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J Appl Physiol 101: 1215–1221, 2006.
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Inhibited skeletal muscle healing in cyclooxygenase-2 gene-deficient mice: the role of PGE_2 and $PGF_{2\alpha}$

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Submitted 19 October 2005; accepted in final form 9 June 2006

Shen, Wei, Victor Prisk, Yong Li, William Foster, and Johnny Huard. Inhibited skeletal muscle healing in cyclooxygenase-2 gene-deficient mice: the role of PGE_2 and $PGF_{2\alpha}$. *J Appl Physiol* 101: 1215–1221, 2006. First published June 15, 2006; doi:10.1152/japplphysiol.01331.2005.—Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat skeletal muscle injury. However, studies have shown that NSAIDs may be detrimental to the healing process. Mediated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2), the cyclooxygenase-2 (COX-2) pathway plays an important role in muscle healing. We hypothesize that the COX-2 pathway is important for the fusion of muscle cells and the regeneration of injured muscle. For the in vitro experiments, we isolated myogenic precursor cells from wild-type (Wt) and COX-2 gene-deficient (COX-$2^{-/-}$) mice and examined the effect of PGE_2 and $PGF_{2\alpha}$ on cell fusion. For the in vivo experiments, we created laceration injury on the tibialis anterior (TA) muscles of Wt and COX-$2^{-/-}$ mice. Five and 14 days after injury, we examined the TA muscles histologically and functionally. We found that the secondary fusion between nascent myotubes and myogenic precursor cells isolated from COX-$2^{-/-}$ mice was severely compromised compared with that of Wt controls but was restored by the addition of $PGF_{2\alpha}$ or, to a lesser extent, PGE_2 to the culture. Histological and functional assessments of the TA muscles in COX-$2^{-/-}$ mice revealed decreased regeneration relative to that observed in the Wt mice. The findings reported here demonstrate that the COX-2 pathway plays an important role in muscle healing and that prostaglandins are key mediators of the COX-2 pathway. It suggests that the decision to use NSAIDs to treat muscle injuries warrants critical evaluation because NSAIDs might impair muscle healing by inhibiting the fusion of myogenic precursor cells.

muscle injury; inflammation; nonsteroidal anti-inflammatory drugs; prostaglandin $F_{2\alpha}$; prostaglandin E_2; fusion

SKELETAL MUSCLE INJURY IS a common occurrence in sporting events and athletic endeavors. Such injuries may range in severity from common and relatively minor ones, like strains and contusions, to much more severe ones, like lacerations. Injury to skeletal muscle induces a healing response that involves various stages. The traumatized muscle undergoes a sequential process of degeneration, inflammation, and regeneration. Additionally, when injuries are severe, the muscle-healing process may culminate in fibrosis (12, 18). Various cytokines and growth factors connect these stages of healing. For instance, mediators such as insulin-like growth factor-1 and transforming growth factor-β1, released by both myofibers and inflammatory cells, induce the processes of muscle regeneration and fibrosis, respectively (19, 26).

Prostaglandins, which are potent modulators of inflammation, appear to be involved in multiple aspects of muscle regeneration after injury (30). Prostaglandins are signaling molecules synthesized from arachidonic acid that is released from membrane phospholipids by phospholipase. The arachidonic acid is converted into the prostaglandin PGH_2 by the enzyme cyclooxygenase (COX). Specific synthases can then convert the COX product PGH_2 into various other prostaglandins. For example, in injured skeletal muscle the prostaglandins PGE_2 and $PGF_{2\alpha}$ play receptor-mediated roles in nociception, inflammation, and regeneration (8, 21, 24, 30). Prostaglandins are important for regulating the control of muscle protein synthesis and degradation (29). In particular, $PGF_{2\alpha}$ can promote the growth of skeletal muscle by stimulating the secondary fusion between nascent myotubes (contains <3 nuclei) and more single myogenic cells (10, 11).

COX exists in three known isoforms: COX-1, COX-2, and COX-3. COX-1 is constitutively expressed and affects homeostasis in many tissues, including the gastric mucosa and circulating platelets. COX-2 is an inducible isoform of COX that is expressed in injured or inflamed locations (22), although in normal adult mammalian kidney a low level of COX-2 expression was observed in the cells of the macula densa and adjacent cortical thick ascending limb and a subset of medullary interstitial cells (6, 7). COX-3, which might be a splice variant of COX-1, is thought to be involved in the central nervous system's control of fever and is responsive to acetaminophen (42).

Treatments prescribed for muscle injuries often include the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) to help alleviate pain and inflammation. NSAIDs reduce prostaglandin synthesis by selectively or nonselectively inhibiting the COX enzymes. Studies have shown that nonselective NSAIDs delay muscle regeneration and impede the long-term functional recovery of injured muscle (1, 25, 28). Recently, physicians have begun to prescribe COX-2-selective inhibitors (such as celecoxib and valdecoxib) for muscle trauma because of these drugs' analgesic and anti-inflammatory properties and reduced side-effect profiles. By avoiding COX-1 inhibition, clinicians can essentially avoid inhibiting homeostatic functions and thus can limit platelet inhibition and gastric mucosal injury. However, some studies have indicated that COX-2-selective NSAIDs may also have detrimental effects on the recovery of injured muscle (2, 35). Research has shown that the COX enzyme and prostaglandins are upregulated after repetitive use of skeletal muscle and that NSAIDs

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can suppress the upregulated synthesis of prostaglandins (38, 40). Thus it is likely that the COX enzyme and its downstream products, the prostaglandins, are integral to the healing process of skeletal muscle.

To date there have been very few studies performed to directly examine the role of COX-2 on muscle healing. We performed in vitro experiments to specifically examine the effects of PGE_2 and $PGF_{2\alpha}$, the COX-2 pathway products, on the fusion of myogenic precursor cells. With the use of a muscle-laceration injury model in both wild-type (Wt) and COX-2 gene-deficient (COX-$2^{-/-}$) mice, our in vivo experiments enabled us to examine the possible interactions between the COX-2 pathway and muscle healing.

MATERIALS AND METHODS

Cell isolation and culture. A previously described preplate technique was used to isolate myogenic precursor cells (16, 31). The gastrocnemius muscles were removed from both COX-$2^{-/-}$ mice (Taconic Farms, Germantown, NY) and their Wt control mice (Taconic Farms). The muscles first were minced with a surgical blade and then were enzymatically digested by sequential treatment with collagenase XI, dispase, and trypsin. The muscle cell extracts were plated on collagen-coated flasks. Different populations were isolated by replating the cells at different time intervals. Cells in the late preplate (LP) population have been shown to possess higher myogenic potential than exhibited by cells in the earlier preplate population (31). The LP cells used in the in vitro experiments were isolated from multiple mice to consider the variability of independent subjects, and these cells were maintained and expanded in proliferation medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10% horse serum, and 0.5% chicken embryo extract). Cells of passage 3–4 were used in all in vitro experiments. The expression of desmin and MyoD, well-known markers of early myogenesis (13, 16), was used to verify the status of LP cells as myogenic precursor cells.

Cell fusion. To observe the fusion ability of LP cells isolated from COX-$2^{-/-}$ mice and Wt control mice, LP cells from each source were plated at the same cell density (20,000 cells per well) in 12-well plates. On *day 0*, cells were grown overnight in serum-free medium to synchronize the cells by starvation. On *day 1*, the serum-free medium was replaced with differentiation medium (Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum and 1% horse serum). The LP cells then were cultured until *day 18*, with replacement of differentiation medium every 3 days. The culture medium was collected for analysis by enzyme-linked immunosorbent assay (ELISA). To observe the effect of PGE_2 and $PGF_{2\alpha}$ on cell fusion, LP cells from COX-$2^{-/-}$ mice were starved on *day 0* for synchronization (as detailed above). On *day 1*, the serum-free medium was replaced with differentiation medium containing different concentrations of PGE_2 and $PGF_{2\alpha}$ [0.28 μM (100 ng/ml), 2.8 μM (1,000 ng/ml), 28 μM (10,000 ng/ml); Sigma, St. Louis, MO]. The differentiation medium containing PGE_2 and $PGF_{2\alpha}$ was replaced every 3 days during the 18-day culture period.

ELISA. ELISA was used to evaluate the differentiation medium collected from the fusion experiments every 3 days. Analysis of PGE_2 and $PGF_{2\alpha}$ was performed as suggested by the manufacturer's protocols (DE0100 PGE_2 ELISA kit, DE1150 $PGF_{2\alpha}$ ELISA kit, R & D Systems, Minneapolis, MN).

Immunohistochemistry. The cultured cells were fixed with cold methanol (−20°C) for 5 min. After administration of 10% horse serum to block nonspecific binding sites for 1 h, the cells were incubated overnight with the primary antibody monoclonal mouse anti-myosin heavy chain-developmental (1:100; Novocastra Laboratories, Newcastle, UK), polyclonal rabbit anti-desmin (Sigma, D8281; 1:200), and mouse anti-MyoD (Pharmingen 554130; 1:250) at 4°C. The cells then were exposed to a Cy3-conjugated secondary antibody (1:250; Sigma) for 50 min at room temperature. 4,6-Diamidino-2-phenylindole staining (Sigma) was used to reveal the nuclei within the cultures. Between each step, cells were washed by PBS for 3 times. Negative controls (staining without the primary antibody) were performed concurrently with all immunohistochemical stainings. Fluorescent microscopy was used to visualize immunofluorescence results (E800, Nikon, Tokyo, Japan). The fusion of LP cells was evaluated at 6, 12, and 18 days, for Wt, COX-$2^{-/-}$, and COX-$2^{-/-}$ + prostaglandin cell cultures. Three hundred nuclei per well (from three fields) for four wells were counted within each group under ×100 magnification. The nuclei were observed to determine whether they belong to a

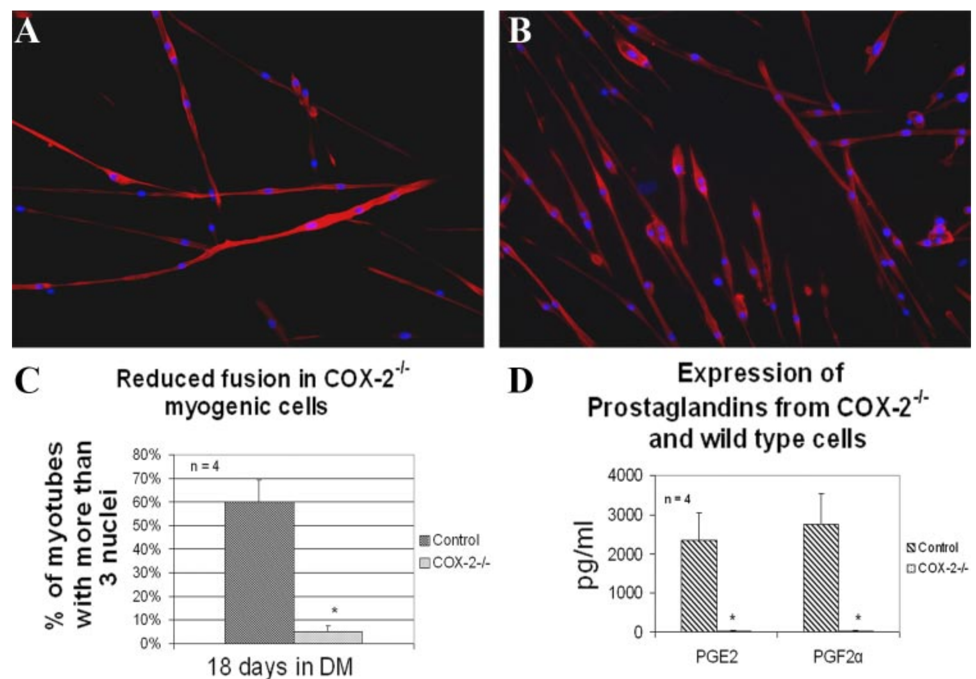

Fig. 1. Compared with the fusion of wild-type (Wt) late preplate (LP) cells (*A*), the fusion of LP cells derived from COX-2 gene-deficient (COX-$2^{-/-}$) mice (*B*) is severely compromised. After COX-$2^{-/-}$ cells and Wt cells were cultured for 18 days in differentiation medium, the percentage of myotubes containing >3 nuclei was significantly lower in the COX-$2^{-/-}$ cell culture than in the Wt cell culture ($*P < 0.05$; *C*). ELISA results indicate that the expression of both PGE_2 and $PGF_{2\alpha}$ was significantly lower in COX-$2^{-/-}$ cell cultures than in Wt cell cultures ($*P < 0.05$; *D*). Error bars represent standard deviation.

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Table 1. *Fusion of LP cells was evaluated at 6, 12, and 18 days*

	% of Single Cells	% of Nascent Myotubes (<3 nuclei)	% of Mature Myotubes (≥3 nuclei)
6 days	28.4/35.7/30.9	61.5/64.3/60.3	10.1/0/8.8
12 days	7.8/10.7/8.0	50.6/86.6/59.9	41.6/2.7/32.1
18 days	5.1/6.9/5.3	34.7/88.0/40.5	60.2/5.1/54.2

Three hundred nuclei per well for 4 wells were counted for each group and were divided into single cells, nascent myotubes (<3 nuclei), and mature myotubes (≥3 nuclei) categories. The percentage of each category was shown in the table in a manner of wild-type (Wt) cells/cyclooxygenase (COX)-$2^{-/-}$ cells/COX-$2^{-/-}$ cells + 0.28 μM $PGF_{2\alpha}$ culture. LP, late preplate.

single cell, a nascent myotube (<3 nuclei), or a mature myotube (≥3 nuclei). The percentage of each category was calculated.

Animal model. Seventeen COX-$2^{-/-}$ mice and 17 Wt control mice (10–12 wk of age, Taconic Farms) were used for in vivo experiments. Seven mice from each group were used for histological evaluation at 5 days after injury. Seven mice from each group were used for both histological and physiological evaluation at 14 days after injury. The other three mice from each group were used for the flow cytometry experiment. The Animal Research and Care Committee at the authors' institution approved all experimental protocols (protocol no. 5/01). The skeletal muscle laceration model used in this study entailed laceration of the TA muscle on both legs, as described previously (5, 23, 27). The mice were anesthetized by intramuscular injection of ketamine (0.03 ml, 100 mg/ml) and xylazine (0.02 ml, 20 mg/ml). A surgical blade (no. 11; SteriSharps, Mansfield, MA) was used to lacerate each TA muscle at its midpoint from both ends and at its full depth. After laceration, the skin was closed with black silk 4-0 suture (Ethicon, Cornelia, GA). Mice were killed 5 and 14 days after surgery, and the TA muscles were evaluated histologically and physiologically. The weight of the TA muscle was measured before the muscles were fresh-frozen in 2-methylbutane precooled in liquid nitrogen and then were stored at −80°C pending histological analysis.

Histology. The cryosections were fixed in 1% glutaraldehyde for 1 min and then were dipped into hematoxylin for 30 s. After being washed in alcohol acid and ammonia water, the sections were immersed in eosin for 15 s. After each step, sections were rinsed with distilled water. Alcohols of increasing concentration (70, 80, 95, and 100%) were then used for dehydration by dipping slides in each concentration for 1 min. Finally, the sections were treated with xylene and covered with glass slips.

Slides were analyzed manually by bright field microscopy (Eclipse E800, Nikon, Tokyo, Japan) and with Northern Eclipse software (Empix Imaging, Cheektowaga, NY). Sections containing the largest area of injured tissue were selected for analysis. The centronucleated regenerating myofibers were counted under ×100 magnification (7 animals/group). At ×200 magnification, an image of the center of the injured area was taken; the minor axis diameter (the smallest diameter) of 200 centronucleated myofibers in this image was measured.

Flow cytometry analysis. The TAs from Wt and COX-$2^{-/-}$ groups were surgically removed at 3 days after injury. Collagenase, dispase, and trypsin were used to digest the tissue matrix and isolate the cells. Debris was removed via filtration with 100-μm filters. Isolated cells first were treated with 10% mouse serum (Sigma) to block nonspecific binding sites. Primary rat anti-CD-11b (conjugated with FITC, R & D Systems) antibodies was used to identify the inflammatory cells, including neutrophils and macrophages, because CD-11b is expressed by both neutrophils and macrophages (14, 39). 7-Amino-actinomycin D (Pharmingen) was added to all tubes to exclude nonviable cells from the analysis. Marked cell samples then were analyzed with a FACS Caliber flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

Physiological testing. The physiological testing was conducted as described previously (43). Briefly, the mice were euthanized and the TA muscles were isolated. Each TA muscle was mounted in a vertical tissue chamber that was constantly perfused with mammalian Ringer's solution aerated with 95% O_2-5% CO_2 and maintained at 25°C. The TA muscle was secured at both ends and was connected by fine wire to a force transducer and length servo system (model 305B, dual mode, Aurora Scientific, Aurora, ON, Canada). The muscle then was stimulated (Grass model S-88 stimulator and current amplifier) by monophasic rectangular pulses of cathodal current to obtain the specific peak force (N/cm^2) and specific tetanic force (N/cm^2). Fourteen days after laceration injury, TA muscles from the Wt mice and the COX-$2^{-/-}$ mice were tested in this manner. The noninjured Wt mice and COX-$2^{-/-}$ mice also were used to generate baseline values. The values of injured muscles were divided by the baseline values to calculate the percentages of functional recovery.

Statistics. Comparisons among multiple groups were performed by one-way ANOVA. The number of regenerating myofibers at 5 and 14 days after injury was compared by two-way ANOVA. Post hoc multiple comparison tests were performed to determine which means differ. The percentage of functional recovery was evaluated by χ^2. Other comparisons between two groups were made by unpaired Student's *t*-test. For all statistical tests, the 0.05 level of confidence was considered statistically significant. Statistical power analysis (power = 0.80) was performed to determine the sample size.

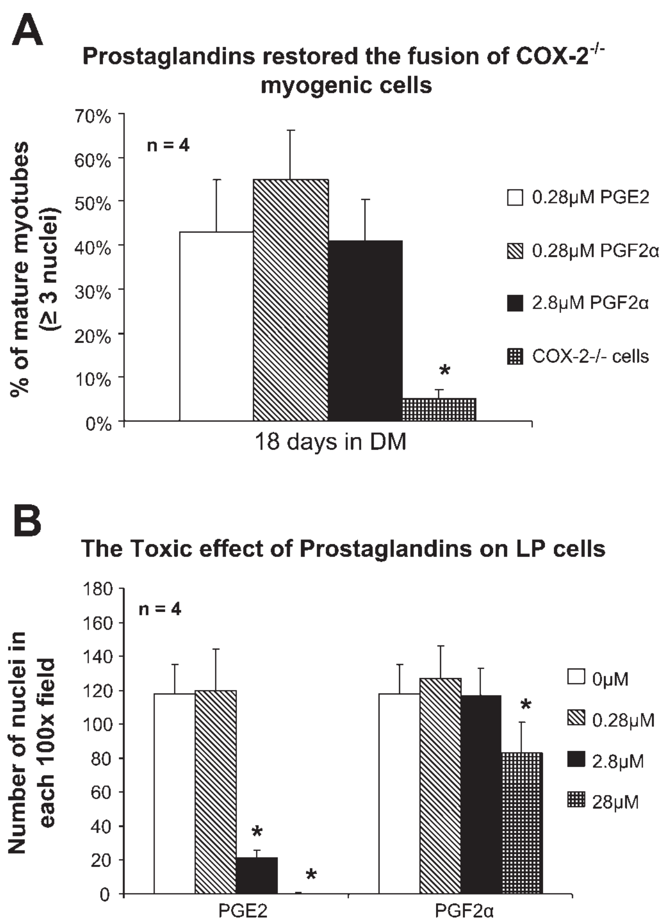

Fig. 2. Addition of PGE_2 (0.28 μM) or $PGF_{2\alpha}$ (0.28 μM, 2.8 μM) significantly improved the fusion ability of COX-$2^{-/-}$ cells to varying degrees (*$P <$ 0.05; *A*). *B*: average number of nuclei in ×100 magnification fields at the end of the culture period. High concentrations of PGE_2 (2.8 μM, 28 μM) or $PGF_{2\alpha}$ (28 μM) were toxic to LP cells and caused cell detachment from the culture surface. Error bars represent standard deviation.

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RESULTS

Fusion of LP cells and the expression of PGE_2 and $PGF_{2\alpha}$. LP cells isolated from Wt mice or COX-$2^{-/-}$ mice were stained for desmin and MyoD to verify their myogenic status. Ninety-nine and 96% of the cells from Wt mice or COX-$2^{-/-}$ mice were positive for desmin, respectively. Seventy and 64% of the cells from Wt mice or COX-$2^{-/-}$ mice were positive for MyoD, respectively. It suggests that these LP populations both contained primarily myogenic precursor cells and were comparable populations. We performed immunostaining to identify myosin heavy chain-developmental expression and cell nuclei in the cell populations cultured for 18 days in differentiation medium. Only 5% of the myotubes in the COX-$2^{-/-}$ LP cell culture contained more than three nuclei, compared with 60% of the myotubes in the Wt LP cell culture (Fig. 1, *A–C*). In Table 1, the percentages of nuclei that belong to single cells, nascent myotubes (<3 nuclei), and mature myotubes (≥3 nuclei) were listed for different time points (6-, 12-, and 18-day culture) and different cell types (Wt cells and COX-$2^{-/-}$cells). The fusion of COX-$2^{-/-}$ cells was probably inhibited at the secondary phase, on the basis of the fact of normal formation of nascent myotubes and limited formation of mature myotubes. However, the total numbers of nuclei were similar in the two groups. At ×100 magnification, averages of 112 and 120 nuclei per field were observed in the Wt cell population and the COX-$2^{-/-}$ cell population, respectively. The expression of PGE_2 and $PGF_{2\alpha}$ was significantly lower in the COX-$2^{-/-}$ LP population than in the Wt LP population at all time points. The expression levels observed on *day 4* are shown as an example (Fig. 1*D*).

PGE_2 and $PGF_{2\alpha}$ restore the fusion of COX-$2^{-/-}$ cells. Both PGE_2 and $PGF_{2\alpha}$ restored the secondary fusion of COX-$2^{-/-}$ cells. Treatment of the COX-$2^{-/-}$ cell cultures with the two different concentrations of $PGF_{2\alpha}$ (0.28 and 2.8 μM) raised the percentages of mature myotubes to 55 and 41% (Fig. 2*A*). In Table 1, the data from the 0.28 μM $PGF_{2\alpha}$ treatment group were used as a sample to show that prostaglandins help to restore the fusion of COX-$2^{-/-}$ cells. Treatment of the COX-$2^{-/-}$ cell cultures with PGE_2 at a concentration of 0.28 μM raised the percentage of mature myotubes to 43% (Fig. 2*A*). At concentrations of 2.8 μM and 28 μM, PGE_2 had a toxic effect on COX-$2^{-/-}$ LP cells over the 18-day period. Most cells gradually detached from the culture surface, and very few cells remained attached at the end of the culture period (Fig. 2*B*). Analysis of the floating cells with a Trypan blue assay demonstrated that the cells were dead.

Muscle regeneration. Our evaluation of TA muscle sections obtained 5 days after laceration revealed fewer regenerating myofibers in the COX-$2^{-/-}$ muscle sections than in the Wt muscle sections ($P < 0.05$). At 14 days after injury, muscle sections from the COX-$2^{-/-}$ mice again contained significantly fewer regenerating myofibers than observed in the Wt muscle sections, and the average diameter of the minor axes of the myofibers in the COX-$2^{-/-}$ sections was significantly lower than that measured in the Wt muscle sections ($P < 0.05$; Fig. 3, *A* and *B*). We did not compare the minor axis diameters in the two groups 5 days after injury because the sections obtained from the COX-$2^{-/-}$ mice contained too few regenerating myofibers.

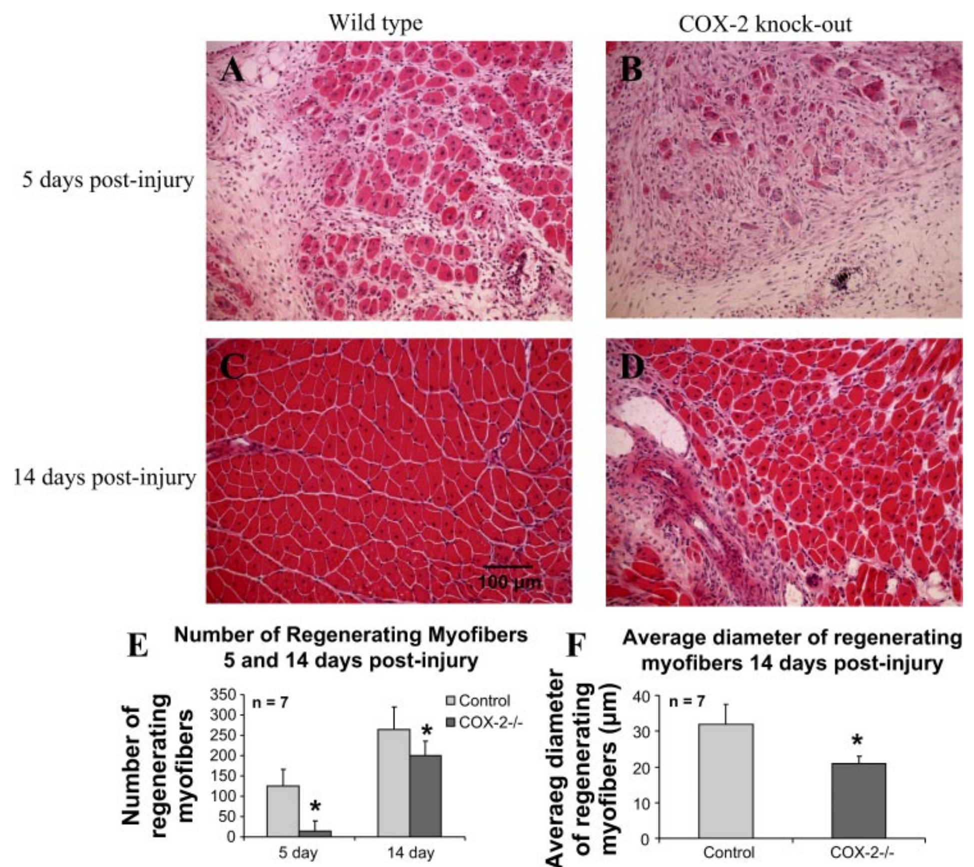

Fig. 3. Recovery of Wt mice (*A*, *C*) and COX-$2^{-/-}$ mice (*B*, *D*) 5 and 14 days after injury is shown by hematoxylin and eosin staining. The number of centronucleated regenerating myofibers per field was significantly lower in COX-$2^{-/-}$ mice 5 and 14 days after injury than in Wt (control) mice (*$P < 0.05$; *E*). The minor axis diameters of regenerating myofibers 14 days after injury were significantly smaller in COX-$2^{-/-}$ mice than in Wt (control) mice (*$P < 0.05$; *F*). Error bars represent standard deviation.

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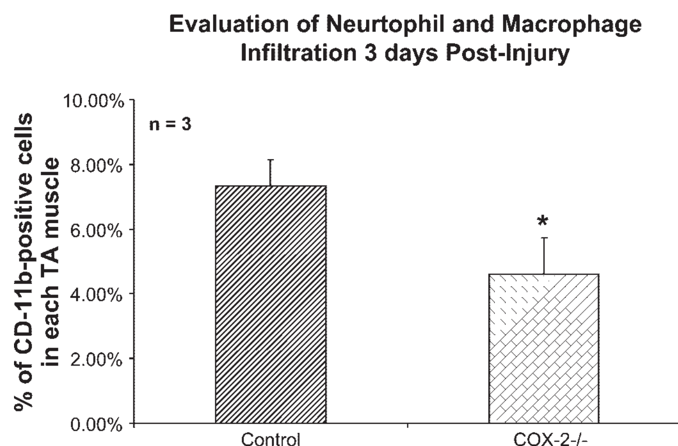

Fig. 4. Flow cytometry revealed significantly reduced infiltration of CD-11b-positive cells, including neutrophils and macrophages, in COX-$2^{-/-}$ mice compared with Wt (control) mice (*$P < 0.05$). Error bars represent standard deviation.

Inflammatory response. We performed flow cytometry to assess the infiltration of inflammatory cells, including neutrophils and macrophages, in the 3-days-postinjury TA muscles. Muscle samples from the COX-$2^{-/-}$ mice contained fewer CD-11b-positive cells than did those from Wt mice at 3 days after injury ($P < 0.05$; Fig. 4). This finding suggests that the COX-$2^{-/-}$ mice exhibited a lower inflammatory response than did the Wt mice.

Physiological testing. We tested the specific peak force (N/cm^2) and the specific tetanic force (N/cm^2) of TA muscles isolated from Wt and COX-$2^{-/-}$ mice before and 14 days after injury. We calculated the functional recovery percentage and used it as an indicator of functional recovery of the injured muscles. The Wt (control) group muscles exhibited better functional recovery than the COX-$2^{-/-}$ muscles, as indicated by a higher functional recovery percentage in the former group of muscles ($P < 0.05$; Fig. 5).

DISCUSSION

Here we used COX-$2^{-/-}$ mice and cells to directly examine the role of COX-2 and prostaglandins in muscle healing. We found that in vitro the fusion of COX-$2^{-/-}$ myogenic precursor cells (LP cells) was compromised just before the secondary fusion phase, a phase during which nascent myotubes (formed by the fusion of 2 myogenic cells) fuse with more single myogenic cells to increase in size and become fully mature (10). The addition of PGE_2 and $PGF_{2\alpha}$, the products of the COX-2 pathway, significantly improved the secondary fusion of the COX-$2^{-/-}$ LP cells. As predicted by the in vitro results, COX-$2^{-/-}$ mice exhibited impaired skeletal muscle healing after laceration injury. On the basis of these results, we conclude that the COX-2 pathway is important in skeletal muscle healing and that PGE_2 and $PGF_{2\alpha}$, two downstream products of COX-2, mediate its effects.

Scientists used severe skeletal muscle injury models, including muscle laceration and contusion, to examine how inflammation affects muscle regeneration (1, 2, 18, 32). Various studies focused on nonselective NSAIDs have yielded conflicting data regarding the importance of inflammatory prostaglandin synthesis during muscle regeneration. These inconsistencies are partly due to the use of different NSAIDs with varied COX-1/COX-2 selectivity or different COX-independent effects (9, 30, 44, 45). To avoid the variable of different NSAIDs and to examine the role of COX-2 directly, we used COX-$2^{-/-}$ mice and cells for the study reported here.

The results of the in vitro experiments demonstrate the importance of COX-2 and its downstream prostaglandin products in myogenesis. COX-$2^{-/-}$ LP cells had a reduced ability to differentiate into mature myotubes (≥3 nuclei) from nascent

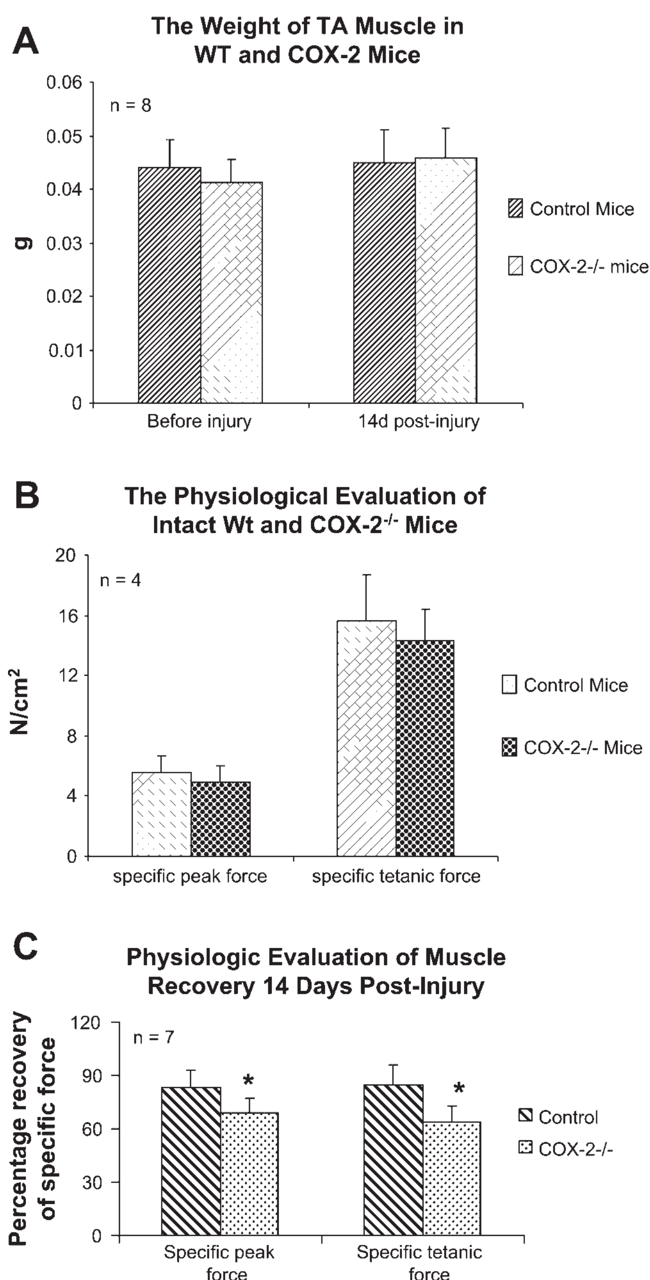

Fig. 5. Weight of TA muscles and physiological testing was measured and performed on Wt (control) and COX-$2^{-/-}$ mice before injury and 14 days after injury. The weight of TA muscle (*A*) and its physiological properties (*B*) showed no difference between Wt and COX-$2^{-/-}$ mice when they were intact. The physiological testing data obtained after injury were divided by the baseline data obtained from intact mice. We revealed significantly reduced percentage of functional recovery in COX-$2^{-/-}$ mice compared with Wt mice (*$P < 0.05$, *C*). Error bars represent standard deviation.

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myotubes (<3 nuclei), and this inhibition of fusion correlated with the cells' significantly reduced expression of PGE_2 and $PGF_{2\alpha}$. The addition of $PGF_{2\alpha}$ and, to a lesser extent, PGE_2 to the COX-$2^{-/-}$ LP cell culture restored the secondary fusion ability of these cells. Earlier studies have demonstrated the involvement of $PGF_{2\alpha}$ in protein synthesis within skeletal muscle and in muscle cell growth (33, 40). Horsley and Pavlath (11) advanced this area of research by showing that $PGF_{2\alpha}$ enhances the fusion of skeletal muscle cells not by stimulating the cells to form myotubes but by recruiting additional cells to fuse with the preexisting nascent myotubes. It is postulated that $PGF_{2\alpha}$ is more important for the secondary fusion of myofibers into larger myofibers than for the initial fusion of 2 single myogenic cells (10). In accordance with this theory, our observations revealed a higher percentage of mature myotubes in the Wt LP cell cultures than in the COX-$2^{-/-}$ LP cell cultures (in which the vast majority of myotubes contained <3 nuclei). However, the total numbers of nuclei within these two types of cell cultures at the end of the culture period were similar, which suggests that the higher percentage of large myotubes in the Wt cell culture was due to increased fusion rather than heightened cell proliferation. Many scientists view PGE_2 as an important inflammatory mediator; however, research also has shown that PGE_2 can influence the rate of protein turnover in skeletal muscle (33). In addition, PGE_2 appears to induce the fusion of other cell types, such as osteoclasts (41). Here we found that PGE_2 administered at a concentration of 0.28 μM restored the secondary fusion ability of COX-$2^{-/-}$ LP cells. At higher concentrations (2.8 μM, 28 μM), however, PGE_2 had a toxic effect on the growing LP cells. Our results indicate that $PGF_{2\alpha}$ is less toxic than PGE_2, but high concentrations of $PGF_{2\alpha}$ (at 28 μM) also significantly inhibited cell growth. A comparison of $PGF_{2\alpha}$ and PGE_2 delivered at the same concentration (0.28 μM) reveals that $PGF_{2\alpha}$ administration resulted in better restoration of the fusion ability of COX-$2^{-/-}$ LP cells.

The results of our in vivo experiments revealed that COX-2 gene deficiency had a significant effect on both inflammation and early muscle regeneration. We found significantly fewer regenerating myofibers in the COX-$2^{-/-}$ mice 5 and 14 days after muscle laceration injury, and those regenerating myofibers had smaller diameters than did myofibers in the Wt mice. The COX-$2^{-/-}$ mice also showed significantly reduced functional recovery of skeletal muscle force 14 days after injury. Our in vivo results are consistent with the findings from a previous study done by Bondesen et al. (2). They showed that the size of regenerating myofibers was significantly smaller in the COX-$2^{-/-}$ mice 10 days after a freeze-induced injury. Our study further proved this notion by showing an inhibited functional recovery in COX-$2^{-/-}$ mice.

PGE_2 appears to play multiple roles in the muscle inflammatory process, including induction of proinflammatory cytokine synthesis and nitric oxide synthase expression, vasodilation with increased vascular permeability, and chemotaxis of inflammatory cells (34, 36, 37). The reduced PGE_2 synthesis in COX-$2^{-/-}$ mice appeared to markedly limit the inflammatory responses. CD-11b is a cell surface marker of both macrophages and neutrophils (14, 15). Our counting of CD-11b-positive cells in the injured muscle revealed significantly reduced infiltration of macrophages and neutrophils 3 days after injury in COX-$2^{-/-}$ mice compared with Wt mice. These inflammatory cells, especially macrophages, are important mediators of the regenerative process. Macrophages secrete growth factors and cytokines that act in a paracrine fashion to stimulate myoblast regenerative events (3, 17, 20). Furthermore, direct contact with macrophages can rescue myogenic precursor cells from apoptosis after muscle injury. The rescued cells can act synergistically with macrophages to amplify chemotaxis and enhance muscle growth (4). In the case of COX-$2^{-/-}$ mice, the clearing of necrotic debris and growth factor-stimulated satellite cell and myoblast proliferation by infiltrating macrophages may be delayed. COX-$2^{-/-}$ mice may also exhibit limited synthesis of $PGF_{2\alpha}$, a product of the COX-2 pathway that appears to be important for the secondary fusion of myoblasts (11). All of these factors may help to explain the limited muscle regeneration observed in COX-$2^{-/-}$ mice. The study reported here provides evidence of this delayed muscle regeneration and subsequent reduction in functional recovery of muscle, as evidenced both histologically (by reduced numbers and size of regenerating myofibers) and physiologically (by limited recovery of muscle force).

Previously, by using NS-398, a COX-2-specific inhibitor, we have shown that the production of prostaglandins was inhibited in LP cell culture and muscle regeneration was delayed in laceration-injury mice (35). In this COX-2 null study, we showed that the COX-$2^{-/-}$ animal had an inhibited muscle healing process, and prostaglandins promoted secondary fusion of COX-$2^{-/-}$ LP cells. On the basis of these results, we conclude that COX-2 pathway probably plays an important role in the healing of skeletal muscle, and prostaglandins are the key mediators of COX-2 pathway in regulating muscle healing. Our results indicate that athletes or patients who are taking NSAIDs to treat acute or chronic pain due to muscle injuries might have an increased risk of reduced functional healing or prolonged rehabilitation. Until researchers generate additional results on this topic, clinicians should weigh the benefits of NSAID-induced pain relief against the possibility of reduced muscle regeneration.

ACKNOWLEDGMENTS

We thank Ying Tang for technical support, Terry O'Day for tremendous help with the physiological testing, Freddie Fu and James Cummins for helpful discussions, and Ryan Sauder for excellent editorial assistance during manuscript preparation.

GRANTS

Supported by funding from the National Institutes of Health (1R01 AR 47973-01 to J. Huard), the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, and the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh. This investigation was conducted in a facility constructed with support from Research Facility Improvement Program Grant Number C06 RR-14489 from the National Center for Research Resources, National Institutes of Health.

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