

p66^{ShcA} and Oxidative Stress Modulate Myogenic Differentiation and Skeletal Muscle Regeneration after Hind Limb Ischemia^{*S}

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Oxidative stress plays a pivotal role in ischemic injury, and p66^{ShcA}ko mice exhibit both lower oxidative stress and decreased tissue damage following hind limb ischemia. Thus, it was investigated whether tissue regeneration following acute hind limb ischemia was altered in p66^{ShcA}ko mice. Upon femoral artery dissection, muscle regeneration started earlier and was completed faster than in wild-type (WT) control. Moreover, faster regeneration was associated with decreased oxidative stress. Unlike ischemia, cardiotoxin injury induced similar skeletal muscle damage in both genotypes. However, p66^{ShcA}ko mice regenerated faster, in agreement with the regenerative advantage upon ischemia. Since no difference between p66^{ShcA}wt and knock-out (ko) mice was found in blood perfusion recovery after ischemia, satellite cells (SCs), a resident population of myogenic progenitors, were examined. Similar SCs numbers were present in WT and ko mice. However, *in vitro* cultured p66^{ShcA}ko SCs displayed lower oxidative stress levels and higher proliferation rate and differentiated faster than WT. Furthermore, when exposed to sublethal H₂O₂ doses, p66^{ShcA}ko SCs were resistant to H₂O₂-induced inhibition of differentiation. Finally, myogenic conversion induced by MyoD overexpression was more efficient in p66^{ShcA}ko fibroblasts compared with WT. The present work demonstrates that oxidative stress and p66^{ShcA} play a crucial role in the regenerative pathways activated by acute ischemia.

Reactive oxygen species (ROS)³ balance is essential to cellular homeostasis, and ROS are implicated in cell growth, metab-

olism, and apoptosis (1, 2). Mitochondria are one of the main sources of intracellular ROS, which are generated as an unavoidable consequence of aerobic metabolism. Pathologic events or exogenous insults can affect mitochondrial electron transfer chain and increase ROS production. ROS imbalance leads to cellular oxidative damage and impairment of physiologic functions and is presently regarded as a mechanism for aging and aging-related diseases (3–5). Specifically, increased ROS production during ischemia plays a causal role in tissue damage, leading to cell death by both apoptosis and necrosis (6–9). The mammalian adaptor protein ShcA has three isoforms, p46, p52 and p66, which share a common structure, but p66^{ShcA} has an additional domain at the N terminus. This domain contains a serine residue at position 36 (Ser-36) that is phosphorylated in response to several stimuli, including UV irradiation and H₂O₂ (10). While p52/p46 are cytoplasmic signal transducers involved in mitogenic signaling from activated tyrosine kinase receptors to Ras, p66 isoform is not involved in Ras activation and regulates ROS metabolism and apoptosis (10–13). A fraction of p66^{ShcA} is localized in the mitochondria and functions as a redox enzyme that generates mitochondrial ROS as signaling molecules for apoptosis (14, 15). According to these data, both p66^{ShcA}ko cells and mice display lower levels of intracellular ROS (13, 16, 17) and are resistant to apoptosis induced by a variety of different stimuli (10, 16, 18, 19). Likewise, p66^{ShcA}ko mice are resistant to ischemia-induced apoptosis and show decreased vascular and muscle damage in response to hind limb ischemia (6). Interestingly, the incidence of atherosclerotic plaque formation and vascular dysfunction, cardiac and renal disease in experimental diabetes, is strongly decreased in p66^{ShcA}ko mice, which also display a prolonged life span (10, 16, 17, 20–23).

Adult skeletal muscles possess a remarkable capacity to regenerate myofibers after damage. This rapid repair process is mainly carried out by satellite cells (SCs) (24). SCs are quiescent myogenically specified precursor cells, situated between the plasma membrane and the basal lamina of mature muscle fibers. Upon injury, SCs become active and proliferate, and their progeny progress down the myogenic lineage, giving rise

thiobarbituric acid-reactive substance; MHC, myosin heavy chain; WB, Western blot.

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³ The abbreviations used are: ROS, reactive oxygen species; SC, satellite cell; ko, knock-out; ALD, arteriolar length density; GM, growth medium; DM, differentiation medium; MEF, mouse embryonic fibroblast; BrdUrd, bromodeoxyuridine; DCFH-DA, dichlorofluorescein diacetate; TBARS,

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to fusion-competent myoblasts and then to new myofibers. Several authors have shown an increased ROS production after ischemia (6–9); nevertheless, ROS influence on skeletal muscle regeneration that follows ischemic injury is still unknown. In the present study, we investigated the role of oxidative stress and p66^{ShcA} in skeletal muscle regeneration that follows acute hind limb ischemia. Results show that p66^{ShcA} and oxidative stress negatively modulate myogenic differentiation; in contrast, p66^{ShcA} deletion enhances skeletal muscle regeneration after ischemia.

EXPERIMENTAL PROCEDURES

Animal Model and Surgical Procedures—All experimental procedures complied with the Guidelines of the Italian National Institutes of Health and with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee. 129 Sv-Ev p66^{ShcA}wt and knock-out (ko) inbred mice were previously described (10); their genetic background was identical except for the p66^{ShcA} locus. Surgical procedures are described in supplemental data.

Histology and Morphometric Analysis—Hematoxylin/eosine-stained sections were prepared, and both capillary density and arteriolar length density (ALD) were measured as previously described (25, 26). Regenerating myofibers were identified by morphological criteria, central nucleus/i, and <30- μ m diameter in hematoxylin/eosine-stained sections or by Desmin immunofluorescence staining. Immunohistochemistry and immunofluorescence were carried out according to standard procedures (25, 27). A Zeiss Axioplan 2 fluorescence microscope with image analyzer KS300 software was used to acquire images and to measure areas. All histological and morphometric analyses were carried out by two blinded readers with comparable results.

Cell Culture—Primary SCs were isolated as previously described (28) and cultured in Dulbecco's modified Eagle's medium high glucose supplemented with 20% horse serum (Sigma), 3% chicken embryo extract (MP Biomedicals Europe) (growth medium, GM). C₂C₁₂ murine myoblasts were cultured as previously described (29). To induce terminal differentiation, myoblasts were shifted to Dulbecco's modified Eagle's medium with 2% horse serum (differentiation medium, DM). To induce oxidative stress, H₂O₂ was added for 4 h before switching the cells to DM in order to induce differentiation. H₂O₂ was renewed daily. Primary mouse embryo fibroblasts (MEFs) were isolated as previously described (30).

Immunofluorescence, BrdUrd Assay, and Oxidative Stress Measurement—Immunofluorescence stainings were performed as previously described (29). *In vitro* SC proliferation was assessed by anti-BrdUrd (B44; BD Biosciences) immunofluorescence staining of proliferating myoblasts incubated with 20 μ M BrdUrd for 1 h. Intracellular ROS production was evaluated through the oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, 25 μ M DCFH-DA (Eastman Kodak Co.) was added to the cells, and, 30 min later, DCFH-DA fluorescence was revealed by fluorescence microscopy, using the same exposure conditions in each experiment. Images were

taken and analyzed by two blinded readers with comparable results.

To measure oxidative stress levels in adductor muscles, thio-barbituric acid-reactive substances (TBARS) were evaluated. The formation of TBARS was determined using the OXI-TEK kit (ZeptoMetrix Corp.) and a luminescence spectrometer (LS55; PerkinElmer) with excitation set at 530 nm, emission at 550 nm, and slit width at 5 nm (31).

Western Blotting—Cell cultures were lysed with Laemmli buffer, while adductor muscle extracts were derived in radio-immune precipitation buffer containing protease inhibitors (30). Immunoblottings were performed according to standard procedures (30). Membranes were stained with Red Ponceau (Sigma) to assess equal protein loading and even transfer.

Antibodies—Antibodies used throughout the study are described in the supplemental data.

Statistical Analysis—Variables were analyzed by Student's *t* test and one way analysis of variance. A value of $p \leq 0.05$ was deemed statistically significant. Results are reported as mean values \pm S.E.

RESULTS

Faster Skeletal Muscle Regeneration of p66^{ShcA}ko Mice after Hind Limb Ischemia—In order to evaluate the role of p66^{ShcA} and oxidative stress in skeletal muscle regeneration, unilateral hind limb ischemia was induced in p66^{ShcA}wt and ko mice by removing the femoral artery. Regenerating myofibers were identified in hematoxylin/eosin-stained sections of ischemic adductor muscles at 2–50 days after ischemia. Important differences were observed in the density of regenerating myofibers and in the time course of regeneration. Seven days after ischemia, WT adductor muscles showed minimal regeneration and numerous necrotic myofibers with infiltrating cells. In contrast, in p66^{ShcA}ko adductor muscles, wide areas of regenerating myofibers and minimal necrotic damage were observed. In WT muscles the density of regenerating myofibers reached a maximum at 21 days after ischemia when necrotic myofibers were still present. Conversely, in p66^{ShcA}ko muscles 21 days after ischemia, regeneration was almost completed and most fibers displayed mature size and peripheral nucleus, in the absence of overt necrosis (Fig. 1, A and B). As expected, the overall number of regenerating myofibers was lower in ko mice than in WT controls since we previously demonstrated that ko mice display lower tissue damage after ischemia (6). Faster regeneration of p66^{ShcA}ko ischemic adductor muscles at 7 days after ischemia was confirmed by Desmin immunofluorescence staining of regenerating myofibers (Fig. 1C).

In order to analyze muscle regeneration in a model in which p66^{ShcA}wt and ko mice showed a similar amount of tissue damage, tibialis anterior muscles of both p66^{ShcA}wt and ko mice were injected with cardiotoxin. Cardiotoxin induced similar areas of myonecrosis 1 day after injection in p66^{ShcA}wt and ko tibialis anterior muscles (supplemental Fig. S1A). Time course of muscle regeneration showed that also in this experimental model, p66^{ShcA}ko mice displayed faster regeneration 7 days after skeletal muscle damage (supplemental Fig. S1B). Taken together, these data indicate that p66^{ShcA}ko mice regenerate skeletal muscle faster than WT controls.

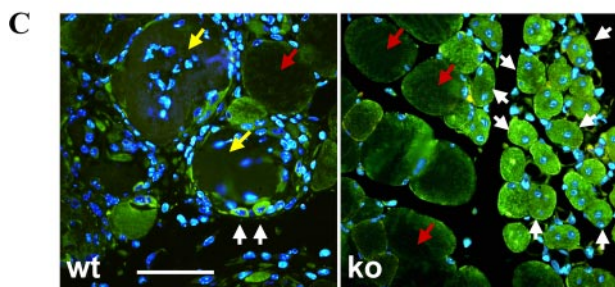
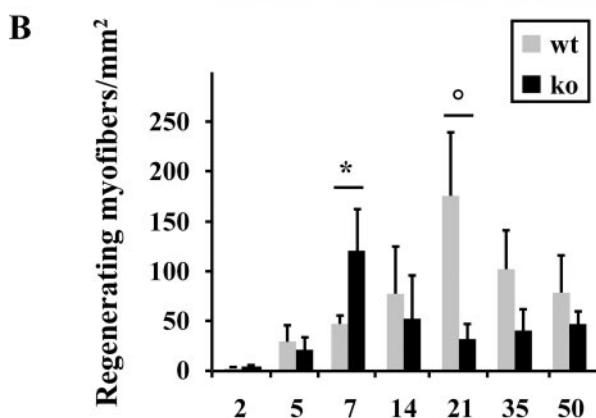
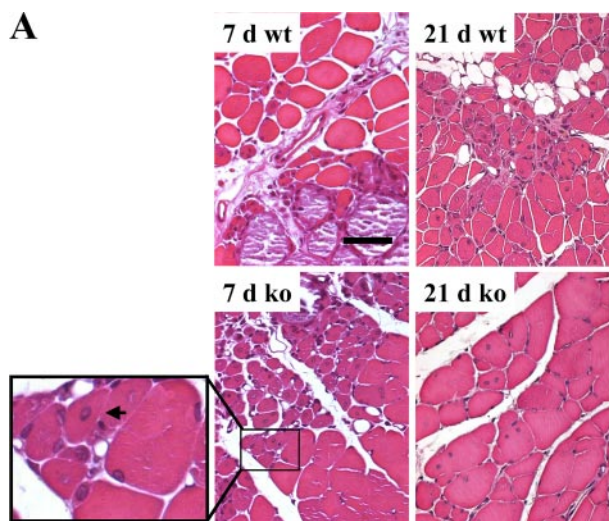


FIGURE 1. Faster regeneration of p66^{ShcA} ko adductor muscles after ischemia. A, representative hematoxylin/eosin-stained sections of p66^{ShcA} wt and ko mice adductor muscles at 7 and 21 days after ischemia. Calibration bar, 50 μ m. Inset shows myofibers at higher magnification; black arrow indicates a regenerating myofiber characterized by the central nucleus and a <30- μ m diameter. B, time course of adductor muscle regeneration. Bar graph represents the mean of regenerating myofibers/mm², WT versus ko; *, $p < 0.03$, °, $p < 0.02$, $n = 6$ /each group. C, representative Desmin immunofluorescences of p66^{ShcA} wt and ko adductor muscles 7 days after ischemia. Calibration size bar, 50 μ m. White arrows show fluorescence-positive regenerating myofibers. Red arrows show fluorescence-negative healthy myofibers. Yellow arrows show fluorescence-negative fibers, most likely necrotic myofibers with infiltrating cells.

Lower Oxidative Stress in p66^{ShcA} ko Mice during Ischemia—In order to clarify whether skeletal muscle regeneration after ischemia takes place in an environment with increased oxidative stress levels, ROS-mediated damage was evaluated. To this aim, nitrotyrosine was detected by immunohistochemistry in ischemic adductor muscle sections. Accumulation of nitrotyrosine was found in necrotic, healthy, and regenerating myofi-

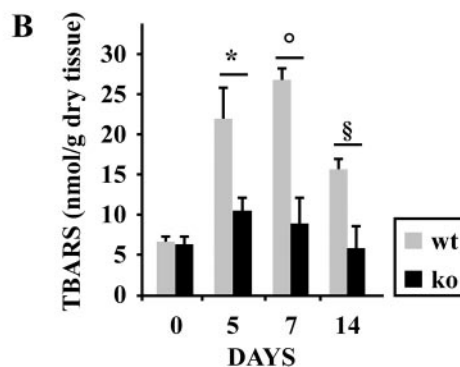
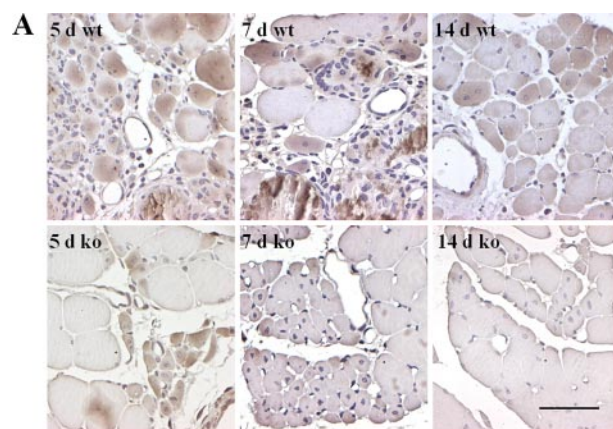


FIGURE 2. Lower oxidative stress in p66^{ShcA} ko mice during ischemia. A, higher level of protein nitrosylation of WT mice adductor muscles 5, 7, and 14 days after ischemia. Representative nitrotyrosine immunostainings of p66^{ShcA} wt and ko mice adductor muscle sections, $n = 6$. Brown nitrotyrosine staining was more intense and diffuse in WT mice. Calibration bar, 50 μ m. B, TBARS were determined in adductor muscle extracts of both normoperfused and ischemic p66^{ShcA} wt and ko mice 5, 7, and 14 days after surgery. Values are expressed as nanomoles/gram of dry tissue (WT versus ko, *, $p < 0.013$, °, $p < 0.002$, §, $p < 0.008$). Error bars = S.E.

bers of p66^{ShcA} wt skeletal muscle sections at 5, 7, and 14 days of ischemia. In contrast, in ko sections nitrotyrosine accumulation was observed only in occasional regenerating myofibers at 5 days after ischemia (Fig. 2A). To confirm these results, tissue levels of TBARS were evaluated in ischemic adductor muscles 5, 7, and 14 days after surgery. Significant increases of TBARS were observed in WT, but not in ko, muscles (Fig. 2B). These data indicate that ischemia leads to oxidative damage that extensively affects p66^{ShcA} wt, but not ko, muscles.

Similar Blood Perfusion Recovery in p66^{ShcA} wt and Ko Mice after Ischemia—In order to assess whether the observed differences in myogenic regeneration were due to increased blood perfusion in p66^{ShcA} ko mice, morphometric and functional measures were performed. Significant differences were observed in capillary density of both normoperfused and ischemic mice. Normoperfused p66^{ShcA} wt adductor muscles showed a higher capillary density than ko. As expected (6, 32), p66^{ShcA} wt capillary density decreased after ischemia, remained low between 2 and 21 days, and returned to initial value between 35 and 50 days. In contrast, no decrease was observed in p66^{ShcA} ko adductor muscles and a transient increment was observed between 7 and 21 days (supplemental Fig. S2A). Interestingly, it was found there was no effect of ischemia on ALD in both genotypes (supplemental Fig. S3, A and B). Because eval-

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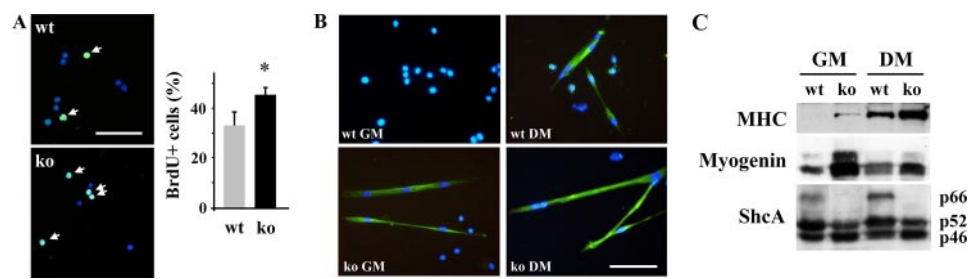


FIGURE 3. Higher proliferation rate and spontaneous differentiation of p66^{ShcA}ko satellite cells *in vitro*. A, higher proliferation rate of cultured p66^{ShcA}ko SCs. Representative anti-BrdUrd immunofluorescences of p66^{ShcA}wt and ko SCs cultured 4 days in growth medium (GM) and incubated for 1 h with 20 μ M BrdUrd. Pictures show the merge of anti-BrdUrd green fluorescence and of Hoechst 33342 blue fluorescence. *White arrows* indicate BrdUrd-positive nuclei. Calibration bar, 50 μ m. *Bar graph* represents percentage of BrdUrd-positive nuclei, WT versus ko; *, $p < 0.02$, $n = 3$. *Error bars* = S.E. B, higher fusion rate and MHC expression of cultured p66^{ShcA}ko SCs. Representative MHC immunofluorescences of p66^{ShcA}wt and ko SCs cultured in GM for 6 days and shifted to DM for 24 h. Pictures show the merge between the MHC green fluorescence and Hoechst 33342 blue fluorescence. Calibration bar, 50 μ m, $n = 6$. C, representative Western blotting of proliferating (6 days) and differentiating (24 h) p66^{ShcA}wt and ko SCs. MHC, myogenin, and the three isoforms of ShcA were detected with specific antibodies. p52^{ShcA} and p46^{ShcA} isoforms indicate similar protein loading in each lane.

p66^{ShcA}wt and ko mice and characterized. Proliferation rate was evaluated by BrdUrd incorporation assay at 4 days from isolation. The percentage of BrdUrd-positive SCs, estimated at the same cell density, was significantly higher in ko than in WT cell cultures, indicating a faster proliferation rate of p66^{ShcA}ko SCs (Fig. 3A).

In order to examine SCs differentiation, cells were allowed to proliferate for 6 days in growth factor-rich medium (GM) and then switched to low growth factor medium (DM). Differentiation was assessed by myosin heavy chain (MHC) immunofluorescence staining

and Western blot (WB) analysis for MHC and for the myogenic transcription factor myogenin. Fig. 3, B and C, shows that ko SCs, at the same cell density as WT cells, were able to differentiate spontaneously, giving rise to small myotubes in GM. In contrast, this condition was not suitable for WT cell differentiation. In agreement with these results, ko cells exhibited higher MHC and myogenin levels in GM. As expected, in DM both WT and ko SCs gave rise to myotubes. Taken together, these results show that p66^{ShcA}ko SCs proliferate faster and exhibit a higher rate of spontaneous differentiation than WT SCs.

Higher Myogenic Potential of p66^{ShcA}ko Mouse Embryo Fibroblasts upon MyoD Overexpression—To confirm the higher myogenic potential of p66^{ShcA}ko cells, the master gene of myogenic differentiation, MyoD, was overexpressed in non-myogenic cells in order to induce myogenic conversion (33). To this aim, p66^{ShcA}wt and ko MEFs were infected, with similar efficiencies, with adenoviral vectors encoding either MyoD or LacZ. Cells were cultured for 32 h in GM and then switched to DM for an additional 48 h. Fig. 4 shows that MyoD overexpression induced a much higher expression of MHC and myogenin in ko than in WT cells. Interestingly, some expression of myogenic markers was observed in ko MEFs transduced with MyoD in GM conditions as well (supplemental Fig. S5).

Lower ROS Production of p66^{ShcA}ko Myotubes—Given that p66^{ShcA}ko mice showed lower basal levels of intracellular and systemic ROS (13, 16, 17, 19), it was tested whether the same was true in SCs culture. Oxidative stress was evaluated by DCFH-DA fluorescent probe. DCFH-DA fluorescence levels were undetectable in proliferating myoblasts and increased in DM according to previously reported data (34). When p66^{ShcA}wt and ko cultures were compared, the number of DCFH-DA-positive myotubes was significantly lower in ko cells, both at 24 and 48 h of differentiation (Fig. 5B).

H₂O₂-induced Oxidative Stress Inhibits Myogenic Differentiation—Given the tight correlation between decreased oxidative stress and increased myogenic potential in p66^{ShcA}ko SCs and mice, it was tested whether oxidative stress affected *in vitro* myogenic differentiation. To this aim, C₂C₁₂ myoblasts were treated with sublethal doses of H₂O₂ and differentiation was induced. Myogenic differentiation was analyzed both by

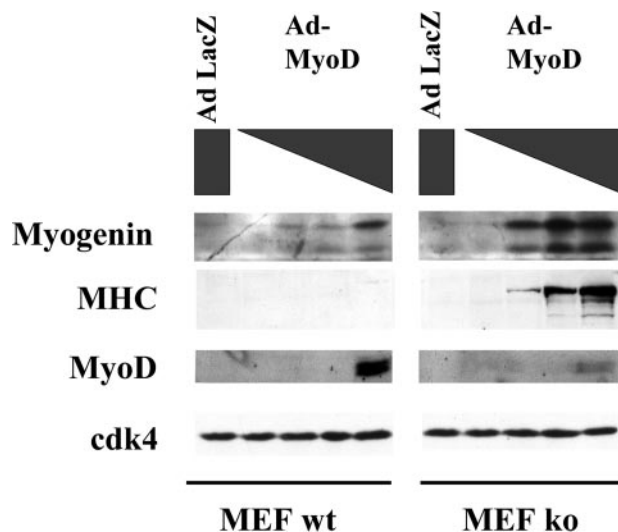


FIGURE 4. Higher myogenic potential of p66^{ShcA}ko MEFs upon MyoD overexpression. MEFs were infected, achieving similar efficiencies between WT and ko, with 100, 300, and 900 multiplicity of infection of Adeno-MyoD to induce myogenic conversion. After infection, cells were cultured in GM for 32 h and then shifted to DM for 48 h. Control infection was performed with 900 multiplicity of infection of Adeno-LacZ. WB was carried out after 48 h of culture in DM. MyoD, myogenin, MHC, and cdk4 (loading control) were detected with specific antibodies. The picture shows representative Western blotting. *Left* (WT) and *right* (ko) panels are part of the same gel that was rearranged to remove not-relevant lanes. MHC light exposure enhances expression differences between WT and ko cells. Longer exposures show sustained MHC expression in WT cells as well.

uation of capillary density and ALD are morphological analyses that give no information about capillary functionality, laser Doppler perfusion imaging was performed to assess hind limb perfusion. Supplemental Fig. S2B shows no significant difference in perfusion recovery between p66^{ShcA}wt and ko ischemic mice.

Higher Proliferation Rate and Spontaneous Differentiation of Cultured p66^{ShcA}ko Satellite Cells—Afterward, it was examined whether SCs may play a role in the accelerated muscle regeneration of p66^{ShcA}ko mice. A similar number of SCs was observed in normoperfused p66^{ShcA}wt and ko adductor muscle sections, as assessed by m-Cadherin and c-Met immunohistochemistry (supplemental Fig. S4, A and B). SCs were then isolated from

MHC immunofluorescence to measure cell fusion and by WB analysis of a series of myogenic markers, including myogenic transcription factors (MyoD, Myf5, and myogenin), MHC, and cell cycle genes that are up-regulated upon myogenic differentiation (pRb and Cyclin D3) (35, 36). MHC immunofluorescence and WB analysis of myogenic markers showed a dramatic inhibition of myogenic differentiation that was dose- and time-dependent (Fig. 6, *A* and *B*).

To assess whether p66^{ShcA} deletion prevented oxidative stress-induced inhibition of myogenic differentiation, the same experiment was performed with SCs (Fig. 6, *C* and *D*). MHC immunofluorescences and WB analysis of myogenic markers showed a dose-dependent inhibition of myogenic differentia-

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tion upon H₂O₂ treatment of p66^{ShcA}wt SCs cultures. In contrast, p66^{ShcA}ko myoblasts were resistant to oxidative stress-induced inhibition of myogenic differentiation.

DISCUSSION

Intracellular redox state modulates many signaling pathways involved in apoptosis, proliferation, and differentiation (1, 2). It is well established that ischemia leads to increased ROS levels that play a causal role in cell death by both apoptosis and necrosis (6–9). However, little is known about the ROS role in myogenic regeneration after damage. To answer this question, we used the p66^{ShcA}ko mouse model. These mice display lower levels of intracellular and systemic ROS in a variety of physiopathologic settings such as diabetes, high fat diet, and aging (16, 17, 20–23). Indeed, we previously showed that the oxidative stress burst triggered by acute ischemia was significantly blunted in ko mice (6). Likewise, we now show that increased levels of protein nitrosylation and TBARS, two markers of oxidative stress (17, 23, 31), were detectable throughout the regenerative process that follows ischemia in p66^{ShcA}wt but not ko skeletal muscles.

We previously demonstrated that p66^{ShcA}ko mice display decreased skeletal muscle damage following acute ischemia (6). The present study provides evidence that p66^{ShcA} deletion enhances skeletal muscle regeneration after femoral artery removal. Although it is possible that part of the regenerative advantage observed in p66^{ShcA}ko mice after ischemia was due to lower initial damage, it is remarkable that faster regeneration was observed upon cardiotoxin injection that induced a similar amount of tissue damage in both genotypes.

Because perfusion strongly influences tissue repair, it was examined whether the faster skeletal muscle regeneration observed in ko mice was due to increased vascularization. The analysis of neoangiogenesis needs to take into account that p66^{ShcA}ko mice exhibit a lower capillary density than WT mice in adductor normoperfused muscles. However, there is no difference in ALD between ko and WT mice. Upon femoral artery dissection, no decrease in capillary density was found in ischemic ko mice, whereas WT mice displayed the expected decrease (6, 32). After the initial demise, capillary density displayed progressive increments in the WT, which started at day 7 and rescued initial value between days 35 and 50. In contrast, in ko mice capillary density displayed a transient increment that rapidly came back to initial value. Therefore, no overt advantage in capillary density kinetic of increase was observed in

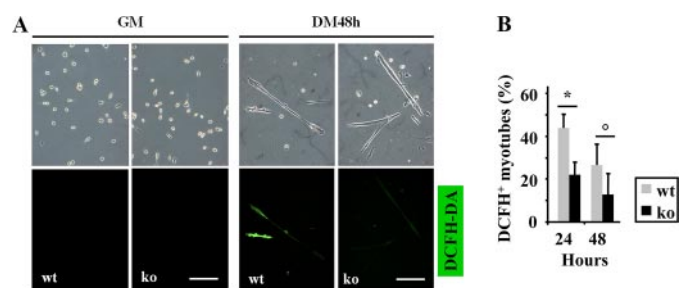


FIGURE 5. Lower oxidative stress in p66^{ShcA}ko myotubes. p66^{ShcA}wt and ko SCs were cultured in GM for 4 days and divided in two groups. The first one received DCFH-DA for the last 30 min. The second one was shifted in DM for 24 or 48 h and DCFH-DA was added for the last 30 min. *A*, top row shows phase contrast microscopy images of p66^{ShcA}wt and ko cells; bottom row shows DCFH-DA green fluorescence in the same cells. Calibration bar, 50 μ m. *B*, bar graph represents percentage of DCFH-DA-positive myotubes at 24 and 48 h of differentiation. WT versus ko, $p < 0.02$; 24 h $n = 4$, 48 h $n = 6$. Error bars = S.E.

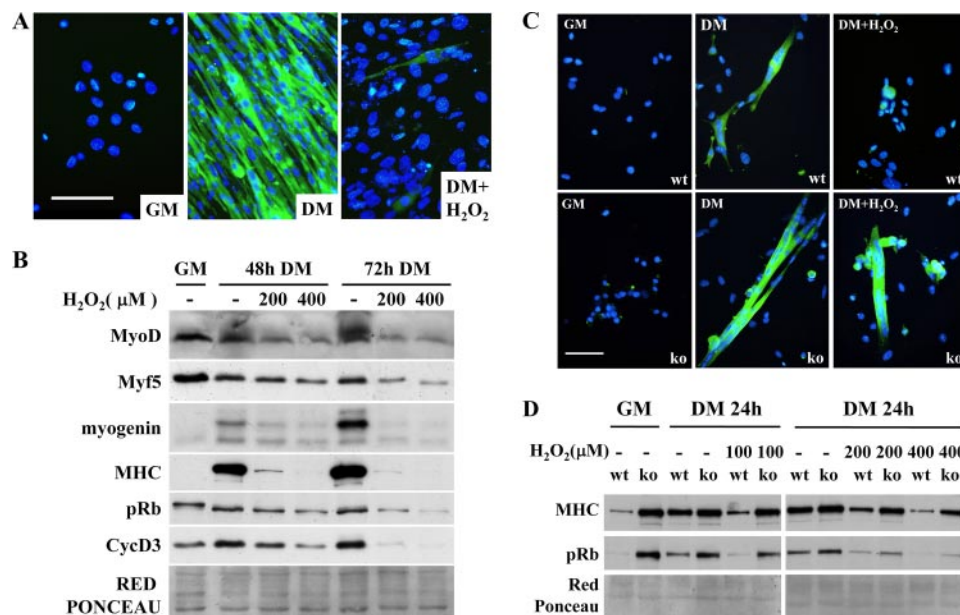


FIGURE 6. H₂O₂ inhibits myogenic differentiation. *A*, representative MHC immunofluorescences of C₂C₁₂ cells. Cells were cultured in growth medium (GM, left panel), shifted to differentiation medium (DM) for 48 h (middle panel), or treated with 200 μ M H₂O₂ for 4 h before induction of differentiation (right panel). Calibration bar, 100 μ m. *B*, Western blotting analysis of C₂C₁₂ cells treated for 4 h with 200 or 400 μ M H₂O₂ before induction of differentiation for 48 or 72 h. GM lane represents proliferating C₂C₁₂ cells. Myogenic markers were detected with the use of specific antibodies. Bottom panel shows Red ponceau staining, indicating similar protein loading in each lane. *C*, representative MHC immunofluorescences of p66^{ShcA}wt and ko SCs. Cells were maintained in GM for 4 days and treated with 200 μ M H₂O₂ for 4 h before induction of differentiation for 24 h (right panels). Calibration bar, 50 μ m. *D*, representative Western blotting of p66^{ShcA}wt and ko SCs treated as in panel *C*. Extracts of proliferating cells (GM) were obtained after 6 days of culture in order to highlight spontaneous differentiation of p66^{ShcA}ko SCs. MHC and pRb were detected with the use of specific antibodies.

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p66^{ShcA}ko mice. In keeping with this interpretation, the ability of p66^{ShcA}ko endothelial cells to form capillary-like structures on Matrigel-coated dishes not only did not increase but was actually decreased.⁴ Finally, ALD was not significantly affected by ischemia in both genotypes.

Perfusion measurement by laser Doppler did not show any significant difference in perfusion recovery between p66^{ShcA}wt and ko ischemic mice. These results are in agreement with the absence of any difference in ALD in normoperfused and ischemic muscles of both genotypes and indicate that different perfusion was not a likely cause of the regenerative advantage of the ko mice.

Thus, the contribution of myogenic precursors in faster skeletal muscle regeneration of p66^{ShcA}ko mice was investigated. We focused our attention on the SCs population, since the contribution of other stem cell populations to skeletal muscle regeneration has been shown to be very limited (37, 38). An equal number of SCs was found in mice of the two genotypes, suggesting that faster regeneration could be due to biological features of ko SCs rather than to their quantity.

In agreement with *in vivo* data, ko SCs displayed a higher proliferation rate at first and spontaneous differentiation when culture was prolonged. These results were confirmed by a totally different approach. Indeed, when MyoD was overexpressed in MEFs, ko cultures differentiated with higher efficiency than WT. Therefore, our experiments strongly indicate that p66^{ShcA} deletion enhances myogenic differentiation both *in vivo* and *in vitro*, indicating that this is a cell-autonomous event.

Natalicchio *et al.* (39) showed that the antisense-mediated reduction of p66^{ShcA} levels in the L6 rat myoblast cell line resulted in marked phenotypic abnormalities, complete disruption of both actin filaments and cell cytoskeleton, and lack of terminal differentiation into myotubes. To reconcile these apparently discordant results, one may keep in consideration the profound differences in the experimental systems adopted and, in particular, the potential off-target effects of the antisense strategy and the fact that immortalized rat myoblasts were used.

Because p66^{ShcA} modulates ROS metabolism and apoptosis, we investigated the relationship between ROS levels and myogenic differentiation. In keeping with previous reports (34, 40, 41), we found that a higher oxidative environment significantly slowed myogenic differentiation and myotube formation and that these morphological observations tightly correlated with decreased expression of differentiation markers. However, p66^{ShcA}ko SCs that genetically lost a crucial element in the machinery transducing oxidative signaling were resistant to H₂O₂ inhibition of myogenic differentiation and maintained the ability to fuse and consequently form multinucleated myotubes. In agreement with *in vivo* results, p66^{ShcA}ko SCs also showed decreased oxidative stress levels, possibly due to different mitochondrial activity (42, 43). Finally, albeit many experimental differences exist, it is worth noting that proliferation of primary SCs is increased when cells are cultured in 6% oxygen,

a condition associated with decreased oxidative stress (44, 45). Conversely, myogenic differentiation was inhibited when myoblasts were exposed to hypoxia (1% oxygen) (29), a condition associated with increased oxidative stress. Although the concentration of H₂O₂ used in our *in vitro* experiments may be considered supra-physiological, up to 800- μ M concentrations of H₂O₂ are widely used in cell culture experiments (46, 47). In addition, in our experimental conditions H₂O₂ concentration up to 400 μ M did not induce cell death, as previously reported (27).

Several models may be proposed to explain the mechanism by which p66^{ShcA} and oxidative stress modulate skeletal muscle regeneration. First, it is possible that nitric oxide plays a role in faster regeneration and higher myogenic potential of p66^{ShcA}ko mice and cells. Indeed, nitric oxide mediates SC activation (48) and it is required for myoblast fusion (49). Because ROS rapidly react with nitric oxide-generating nitrogen species, such as peroxynitrite (5), it is conceivable that p66^{ShcA} deletion enhances nitric oxide bioavailability (17), favoring myogenic differentiation.

Another potential mechanism involves the NAD⁺-dependent histone deacetylase Sir2. The deacetylase activity of Sir2 senses the fluctuation of cytosolic NAD⁺/NADH ratio, *i.e.* the cellular redox state (50). In the presence of high levels of ROS, NAD⁺ levels increase, leading to Sir2 activation, which in turn inhibits MyoD-dependent transcription. Higher myogenic potential of p66^{ShcA}ko mice could be due to lower levels of ROS mediating decreased Sir2 activity.

Finally, Puri *et al.* (51) demonstrated that genotoxic stress may trigger a differentiation checkpoint and cause a reversible inhibition of myogenic differentiation targeting MyoD phosphorylation. It is possible to speculate that increased ROS levels may trigger the differentiation checkpoint and that the checkpoint activation may be attenuated by p66^{ShcA} deletion. While further studies are certainly needed, it is worth noting that these models are not mutually exclusive and the final phenotype might be the product of the combinatorial effect of the modulation of different pathways.

In conclusion, the present study has demonstrated the implication of oxidative stress and p66^{ShcA} in ischemic skeletal muscle damage and regeneration. These findings point to p66^{ShcA} as a key target in novel therapeutic strategies aimed at stimulating tissue regeneration after ischemic events.

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