM-DCF fluorescence increase (0.90 ± 0.13; Fig. 1E, fourth bar; P > 0.05).

Application of H₂O₂ causes HDAC4-GFP and HDAC5-GFP translocation from nucleus to cytoplasm. We next examined whether increasing intracellular ROS by addition of H₂O₂ to the Ringer solution has any effects on the nuclear localization of HDAC4-GFP or HDAC5-GFP fusion protein constructs expressed in adult skeletal muscle fibers. Under control conditions, HDAC4-GFP is concentrated in FDB fiber nuclei (Fig. 2A). During the first 30 min no reagent was added, and the nuclear HDAC4-GFP remained constant (Fig. 2A, open circles, −30 and 0 min). Subsequent application of H₂O₂ (40 μM) caused a continuous decline in nuclear HDAC4-GFP (Fig. 2A). The nuclear mean pixel fluorescence declined by 20 ± 2% (P < 0.01) by the end of the 60-min observation period in the presence of H₂O₂. We also examined the effects of H₂O₂ on the nuclear efflux of HDAC5-GFP. As reported previously (20), HDAC5-GFP is more highly localized in muscle fiber nuclei under resting conditions than is HDAC4 (mean value of n/c for HDAC4-GFP from 10 nuclei from 5 fibers = 2.87 ± 0.24; mean value of n/c for HDAC5-GFP from 12 nuclei from 6 fibers = 6.22 ± 0.41). As was the case for HDAC4, addition of H₂O₂ also caused a net nuclear efflux of HDAC5-GFP (Fig. 2C, open circles). The nuclear mean pixel fluorescence from HDAC5-GFP declined by −22 ± 3% (P < 0.01) by the end of the 60-min observation period in the presence of H₂O₂.

We then investigated the effects of NAC on the H₂O₂-induced nuclear efflux of HDAC4 and 5-GFP. Under resting conditions before H₂O₂ addition, NAC has no effects on the localization of HDAC4-GFP (Fig. 2B) or HDAC5-GFP (Fig. 2D). In the presence of NAC, the H₂O₂-induced nuclear efflux of both HDAC4-GFP and of HDAC5-GFP was completely blocked (Fig. 2, B and D), establishing that oxidation underlies the effect of applied H₂O₂ on nuclear efflux of HDAC4 and 5-GFP.

Intense FDB muscle fiber activity increases intracellular ROS. Skeletal muscle contraction can generate ROS (33, 41). We thus tested whether 100-ms trains at 50 Hz with 2 trains/s (hereafter referred to as “50-Hz trains”), a relatively intense stimulation pattern previously shown to generate ROS in mouse extensor digitorum longus (EDL) muscle bundles (41), can significantly increase the rate of CM-DCF oxidation relative to the resting rate in our single FDB fiber culture system. CM-DCF fluorescence was first recorded every 10 min during a 30-min control period without stimulation. Then, the fibers were repetitively electrically stimulated with the 50-Hz

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train protocol, a relatively intense pattern of activity. CM-DCF fluorescence was recorded every 10 min (between trains of fiber stimulation) during a 60-min stimulation period. During the 30-min resting period, the CM-DCF fluorescence increased constantly with a slope of 1.15 ± 0.24%/min (7 fibers, P < 0.01). This indicates that the simulation pattern used here (50-Hz trains) causes generation of more ROS than is produced under resting conditions in our FDB cultures. This increase is clearly seen by comparison of the extrapolation (Fig. 3A, dashed line) of the fit to the 30-min control period to the period of fiber stimulation. The rate of increase of CM-DCF fluorescence during fiber stimulation clearly exceeds the extrapolated control rate of increase.

In another group of FDB fibers, we tested whether NAC can block the increase in the slope of CM-DCF fluorescence during 50-Hz train electrical stimulation. In the presence of NAC, the slopes for the 30-min resting period and for the 60 min with 50-Hz train electrical stimulation were not significantly different (2.48 ± 0.24 and 2.24 ± 0.23%/min, respectively; 6 FDB fibers; P > 0.05). As shown in Fig. 3B, preincubation with the...
anti oxidant NAC essentially eliminated the difference in the slopes between the 30-min resting period and the 60-min electrical stimulation period.

We also measured the ROS generation produced by 10-Hz continuous stimulation. Figure 3C shows that in the first 30-min resting period, the slope of the normalized fluorescence was 1.79 ± 0.63%/min. In the following 60 min, when the same group of fibers were stimulated continuously at 10 Hz, the slope of the normalized fluorescence was not significantly different (1.53 ± 0.60%/min; 6 FDB fibers; \( P > 0.05 \)). This indicates that 10-Hz continuous electrical stimulation does not produce more ROS than is produced at rest. In this regard, it is of interest to note that both 50-Hz train and 10-Hz continuous stimulation deliver the same overall number of pulses averaged over time (i.e., 10 pulses over a 1-s period), but grouped differently.

In Fig. 3D, we normalized the slope of CM-DCF fluorescence during the 60-min muscle stimulation period in each fiber to the slope determined in the same fiber in the control period. When 50-Hz trains were applied in the second interval, the slope increased to 2.58 ± 0.45 times that of control (Fig. 3D, left bar; \( P < 0.01 \) compared with slopes of before stimulation). This increase was eliminated in the presence of NAC during both the control and stimulation intervals (Fig. 3D, middle bar; \( P > 0.05 \) compared before stimulation). The 10-Hz continuous stimulation did not significantly change the rate of CM-DCF fluorescence increase (Fig. 3D, right bar; \( P > 0.05 \) compared with before stimulation).

Fig. 3. Effects of 50-Hz train or 10-Hz continuous stimulation on the production of endogenous ROS. A: FDB fibers were loaded with ROS sensitive dye CM-H2DCFDA and monitored by imaging every 10 min. Fibers were first monitored for 30 min without stimulation. Electrical stimulation of 50-Hz trains for 60 min significantly increased the rate of ROS generation compared with the 30-min resting period of the same group of muscle fibers. Data were from 7 FDB fibers. B: same pattern of electrical stimulation was applied to FDB fibers preincubated with 10 mM NAC for 30 min. Electrical stimulation for 60 min did not result in significant increase of the slope of linear fit, suggesting that there is no significant increase in ROS generation. Data were from 6 FDB fibers. C: FDB fibers were loaded with CM-H2DCFDA and monitored by imaging every 10 min, while continuous electrical stimulation at 10 Hz for 60 min. There is no significant difference in slope of fluorescence increase in the 60-min stimulation period comparing to the 30-min resting period, suggesting the electrical stimulation at 10 Hz continuously does not generate more ROS than without stimulation. Data were from 6 FDB fibers. Dashed lines in A–C are extrapolation of the linear fit of the first 30 min without electrical stimulation. In B and C, the dotted lines are nearly superimposed on the measured data. However, in A the measured data are significantly higher than the extrapolation. D: ratio of slopes of the 60-min stimulation period over the 30-min resting period. Columns from left to right are data from A–C, respectively. **\( P < 0.01 \).
HDAC5 nuclear efflux responds only to a muscle activity pattern that generates significant amounts of ROS. Next, the 50-Hz train stimulation pattern was applied to FDB cultures expressing HDAC5-GFP, and HDAC5-GFP nuclear efflux was monitored. The nuclear level of HDAC5-GFP declined continuously during 50-Hz train stimulation (Fig. 4A, circles), dropping by 18 ± 5% after 60 min of 50-Hz train stimulation.

It was shown in Fig. 3, A and B, that the 50-Hz train stimulation pattern generates more ROS than is produced under resting conditions and that this increase in ROS production can be eliminated by preexposure to NAC. To test for a possible role of ROS in the HDAC5-GFP nuclear efflux during 50-Hz train stimulation, in another group of FDB fibers expressing HDAC5-GFP, we first incubated fibers with NAC for 30 min after which the fibers were stimulated with the same 50-Hz train pattern. In the presence of NAC (Fig. 4A, triangles), electrical stimulation with 50-Hz train for 60 min has no effects on the nuclear level of HDAC5-GFP (14 nuclei from 10 FDB fibers). Thus, compared with the 18% decrease of nuclear HDAC5-GFP in the absence of NAC, the presence of NAC essentially abolished the effects of muscle activity on HDAC5-GFP nuclear efflux. This demonstrates that ROS generated by muscle activity played an essential role in HDAC5-GFP nuclear efflux.

In another group of HDAC5-GFP-expressing FDB fibers, we tested the effects of 10-Hz continuous stimulation, which does not increase ROS production (Fig. 3C), on the nuclear efflux of HDAC5-GFP. As noted above, 10-Hz continuous stimulation delivers the same number of stimuli as the 50-Hz train pattern but grouped differently. However, during the 60-min 10-Hz continuous stimulation, there is no noticeable decrease in nuclear HDAC5-GFP (Fig. 4B; 1.8 ± 0.9% decline; P > 0.05 for significant difference from 0 min). Taken together, these results show that a muscle activity pattern that does not increase ROS production will not promote HDAC5-GFP nuclear efflux, further strengthening the conclusion that HDAC5-GFP nuclear efflux during fiber stimulation exclusively depends on ROS produced during muscle activity.

HDAC4-GFP nuclear efflux responds to both ROS production and CaMK activation during repetitive fiber stimulation. Previously, we reported that both “10-Hz train” (one 5-s duration train at 10 Hz delivered every 50 s) electrical stimulation and 1-Hz continuous stimulation did not lead to any HDAC5 nuclear efflux but did cause nuclear efflux of HDAC4-GFP and that the HDAC4-GFP nuclear efflux was accomplished via activation of CaMK II (20). However, a possible role of ROS in HDAC4 nuclear efflux was not investigated. We therefore now tested the effects of 50-Hz train stimulation, a muscle activity pattern that increases ROS production in FDB cultures (Fig. 3A), on the nuclear efflux of HDAC4-GFP. Figure 5A (circles) shows that 60-min muscle activity with 50-Hz train stimulation promoted continuous HDAC4-GFP nuclear efflux throughout the stimulation, with HDAC4-GFP nuclear fluorescence declining by 25 ± 5% (significantly different from 0 min; P < 0.01) by the end of the 60-min stimulation period. Since 50-Hz train stimulation generates ROS in these fibers (Fig. 3A), we next investigated whether ROS produced by muscle activity plays any roles in the nuclear efflux of HDAC4-GFP induced by 50-Hz train stimulation by using the ROS scavenger NAC. In the presence of 10 mM NAC, 50-Hz train stimulation still can cause continuous nuclear efflux of HDAC4-GFP during stimulation (Fig. 5A, squares), with nuclear HDAC4 declining by 15 ± 3% by the end of 60-min 50-Hz train stimulation, which was significantly less decline than in the absence of NAC. Thus NAC decreased, but did not completely block, the nuclear efflux of HDAC4-GFP during 50-Hz train stimulation (Fig. 5A, circle and squares).

KN-62 is a widely used inhibitor of CaMK that was found to completely eliminate the nuclear efflux of HDAC4-GFP caused by 10-Hz train electrical stimulation in a previous study from our laboratory (20). Here, we found that KN-62 only partially blocked the nuclear efflux in response to 50-Hz trains (Fig. 5A, circles). After 60 min of 50-Hz train stimulation in the presence of KN-62, nuclear HDAC4-GFP concentration was decreased significantly less than in control (P < 0.01) but
was also significantly decreased compared with the starting level \( (P < 0.01) \). This is different from the previously observed effects of KN-62 on the nuclear efflux due to 10-Hz train stimulation or 1-Hz continuous stimulation, where KN-62 completely eliminated HDAC4 nuclear efflux \( (20) \). This suggests that different muscle activity patterns may promote HDAC4 nuclear efflux through different mechanisms, with 10-Hz trains or 1-Hz continuous stimulation acting on HDAC4 nuclear efflux exclusively via CaMK but with 50-Hz trains acting on HDAC4 via both mechanisms. Treatment with a combination of both KN-62 and NAC fully abolished the nuclear efflux of HDAC4-GFP by 50-Hz trains \( (\text{Fig. 5A, triangles}) \), which is not significantly different from 0 \( (P > 0.05) \), showing that both ROS and CaMK are involved in the nuclear efflux of HDAC4-GFP caused by 50-Hz trains.

We next tested the effects of 10-Hz continuous stimulation, which delivers the same average number of pulses per minute as 50-Hz trains but with the pulses grouped differently, on HDAC4-GFP nuclear efflux. The 10-Hz continuous stimulation also caused nuclear efflux of HDAC4-GFP \( (\text{Fig. 5B, circles}) \), with nuclear concentration declining by 16 ± 4% after 60 min. However, this decline in nuclear HDAC4-GFP fluorescence in response to 10-Hz continuous stimulation is not blocked by 10 mM NAC incubation \( (\text{Fig. 5B, squares}) \), which is not significantly from the percent decline without any blockers; \( P > 0.05 \) but is completely eliminated by the CaMK inhibitor KN-62 \( (\text{Fig. 5B, triangles}) \). The change in nuclear fluorescence during 10-Hz continuous stimulation in the presence of KN-62 was not significantly different from 0 min \( (P > 0.05) \). Taken together, the current results suggest that 10-Hz continuous stimulation, which activates CaMK without affecting ROS production, enhances HDAC4-GFP nuclear efflux exclusively through HDAC4 phosphorylation by CaMK but has no effect on the nuclear movements of HDAC5. Thus in the absence of ROS generation, fiber activity can promote HDAC4 but not HDAC5 nuclear efflux. The latter requires ROS production, which is not detectable during the 10-Hz train stimulation.

**NOX2 is the main source of ROS generated by intense muscle activity.** Since NAC indiscriminately scavenges ROS and reactive nitrogen species from various sources, we further examined the specific source of the ROS that generates ROS dependent HDAC nuclear efflux due to intense (50-Hz trains) muscle activity. NOX are considered as a potential intracellular source of ROS in skeletal muscle (both normal and dystrophic; Refs. 36, 42, 44). FBD fibers were isolated from NOX2 KO mice \( (\text{as well as from matched wild-type mice}) \) and were cultured and loaded with CM-H2DCFDA. Electrical stimulation with 50-Hz trains resulted in significant increase of CM-DCF fluorescence slope in muscle fibers from wild-type control mice \( (\text{Fig. 6, A and C, left bar}) \); slope increased from 1.36 ± 0.39 to 2.43 ± 0.24%/min; \( P < 0.01 \), which is similar to results from CD-1 mouse \( (\text{Fig. 3, above}) \). In contrast, in cultures from NOX2 KO mice, the same pattern of electrical stimulation did not change the slope of fluorescence increase during the 60-min stimulation interval compared with the 30-min resting period \( (\text{Fig. 6, B and C, right bar}) \); slope was 1.82 ± 0.16 in control and 1.85 ± 0.19%/min during stimulation; \( P > 0.05 \). These results with muscle from NOX2 KO mice strongly suggest that NOX2 is the source of ROS production during stimulation with 50-Hz trains in FDB muscle fibers under our culture and experimental conditions.

We also examined whether there were any differences in calcium handling in NOX2 KO mice compared with wild-type controls. Using indo-1-loaded fibers, we found that there was no difference in indo-1 fluorescence ratio in resting fibers.
(0.76 ± 0.01 in 24 wild type and 0.72 ± 0.01 in 18 NOX2 KO; \( P > 0.05 \)) indicating that the resting Ca\(^{2+}\) concentration was the same in both types of fibers. As shown in Fig. 6, Ca\(^{2+}\) transients in response to both single action potentials (Fig. 6D) and during tetanic contraction in response to 400-ms trains of action potentials at 100 Hz (Fig. 6E) measured with fluo-4 were very similar. For the single action potential, there was no significant difference between NOX2 KO and wild-type control fibers in either peak of calcium transient (11.3 ± 0.5 in 12 wild type and 11.1 ± 0.5 in 15 NOX2 KO; \( P = 0.8 \)) or the time to peak (3.42 ± 0.28 in the wild type and 3.01 ± 0.17 in the NOX2 KO; \( P = 0.2 \)), but the half duration was slightly shorter in the NOX2 KO fibers (33.0 ± 1.1 in the wild type and 27.0 ± 1.3 in the NOX2 KO; \( P = 0.003 \)). Thus the differences observed for ROS generation in NOX2 KO fibers compared with wild-type control fibers were not due to changes in resting Ca\(^{2+}\) or Ca\(^{2+}\) transients in the NOX2 KO fibers.

Next, we expressed HDAC4-GFP or HDAC5-GFP in FDB fibers from NOX2 knockout (KO) mice or wild-type control mice. FDB fibers from wild-type control (A) or from NOX2 KO (B) mice were loaded with ROS sensitive dye CM-H\(_2\)DCFDA and monitored by imaging every 10 min. Fibers were first monitored for 30 min without stimulation. Electrical stimulation of 50-Hz trains for 60 min significantly increased the rate of ROS generation compared with the 30-min resting period in control fibers (A), but not in NOX2 KO fibers (B). Data were from 12 FDB fibers for A and 16 fibers for B, respectively. C: ratio of slopes of the 60 min stimulation period over the 30-min resting period. **\( P < 0.01 \). D and E: fibers from NOX2 KO mice or from wild-type mice have similar calcium transients. D: superimposed typical single calcium transients from wild-type FDB fiber (black) or from NOX2 KO FDB fiber (gray) with calcium sensitive dye fluo-4. Data are shown as \( \Delta F/F_0 \). E: fibers from wild-type or NOX2 KO were stimulated with a pulse train (400 ms at 100 Hz) and the resulting calcium transients were recorded with fluo-4. Calcium transients recorded from wild-type or NOX2 KO showed similar peak values and decay rates.

Based on the results in Fig. 7, the NOX2 KO fibers provide a means of characterizing the Ca\(^{2+}\)-dependent nuclear efflux of HDAC4 and 5. However, the efflux of HDAC5-GFP in fibers from wild-type control mice but that the efflux of HDAC5-GFP was completely lacking in the fibers from NOX KO mice, with the relative nuclear fluorescence not significantly different from 0 min after 60-min stimulation (\( P > 0.05 \)). For the experiments on HDAC4-GFP (Fig. 7B), fibers from wild-type or NOX KO mice were first incubated with CaMK inhibitor KN-62 for 30 min to eliminate any effects of CaMK activation during stimulation. The fibers were then stimulated with 50-Hz trains. In the presence of KN-62, which selectively eliminates CaMK activation, 50-Hz train stimulation again led to nuclear efflux of HDAC4-GFP in wild-type fibers but not in NOX KO fibers. Taken together, our results suggest that ROS generated by NOX2 is the main source of ROS production during 50-Hz train muscle activity and that ROS generated by NOX2 causes nuclear efflux of HDAC4 and 5.
stimulation gave the same rate of decline of nuclear HDAC4, indicating that CaMK was already maximally activated by the 10-Hz continuous stimulation and that concentrating the pulses into 50-Hz trains caused no further HDAC4 nuclear efflux (Fig. 8A). This is in contrast to wild-type fibers, where 50-Hz trains activate NOX2-dependent ROS generation, resulting in a component of HDAC4-GFP nuclear efflux dependent on ROS production, which is not seen with 10-Hz continuous stimulation (Fig. 5). In NOX2 KO fibers in the presence of pharmacological block of CaMK by KN-62, neither 10-Hz continuous stimulation (Fig. 8B) nor 50-Hz train stimulation (Fig. 7B, open circles) causes nuclear efflux of HDAC4-GFP.

**ROS production by muscle activity activates MEF2.** Stimulation of FDB cultures also resulted in changes in expression of a MEF2-element-driven luciferase reporter construct. FDB fibers were infected with adenovirus containing a luciferase cDNA driven by a promoter containing six consecutive MEF2 elements (45) and incubated for 2 days. Fibers were stimulated for 1 h with 50-Hz trains and then cultured for another 24 h. This stimulation caused the luciferase activity in fiber culture extracts to increase to 2.14 times compared with extracts from unstimulated fiber cultures (Fig. 9; \( P < 0.01 \)). Pretreatment with 10 mM NAC partially blocked the enhancement of MEF2 reporter activity induced by stimulation (Fig. 9), indicating that at least part of the increase in MEF2-driven luciferase reporter activity during intense muscle activity was mediated by ROS.

**DISCUSSION**

We (20) have previously reported that both 10-Hz train and 1-Hz continuous electrical stimulation of FDB muscle fibers

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**Fig. 7.** Effects of 50-Hz train stimulation on HDAC5-GFP or HDAC4-GFP nuclear efflux in NOX2 KO mice and wild-type control mice. A: FDB fibers from NOX2 KO mice or control mice expressing HDAC5-GFP were stimulated. Stimulation in fibers from control mice enhanced nuclear efflux of HDAC5-GFP, but not in fibers from NOX2 KO mice. Data were from 11 nuclei of 9 FDB fibers for NOX2 KO mice. Data were from 11 nuclei of 9 FDB fibers for control mice. B: FDB fibers from NOX2 KO mice or control mice expressing HDAC4-GFP were first treated with KN-62, then stimulated. Stimulation in fibers from control mice enhanced nuclear efflux of HDAC4-GFP but not in fibers from NOX2 KO mice. Data were from 12 nuclei of 8 FDB fibers for NOX2 KO mice. Data were from 11 nuclei of 7 FDB fibers for control mice. \(* P < 0.05, \text{**} P < 0.01.\)

**Fig. 8.** Effects of 50-Hz train or 10-Hz continuous stimulation on HDAC4-GFP nuclear efflux in NOX2 KO mice. A: FDB fibers from NOX2 KO mice were stimulated with 50-Hz trains or with 10 Hz continuously. Both patterns of stimulation caused similar nuclear efflux of HDAC4-GFP. Data were from 8 nuclei of 6 fibers for 50-Hz train and 12 nuclei of 6 fibers for 10-Hz continuous stimulation. B: 10-Hz continuous stimulation did not result in significant nuclear efflux of HDAC4-GFP if muscle fibers from NOX2 KO mice were incubated with 5 \( \mu \text{M} \) KN-62. Data were from 9 nuclei of 5 fibers. \(** P < 0.01.\)
Sparked by the recent reports that ROS can regulate distribution of both HDAC4 and HDAC5 when expressed as GFP fusion proteins in isolated muscle fibers, we find that 1) ROS promotes the nuclear efflux of both HDAC4 and HDAC5 when expressed as GFP fusion proteins in isolated muscle fibers; 2) 50-Hz train stimulation causes an increase in ROS production that leads to nuclear efflux of both HDAC4 and 5; 3) HDAC4 and HDAC5 respond to electrical stimulation differentially: HDAC5 responds only to stimulation patterns (e.g., 50-Hz trains) generating an increased rate of ROS production, whereas HDAC4 responds both to an intense stimulation pattern generating increased rate of ROS production (50-Hz trains), as well as to less intense stimulation patterns (10-Hz trains and 1- and 10-Hz continuous) that do not increase ROS production but that do activate CaMK; 4) the ROS production that leads to HDAC4 and 5 nuclear efflux during intense fiber stimulation patterns requires NOX2; but 5) NOX2 does not modulate resting Ca\(^{2+}\) or the amplitude of the Ca\(^{2+}\) transients due to single action potentials or during trains of action potentials. These results clearly implicate ROS and NOX2 in the intense activity-dependent nuclear efflux of HDAC4 and 5 in isolated FDB muscle fibers under the conditions of our experiments. The extent to which ROS and NOX2 are also involved and activated by intense activity in muscle fibers in muscles in vivo remains to be determined.

**Production of ROS cause both HDAC4 and HDAC5 nuclear efflux.** Two recent elegant reports (1, 31) studied the role of redox–dependent pathways in the regulation of class II HDACs and cardiac hypertrophy. They found that Cys-667 and Cys-669 in HDAC4 could be oxidized by ROS generated by hypertrophic stimuli. Such oxidation led to the formation of intramolecular disulfide bonds and exposed the nuclear export signal in HDAC4. The two cysteines in HDAC4 are conserved in other class II HDAC members. The redox pathway for regulation of HDAC4 is reported to be independent of the phosphorylation-dependent pathway for HDAC regulation that has been extensively studied both in cardiac (3) and skeletal (20, 27) muscles. Both cysteines are conserved in HDAC5 as Cys-696 and Cys-698. In our study with skeletal muscle, both HDAC4-GFP and HDAC5-GFP respond with nuclear efflux due to an increased rate of ROS production either generated by addition of H\(_2\)O\(_2\) to the bath solution or by intense electrical stimulation.

**HDAC4 and HDAC5 respond differentially to electrical stimulation.** One of our novel findings is that nuclear efflux of HDAC5 is increased only by stimulation patterns generating an increased rate of production of ROS. HDAC5 nuclear efflux was not increased by patterns of muscle activity that only activate CaMK but do not increase the rate of ROS production. This further strengthens the notion that HDAC5 is not a substrate of CaMK II (2). As for HDAC4, since it is a substrate of CaMK II, it responds to electrical stimulation via both ROS and CaMKII. Figure 10 presents a signaling schematic summary for the differential regulation of HDAC4 and 5 nuclear efflux, which is represented functionally in Fig. 10 as an inhibition of the inhibitory influence of HDAC4 or 5 on MEF2 transcriptional activity. Only 50-Hz trains lead to the nuclear efflux of HDAC5 (bottom line), which is mediated exclusively by ROS generated by NOX2. In contrast, all four of the stimulation patterns lead to HDAC4 nuclear efflux, which is mediated by both CaMK and ROS.

Fig. 9. Effects of electrical stimulation and NAC on MEF2 activity. Luciferase activity was increased in fibers infected with MEF2-luciferase reporter and stimulated with 50-Hz trains (middle bar; **P < 0.01 compared with control). The presence of 10 mM NAC partially blocked the increase in luciferase activity stimulated with electrical pulses (right bar, ###P < 0.01 compared with stimulated in the absence of NAC). Results represent triplicate measurements from each of four independent experiments.

Fig. 10. Working hypothesis: 1-Hz continuous stimulation, 10-Hz train, 10-Hz continuous stimulation, and 50-Hz train trigger calcium transients and activate CaMK, the latter phosphorylates only HDAC4 but not 5, promoting the nuclear efflux of HDAC4. Only 50-Hz train stimulation generates ROS, resulting in nuclear efflux of HDAC4 and 5. Nuclear efflux of HDACs will remove the inhibition on transcription factor MEF2. 1): Liu et al. (20); 2): this study.
NOX2 generates the ROS that causes nuclear efflux of HDAC4 or 5. NOX2 uses NADPH as a substrate to convert molecular oxygen to generate superoxide, which is then rapidly converted to H₂O₂. Recently, NOX2 has been reported to be localized mainly in the sarcolema in skeletal muscle (44) and is considered to be a major source of ROS contributing to muscle damage in dystrophic skeletal muscle (42, 44). During activity of normal skeletal muscle, although NOX was implicated as being a more important source of ROS than mitochondria (28), the exact source and location of ROS generation have not been established (36). Here by using NOX2 KO mice we found that during intense muscle activity, ROS is mainly generated by a NOX2-dependent process. These findings with NOX2 KO mice should be more specific than results with the NOX inhibitors DPI or apocynin. In contrast, in cardiac muscle NOX4 is a major source of oxidative stress (16). According to very recent reports, in cardiac muscle NOX4 plays an essential role in mediating phenylephrine-induced superoxide production and nuclear export of HDAC4 (23), whereas NOX2 is responsible for the ROS generation during mechano-stress in cardiac cells (37). Thus it seems that in skeletal muscle or cardiac muscle, different isoforms of NOX produce ROS in response to different stress signals.

In addition to ROS produced by NOX2, nitric oxide (NO) is generated by nitric oxide synthase during skeletal muscle activity (12, 40) and the NO-sensitive dye CM-DCFH can react with NO (30). NO production as monitored with an NO-specific dye is increased in FDB fibers stimulated by electrical pulses (500-ms 50-Hz trains, 1 every second; Ref. 40). This is 2.5 times more stimuli per second, on average, than in our 50-Hz train stimulation pattern. However, our results with NOX2 KO mice demonstrate that the entire effect of the 50-Hz train stimulation used in our studies requires NOX2, which does not produce NO. Thus any role of NO in the observed nuclear efflux of HDAC5 or in the NAC-sensitive nuclear efflux of HDAC4 during our 50-Hz train stimulation protocol would have to be via NOX2-generated ROS in conjunction with NO from another source.

Downstream and upstream signaling from and to NOX2. Assuming that genetic global knockout of NOX2 does not result in compensatory changes resulting in reduced non-NOX2 ROS production during intense muscle activity, our results with muscle fibers from NOX2 KO mice implicate NOX2 as the main source of ROS production during 50-Hz train stimulation. However, the downstream pathway linking this ROS production to HDAC nuclear efflux has not been examined here. One possible mechanism would be direct oxidation and resulting nucleus to cytoplasm translocation of HDAC4 and 5, as recently found for HDAC4 in cardiac muscle but not yet investigated in skeletal muscle (1). Another possible mechanism would be direct or indirect ROS-dependent activation of a kinase that then phosphorylates HDAC4 and 5. Oxidative activation of AMPK (46) could lead to phosphorylation of HDAC4 and 5, resulting in nuclear efflux (24, 25). In contrast, oxidative activation of CaMKII (9) could only phosphorylate HDAC4, but not HDAC5 since CaMKII does not phosphorylate HDAC5. Thus at least the observed nuclear efflux of HDAC5 during 50-Hz train stimulation must be independent of CaMKII.

We have also not investigated the upstream signaling mechanism leading from 50-Hz trains of action potentials to activation of NOX2. Direct Ca²⁺ activation of NOX2 does not seem to occur (5, 8), but Ca²⁺ binding to some other direct or indirect regulator of NOX2 might be a possibility. Alternatively, NOX2 could be activated by the mechanical stretch/deformation occurring during the fiber contraction generated by the 50-Hz train stimulation Ca²⁺ transients (39). In this case, the mechanical changes in response to less intense stimulation patterns, which do not generate ROS, would be insufficient to activate NOX2.

Stimulation pattern-dependent ROS production. We have found that 50-Hz trains, but not 10-Hz continuous stimulation, generate an increased rate of ROS production in muscle fibers compared with that found in resting fibers as measured with the ROS-sensitive fluorescent dye CM-DCFH. The 50-Hz train pattern of electrical stimulation that we use gives the same average number of pulses per minute as 10-Hz continuous stimulation but grouped at a higher frequency (50 Hz) and in shorter bursts. How an equal number of pulses but grouped into bursts of different frequencies generates different amounts of ROS is an interesting open question for further study. To the best of our knowledge, there is no previous study published on the relationship between stimulation pattern and frequency and the rate of ROS generation in skeletal muscle.

Implications for muscle remodeling and denervation atrophy. Our findings have potential implications for improved understanding of the connections between ROS production and regulation of muscle function via HDACs. Oxidative stress is implicated in a wide variety of physiological and disease processes in skeletal muscle (34). ROS generated in skeletal muscle is involved in osmotic stress-induced Ca²⁺ release in normal and dystrophic skeletal muscle fibers (22, 44). Paradoxically, ROS production is increased in skeletal muscle both in response to muscle use as in exercise training and during prolonged periods of disuse (35). In normal skeletal muscle, ROS act as a signaling system during muscle activity by regulating redox sensitive kinases, phosphatases, and transcription factors (35). Of particular importance related to skeletal muscle remodeling is the phosphatase calcineurin, which activates the transcription factor nuclear factor of activated T cells and which is inhibited by oxidation (7). NF-κB and proliferator-activated receptor-γ coactivator-1α are two other muscle transcriptional regulators previously found to be affected by redox status (11, 14). Here we report for the first time that the nuclear cytoplasmic distribution of class II HDAC4 and 5 are also both regulated by ROS. Enhanced ROS-dependent nuclear efflux of HDAC4 and 5 would relieve the inhibition of these class II HDACs on the transcription factor MEF2 and result in the upregulation of MEF2 transcriptional activity leading to enhanced slow fiber-type gene expression (10, 43). If HDAC4 and 5 have differential effects on MEF2 transcriptional activity, the effects of HDAC4 would be removed by lower intensity activity without ROS involvement, whereas the effects of HDAC5 would only be removed by the higher intensity activity that generates ROS.

HDAC4 and 5 not only play important roles in skeletal muscle fiber type regulation but also are implicated in denervation induced muscle atrophy (29). Dual knockout of HDAC4 and 5 strongly suppresses the expression of MuRF1 and atrogin-1 during denervation and essentially eliminates muscle denervation atrophy (29). On the other hand ROS production, which we show here can promote HDAC nuclear efflux thus
decreasing the transcriptional repressor effects of HDACs, is enhanced in disuse muscle atrophy (34), in which both MurF1 and atrogin-1 are increased (18). It will thus be interesting in future studies to quantitatively compare ROS production under different physiological and pathological conditions and to determine the resulting effects on the sub cellular localization of HDACs. It is also important to further investigate how ROS production is fine-tuned under different muscle conditions and to determine whether localization of HDACs is affected. For example, we show here that during intense fiber stimulation the ROS causing HDAC4 and 5 nuclear efflux is generated by NOX2. It is possible that other sources of ROS production and/or other subcellular locations for particular sources of ROS production may ultimately account for the diverse effects of ROS in skeletal muscle. Finally, the possibility of distinct vs. redundant effects of HDAC4 and 5 in muscle fibers requires further investigation.

In conclusion, our results show that NOX2-dependent generation of ROS causes nuclear efflux of HDAC5 and contributes to nuclear efflux of HDAC4 during intense skeletal muscle activity but does not alter Ca²⁺ transients.

ACKNOWLEDGMENTS

We thank Dr. M. Williams for NOX2 KO and control mice, Dr. S. L. Schreiber for HDAC4-GFP cDNA, Dr. J. D. Molkentin for adenovirus encoding MEF2-luciferase reporter, and Dr. T. A. McKinsey for adenovirus encoding HDAC5-GFP.

GRANTS

This work is supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant R01-AR056477.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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*AJP-Cell Physiol* • doi:10.1152/ajpcell.00152.2012 • www.ajpcell.org