The COX-2 pathway is essential during early stages of skeletal muscle regeneration

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SKELETAL MUSCLE REGENERATION is initiated by muscle injury and involves the degeneration of damaged myofibers, inflammation, and the formation of new myofibers through myogenesis. Because the nuclei within myofibers are postmitotic, myogenesis primarily depends on satellite cells, which are quiescent muscle precursor cells that lie beneath the basal lamina surrounding each myofiber. In response to injury, satellite cells reenter the cell cycle and proliferate. These activated precursor cells, or myoblasts, then undergo differentiation and fusion to restore normal muscle structure. Muscle regeneration also depends on other cellular processes involving nonmuscle cells. One of the earliest of these is the inflammatory response, which facilitates myogenesis via phagocytosis of cellular debris and the release of chemotacticants and growth factors (7, 26, 44, 55). The cellular events that occur during muscle regeneration are orchestrated by a number of growth factors and cytokines. However, many aspects of the regulation of muscle regeneration remain unclear and most likely involve molecules that have yet to be defined. Prostaglandins (PGs) are one such candidate group of molecules, as they have been implicated in various stages of myogenesis and are synthesized by regenerating muscle (28, 30, 37, 56). PGs are involved in myoblast proliferation (63), differentiation (48), and fusion (10, 13, 18, 46, 62) in vitro and appear to play an important role in myofiber growth during development (30). PGs also regulate protein synthesis and degradation in muscle, both in vivo and in vitro (37, 50, 58, 60). In addition to their roles in myogenesis, PGs are also potent modulators of inflammation, as evidenced by the ability of inhibitors of PG synthesis known as nonsteroidal anti-inflammatory drugs (NSAIDs) to ameliorate pain and inflammation following muscle injury and other types of tissue damage.

PG synthesis begins with phospholipase-mediated release of arachidonic acid from membrane phospholipids. Cyclooxygenase (COX) converts arachidonic acid to PGH2, which is subsequently metabolized by specific synthases to the bioactive PGs: PGE2, PGF2α, PGI2, and PGD2. Once synthesized, PGs are secreted from the cell and mediate diverse processes via both specific G-protein-coupled receptors and nuclear peroxisome proliferator-activated receptors (15). COX exists as multiple isoforms. COX-1 is constitutively expressed, and COX-1-derived PGs have primarily homoeostatic functions but also appear to be involved in acute inflammation (23). A recently discovered splice variant of COX-1, COX-3, is expressed in the central nervous system and is involved in fever but does not appear to play a significant role in inflammation (6). In contrast to COX-1 and COX-3, COX-2 is an immediate/early gene whose expression in most tissues is low or absent but is transiently induced by mitogens and cytokines. Whereas COX-2 is generally considered proinflammatory, it also appears to play a role in the resolution of inflammation (16, 23). Despite catalytic and structural similarities, COX-1 and COX-2 have distinct biological functions due to differences in gene expression, mRNA stability, translational efficiency, substrate pool preference, and subcellular localization (11). Although COX-1 and COX-2 both produce PGH2, each isoform is associated with a different array of final PG products, which is likely due to differential functional coupling with downstream PG syntheses (59). In addition, the phenotypes of COX-1−/− mice are distinct from those of COX-2−/− mice (23), providing further evidence that the functions of these isoforms are nonredundant.

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NSAIDs are valuable tools for studying the involvement of PGs in physiological processes, as they are competitive inhibitors of COX activity. Nonselective NSAIDs decrease PG synthesis by inhibiting both COX-1 and COX-2, many with higher selectivity for COX-1. In contrast, selective NSAIDs specifically inhibit either COX-1 or COX-2. Previous studies analyzing the effect of NSAID treatment on various models of skeletal muscle injury employed nonselective NSAIDs (1, 21, 24, 25, 32, 35, 54). However, due to the inconsistency of experimental methods, type of injury, drug types and doses, and species used in these studies, the function of PGs in skeletal muscle regeneration remains unclear. The use of nonselective NSAIDs did not allow for the individual roles of the COX-1 and COX-2 pathways to be distinguished, which is a significant limitation in light of abundant evidence from other tissues that the COX pathways have nonredundant biological functions.

Here, we tested the hypothesis that COX activity is required for muscle regeneration and investigated the potentially distinct roles of COX-1 and COX-2. Through the use of selective COX-1 and COX-2 inhibitors as well as COX-1 −/− and COX-2 −/− mice, we show that the COX-2 pathway, but not the COX-1 pathway, is crucial for normal muscle regeneration. These results are the first evidence of distinct roles for these two pathways in skeletal muscle. Furthermore, these results suggest that PGs derived from COX-2, but not COX-1, are critical for muscle regeneration, which is consistent with their role in wound repair in other tissues, including bone and liver (2, 12, 47, 49, 52).

MATERIALS AND METHODS

Animals and drugs. Male C57BL/6 mice (8–11 wk) were purchased from Charles River. Male COX wild-type, COX-1 −/−, and COX-2 −/− mice (B6;129P2, 8–11 wk) were purchased from Taconic Farms. Mice were housed under a 12:12-h light-dark cycle, with food and water provided ad libitum. All animals were handled in accordance with the guidelines of Emory University’s Institutional Animal Care and Use Committee. SC-560 and SC-236 were generously provided by Pharmacia.

Muscle injury and histological analyses. Mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 15 mg/kg xylazine. Tibialis anterior (TA) muscles were subjected to a standardized intraperitoneal injection of 100 mg/kg ketamine and 15 mg/kg xylazine. Sections containing the largest area of damage were frozen muscles at 4°C to −80°C until analysis. TxB2 was quantified by enzyme immunoassay (Assay Designs), according to the manufacturer’s instructions.

Immunoblotting. Control and injured TA muscles were homogenized in 300 μl of 250 mM sucrose, 100 mM potassium chloride, 20 mM imidazole, and 5 mM EDTA (pH 6.8) containing protease inhibitors (Mini Complete, Boehringer Mannheim). Homogenates were centrifuged for 15 min at 5,000 g at 4°C to separate insoluble myofibrillar proteins, which were discarded. The remaining supernatant was centrifuged for soluble proteins (19). Equal amounts of protein (100 μg) were separated by SDS-PAGE (7.5%) and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore). After nonspecific binding was blocked with blocking buffer [5% non-fat dry milk in Tris-buffered saline (TBS) for 30 min], membranes were incubated in blocking buffer containing a 1:500 dilution of an antibody against COX-2 (Cayman Chemical) overnight at 4°C. Blots were washed in TBS containing 0.1% Tween 20 and then incubated in donkey anti-rabbit HRP-conjugated secondary antibody (Jackson ImmunoResearch) for 1 h at room temperature. After the blots were washed with TBS-Tween 20, antibody binding was detected by using enhanced chemiluminescence. Exposed films were photographed, and protein levels were quantified by densitometry using Kodak 1D Image Analysis Software. Membranes were stained with BioSafe Coomassie (Bio-Rad) to ascertain equal protein loading. The amount of COX-2 protein was normalized against a control sample that was included with every immunoblot.

Isolation and immunostaining of mononucleated cells from injured muscle. Mononucleated cells were isolated from regenerating TA muscles 3 days after injury by mechanical and enzymatic dissociation. Muscles were excised, separated from fat and excess connective tissue, minced into a coarse slurry, and then digested for 1 h with 0.1% pronase (Calbiochem) in DMEM at 37°C with mild agitation. The digest was then mechanically dissociated by repeated trituration followed by filtration through a 100-μm vacuum filter (Millipore). The filtered digest was centrifuged through an isotonic Percoll gradient (60% overlay with 20%), as described (61). Mononucleated cells were collected from the Percoll interface, resuspended in Ham’s F-10 supplemented with 20% fetal bovine serum, 5 ng/ml basic FGF, 100 U/ml penicillin G, and 100 μg/ml streptomycin, and seeded onto collagen-coated Perlemoax chamber slides (Fisher Scientific). After 24 h, cells were washed with PBS and fixed for 10 min in 3.7% formaldehyde.

The following antibodies were used for immunostaining: anti-MyoD (NCL-MyoD1, 1:20; Novocastra), anti-desmin (1:200; Sigma), and FITC-conjugated anti-Mac-1 (1:100; BD Pharmingen). All steps were performed at room temperature. Cells were incubated with blocking buffer containing 5% goat serum and 0.25% Triton X-100 in PBS, followed by either anti-desmin or anti-MyoD antibody for 1 h. For the anti-desmin antibody, cells were washed with PBS containing 0.2% Tween 20 (PBS-T) and then incubated with FITC-conjugated goat anti-rabbit IgG (1:250 in blocking buffer; Cappel Research Products) for 1 h. For the anti-MyoD antibody, cells were washed with PBS-Tween 20 and then incubated in biotin-conjugated goat anti-mouse (1:500; Jackson Immunoresearch) for 1 h followed by Texas Red-conjugated streptavidin (1:2,000; Jackson Immunore-
search) for 30 min. Cells were then washed and incubated with 4',6-diamidino-2-phenylindole (0.25 μg/ml; Sigma) for 5 min to visualize nuclei. Slides were mounted in Vectashield mounting media (Vector Laboratories). For the Mac-1 antibody, cells were incubated with FITC-conjugated anti-Mac-1 for 1 h. Cells were then washed and nuclei visualized with 4',6-diamidino-2-phenylindole before mounting as above. Cells in 7–21 random fields (150–650 cells) were analyzed by using fluorescence microscopy. The total number of cells expressing each protein of interest was calculated for each sample by multiplying the total number of cells isolated by the percentage of immunopositive cells. Specificity of immunostaining was verified by using appropriate secondary antibody and isotype-specific controls.

**RNA isolation and RT-PCR.** For real-time RT-PCR analysis of gene expression during muscle regeneration, total RNA was isolated from TA muscles by homogenization in TRIZol Reagent (Life Technologies) following the manufacturer’s protocol. Total RNA (2 μg) was reverse transcribed in a 20–60 μl final reaction volume by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reaction was incubated at 25°C for 10 min, 42°C for 50 min, followed by 72°C for 10 min, to inactivate the reverse transcriptase.

Real-time PCR was performed, and results were analyzed by using the iCycler iQ Real-Time Detection System and software (Bio-Rad). cDNA (2 μl from each sample) was amplified by using gene-specific primers (Table 1) in a 25-μl reaction containing the appropriate primer pair (400 nM each primer) and iQ SYBRgreen Supermix (Bio-Rad). Samples were incubated at 95°C for 4 min, followed by 35–40 cycles (30 s each) of denaturation, annealing, and extension at 95°C, 53°C (except 55°C for Mac-1 and 58°C for MyoD), and 72°C, respectively. SYBRgreen fluorescence was measured at the end of the extension step of each cycle. All reactions were run in triplicate, and PCR product size was verified both by melt curve analysis and agarose gel electrophoresis. Real-time PCR reactions were also performed on 2 μl of each RNA sample to confirm the absence of contaminating genomic DNA.

To quantify each gene of interest, the starting quantity of cDNA template in each sample was determined by comparing the threshold cycle for PCR amplification (C_T value) to a standard curve. The C_T value is the cycle number at which the fluorescence intensity crosses a threshold value set in the exponential phase of the PCR reaction. If necessary, samples were diluted so that the C_T value was within the standard curve range. Starting quantity values for each gene were normalized to that of 18S rRNA from the same sample. Gene-specific standards were prepared by conventional RT-PCR and purified by agarose gel electrophoresis, followed by extraction with the QiAquick gel extraction kit (Qiagen). Denaturation, annealing, and extension steps of conventional PCR reactions (50 μl) were the same as those in real-time PCR reactions with the use of the appropriate annealing temperature for each primer pair. Standard curves comprised six to seven serial 1:10 dilutions of purified PCR product, starting at 100- to 1,000-pg DNA. Primers used for real-time PCR reactions were designed to generate an amplicon located within the corresponding standard amplicon (Table 1).

For conventional RT-PCR analysis of COX-2 expression, primary myoblast cultures were prepared from hindlimb muscles of three wild-type and three COX-2−/− mice, based on previously described methods (40). Total RNA was isolated from myoblasts by using TRIZol Reagent, and 1 μg total RNA was reverse transcribed as above. cDNA (2 μl) was amplified in a 50-μl PCR reaction containing 62.5 μM real-time COX-2 primers (Table 1) or Quantum RNA 18S primers (Ambion) as a control. Amplification cycles comprised 94°C for 5 min followed by 28 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR products were resolved by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide staining.

**Statistics.** To determine significance between two groups, comparisons were made by using the Student’s t-test. Analyses of multiple groups were performed by using one-way ANOVA with Bonferroni’s posttest using GraphPad Prism version 4.0a (GraphPad Software). For all statistical tests, P < 0.05 was accepted for significance.

**RESULTS**

**COX-2 expression is induced early after muscle injury.** To analyze the expression of both COX isoforms in skeletal muscle during regeneration, COX-1 and COX-2 mRNA was quantified at various times after injury by using real-time RT-PCR. As shown in Fig. 1A, COX-2 mRNA is induced approximately eightfold in response to muscle injury, whereas COX-1 mRNA remains relatively constant. The induction of COX-2 protein was confirmed by immunoblotting (Fig. 1B). Induction of COX-2 protein has also been observed within paraspinal myofibers after retraction injury (27). The maximum amount of COX-2 protein is observed 1 day after injury,

**Table 1. Real-time RT-PCR primer sequences**

<table>
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<th>Gene Name</th>
<th>Accession No.</th>
<th>Primer Sequences (5'–3')</th>
<th>Amplicon Size, bp</th>
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<td>F: GAAACGCCTACCCACAGTCGAGG</td>
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<td></td>
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<td>Standard</td>
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<td></td>
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<td>482</td>
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<td>R: TGTGCTACCCAGGAGGAGAATC</td>
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F, forward primer; R, reverse primer.
not affect the XSA of uninjured TA myofibers (Fig. 2D), suggesting that COX-2 activity is not required for the ongoing maintenance of myofiber size. In contrast to SC-236, the COX-1 inhibitor SC-560 does not affect myofiber size during the first week after injury (Fig. 3A). This result is not due to insufficient COX-1 inhibition by SC-560, because the dose of SC-560 used inhibited COX-1 activity by 75% (Fig. 3B). Furthermore, the size of regenerating myofibers is normal in COX-1−/− mice (data not shown). Together, these data indicate that COX-2 activity, but not COX-1 activity, is required for normal growth of regenerating myofibers and provide the first evidence for distinct roles of the COX-1 and COX-2 pathways in skeletal muscle.

The attenuating effects of SC-236 on myofiber growth are already apparent within the first week after muscle injury. Along with the early induction of COX-2 expression, these data suggest that COX-2 activity is crucial during early stages of regeneration. To test this hypothesis, mice were treated with SC-236, starting 7 days after injury, and myofiber CSA was subsequently analyzed on day 21. Figure 2E indicates that myofiber CSA is unaffected under these conditions, demonstrating a requirement for COX-2 activity during the early stages of regeneration.

Muscle regeneration is also attenuated in COX-2−/− mice. Several studies have provided evidence for COX-independent actions of selective and nonselective NSAIDs (53). To determine whether the effect of SC-236 on muscle regeneration is COX-2 specific, muscle regeneration was analyzed in COX-2−/− mice. The absence of COX-2 expression in COX-2−/− muscle cells was confirmed by RT-PCR (Fig. 4A). Representative sections from wild-type and COX-2−/− TA muscles 10 days after injury are shown in Fig. 4B. The XSA of regenerating COX-2−/− myofibers is 31% smaller at this time point (Fig. 4C). This difference in size between wild-type and COX-2−/− myofibers is comparable to that observed with SC-236 treatment during this early period of regeneration (Fig. 2B). Furthermore, myofiber CSA is normal in uninjured COX-2−/− muscles, suggesting that attenuation of myofiber growth after injury in COX-2−/− muscles is not due to an initial difference in uninjured myofiber size. Thus the effects of SC-236 on muscle regeneration are likely due to a specific effect on COX-2.

Adverse effects of COX-2 inhibition and deficiency on myoblasts in regenerating muscle. The earliest cellular events in muscle regeneration include satellite cell activation and subsequent myoblast proliferation, both of which are marked by increased MyoD expression. To address further the hypothesis that COX-2 is crucial during early stages of regeneration, the effect of SC-236 on MyoD mRNA expression after injury was determined by using real-time RT-PCR. As shown in Fig. 5A, SC-236 treatment decreases the amount of MyoD mRNA at all time points examined. To determine whether this decrease reflects actual changes in the number of myoblasts, mononucleated cells were isolated from regenerating TA muscles 3 days after injury from mice treated with vehicle or SC-236, plated, and immunostained for two markers of myoblasts, MyoD and desmin. This time point was chosen because it corresponds to the peak of MyoD expression in normal regenerating muscle (data not shown). The total number of mononucleated cells isolated from SC-236-treated muscles is reduced by 53%, and the number of MyoD+ and desmin+ cells

![Fig. 1. Cyclooxygenase (COX)-2 mRNA and protein are induced during muscle regeneration. A: COX-1 (○) and COX-2 (■) mRNA were quantified by real-time RT-PCR at various time points after injury. B: representative immunoblot showing that COX-2 protein is nearly undetectable in uninjured muscle [day 0 (d0)] but increases dramatically after muscle injury [day 1 (d1)]. A portion of a Coomassie-stained gel demonstrates relative protein loading. COX-2 protein was quantified at the indicated time points by densitometry and normalized against protein loading. Values are means ± SE; n = 4. *P < 0.05 vs. d0.](http://ajpcell.physiology.org/)

Although mRNA levels peak 4 days later. This apparent discrepancy between COX-2 mRNA and protein may be simply the result of the particular time points analyzed. Alternatively, COX-2 expression in regenerating skeletal muscle may be subject to complex transcriptional, posttranscriptional, and translational regulation, as observed in other tissues (8), such that mRNA and protein levels do not always correlate with each other. The differential expression patterns of COX-1 and COX-2 in response to muscle injury suggest that these two isoforms may have distinct functions during regeneration.

Muscle regeneration is attenuated by a COX-2 inhibitor but is unaffected by COX-1 inhibition. Selective COX inhibitors were used to determine whether the COX pathways play differential roles in regulating muscle regeneration. TA muscles were collected at different time points after injury, representing early, intermediate, and late stages of regeneration (Fig. 2A). As shown in Fig. 2B, the XSA of regenerating myofibers is decreased significantly (20–33%) by the COX-2 inhibitor SC-236 up to 3 wk after injury. Representative sections are shown 7 and 15 days after injury (Fig. 2C). These data implicate a role for COX-2 in regulating myofiber growth during muscle regeneration. However, SC-236 treatment does affect the XSA of uninjured TA myofibers (Fig. 2D), suggesting that COX-2 activity is not required for the ongoing maintenance of myofiber size. In contrast to SC-236, the COX-1 inhibitor SC-560 does not affect myofiber size during the first week after injury (Fig. 3A). This result is not due to insufficient COX-1 inhibition by SC-560, because the dose of SC-560 used inhibited COX-1 activity by 75% (Fig. 3B). Furthermore, the size of regenerating myofibers is normal in COX-1−/− mice (data not shown). Together, these data indicate that COX-2 activity, but not COX-1 activity, is required for normal growth of regenerating myofibers and provide the first evidence for distinct roles of the COX-1 and COX-2 pathways in skeletal muscle.

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is reduced by 65 and 50%, respectively (Fig. 5B). Similar decreases are observed in COX-2/H11002/H11002 muscles relative to wild-type (Fig. 5C), confirming that the effects of SC-236 on cell number are COX-2 dependent. Differences in the number of cells isolated from C57BL/6 (drug studies) and B6;129P2 (genotype studies) muscles are likely mouse strain dependent (33, 42). Together, these data suggest that both COX-2 inhibition and deficiency adversely affect myoblasts within regenerating muscle.

The inflammatory response to muscle injury is decreased by COX-2 inhibition and deficiency. Inflammation is a crucial early event in muscle regeneration (55). The COX-2 pathway is a key mediator of the inflammatory response to tissue damage and other stimuli. Given that our results suggest an important role for COX-2 during the early stages of muscle regeneration, we also examined the inflammatory response after muscle injury in SC-236-treated and COX-2/H11002/H11002 mice. Mac-1 (CD11b/CD18), a cell-surface protein expressed by macrophages and neutrophils (42), was used as a marker of intramuscular inflammation during regeneration. As shown in Fig. 6A, the amount of Mac-1 mRNA is decreased by SC-236 treatment. This effect on Mac-1 mRNA correlates with a 50% decrease in the number of Mac-1+ cells isolated from injured TA muscles (Fig. 6B). A comparable decrease in the number of Mac-1+ cells is observed in COX-2/H11002/H11002 muscles relative to wild type, confirming the specificity of SC-236 for COX-2 (Fig. 6C). Together, these data suggest that tissue inflammation after muscle trauma is impaired by SC-236 treatment and in COX-2/H11002/H11002 mice.

Fig. 2. Myofiber growth during regeneration is attenuated by the COX-2 inhibitor SC-236. A: schematic representation of experimental procedures with COX inhibitors. B: the cross-sectional area (XSA) of regenerating myofibers with (●) and without (○) SC-236 treatment was determined at early, intermediate, and late time points after injury. Percentages are the difference between vehicle (V) and SC-236 XSA at the indicated time points. C: representative hematoxylin and eosin-stained sections of regenerating tibialis anterior (TA) muscles from vehicle- or SC-236-treated mice at 7 and 15 days after injury. Bar = 50 μm. D: SC-236 has no effect on the XSA of uninjured myofibers after 35 days of treatment. E: SC-236 treatment starting 1 wk after injury has no effect on the XSA of regenerating myofibers at 21 days after injury. Values are means ± SE; n = 5–14. *P < 0.05.

Fig. 3. The COX-1 inhibitor SC-560 has no effect on myofiber growth during early regeneration. A: the XSA of regenerating myofibers was determined 7 days after injury in mice treated with SC-560. Vehicle and SC-236 data are from day 7 in Fig. 2B and are repeated here for comparison (n = 7–14). B: plasma thromboxane B2 (TXB2) was quantified as a measure of COX-1 activity and is inhibited 75% by SC-560 (n = 6). Values are means ± SE. *P < 0.05.
DISCUSSION

Using both pharmacological and genetic approaches, we demonstrate that COX-2, but not COX-1, is critical for normal muscle regeneration following traumatic injury (Figs. 2–4). Thus the COX-1 and COX-2 pathways have distinct roles during muscle repair. This is the first study to quantitatively examine the separate roles of the COX-1 and COX-2 pathways during muscle regeneration, including analyses of both inflammatory and muscle cells. Some previous studies examining the effects of nonselective NSAIDs on muscle regeneration after strain injury noted an early delay in myofiber formation based solely on qualitative evaluation of muscle histology (1, 32, 35). In contrast, other studies observed that muscle regeneration after contusion injury is unaffected by NSAID treatment (21, 54). Discrepancies among these previous studies may be due to differences in the type of muscle injury, species, drug types, drug doses and dosing regimens, time points analyzed, and/or the lack of quantitative measurement of myofiber growth. Some of the effects of NSAIDs are due to COX-independent mechanisms (53), which may also contribute to the variability of the observed effects. Our studies of COX-2−/− mice strongly suggest that the observed effects of the COX-2 inhibitor SC-236 on muscle regeneration are due to COX-dependent mechanisms.

Our results suggest that COX-2 activity is most crucial during the early stages of muscle regeneration, as evidenced by the fact that SC-236 does not adversely affect myofiber growth when administered after the first week of regeneration (Fig. 2E). COX-2 mRNA and protein levels increase early after injury and decline by day 7 (Fig. 1), which likely explains the ineffectiveness of SC-236 when started at later times after injury. The importance of COX-2 at early time points after injury is also evidenced by the adverse effects of SC-236 and COX-2 deficiency on the number of myoblasts within muscle 3 days after injury (Fig. 5, B and C). Attenuated myofiber growth due to SC-236 or COX-2 deficiency, therefore, corre-
lates with the presence of fewer myoblasts, which is consistent with previous reports showing that the efficiency of muscle regeneration is proportional to myoblast number (33, 40). We hypothesize that, without an adequate number of myoblasts, myofiber growth is stunted during the first week but proceeds normally once a “threshold” number of myoblasts is generated. In support of this hypothesis, myofiber growth in the presence of SC-236 is initially attenuated but eventually parallels the rate of myofiber growth in control muscles after 1 wk (Fig. 2B). The effect of COX-2 inhibition on myoblast number suggests that other types of muscle growth that are dependent on myoblasts, such as hypertrophy or recovery from atrophy (34, 45), may also be adversely affected by COX-2 inhibition.

The mechanism by which the number of myoblasts in regenerating muscle is decreased by the lack of COX-2 activity may be either extrinsic or intrinsic to muscle cells. The most likely muscle-extrinsic function of COX-2-derived PGs is in the inflammatory response after muscle injury. Muscle regeneration is impaired when inflammatory cells are depleted (26, 43) and stimulated when they are increased (26), demonstrating the importance of inflammation after muscle damage. Neutrophils and macrophages that infiltrate damaged muscle not only phagocytose cellular debris but also secrete growth factors, chemokine attractants, and PGs, many of which have been implicated in myogenesis (3, 5, 31, 44). A reciprocal relationship between muscle cells and macrophages also exists in that myoblasts recruit macrophages to sites of muscle injury via secreted factors (7). Thus evidence suggests that muscle regeneration involves complex interactions between inflammatory and muscle cells.

Inflammation after muscle injury is decreased by SC-236 treatment and COX-2 deficiency, as indicated by the decrease in intramuscular Mac-1+ cells (Fig. 6). This effect may be due to several mechanisms, such as impaired inflammatory cell proliferation or chemotaxis or impaired vasodilation. The inflammatory cells quantified in this study include both neutrophils and macrophages, as Mac-1 is expressed by both of these cell types. However, the majority of Mac-1+ cells isolated 3 days after injury in this study are most likely macrophages, because they are the predominant inflammatory cell type present within 24–48 h after injury in various models of muscle injury (14, 36, 57). The majority of macrophages in injured muscle are thought to arise from chemotaxis from the circulation and not proliferation of resident macrophages (55). Thus the decrease in Mac-1+ cells is not likely the result of impaired macrophage proliferation. Results from another experimental model of inflammation suggest that macrophage chemotaxis is impaired in the absence of COX-2 activity (23). In addition, the production of vasodilatory PGs, such as PGE2 and PGI2, may be diminished by COX-2 inhibition or deficiency, leading to impaired inflammatory cell infiltration. Thus attenuation of the inflammatory response in our experiments may adversely affect myofiber growth simply by delaying or diminishing inflammatory cell infiltration and the subsequent clearing of necrotic tissue. Alternatively, decreasing the number of inflammatory cells may decrease PG and growth factor release into the injured area, thereby delaying the early phases of myogenesis.

Given the complexity of the interactions between myoblasts and inflammatory cells during regeneration, the possibility that impaired inflammation is the sole cause of the attenuated myofiber growth observed in SC-236-treated and COX-2−/− muscles seems unlikely. Although inflammation clearly plays a crucial role in muscle regeneration, evidence suggests that myogenesis in vivo does occur, albeit more slowly, in the absence of infiltrating inflammatory cells (43). Furthermore, several studies have implicated PGs as regulators of myogenesis in vitro (10, 13, 18, 46, 48, 62, 63), suggesting that PGs have functions that are intrinsic to muscle cells. Thus the decreased number of myoblasts that we observed in the absence of COX-2 activity could be due to the requirement for COX-2-derived PGs during satellite cell activation, myoblast proliferation, and/or myoblast survival. COX-2-dependent PG synthesis may play a role in satellite cell activation, because mitogenic activation of other cell types is associated with upregulation of COX-2 (20, 22, 41). Alternatively, the COX-2 pathway may regulate myoblast proliferation. Proliferation of various cell types is regulated by the COX-2 pathway and is inhibited by COX-2-selective inhibitors during tissue repair (2, 4, 47, 52). Recent evidence also implicates the COX-2 pathway...
in the regulation of apoptosis. Various cancers, which are often characterized by resistance to apoptosis, are associated with constitutive overexpression of COX-2 (51). One potential mechanism for the inhibitory effects of COX-2-selective NSAIDs on tumorigenesis in vivo and the growth of various cancer cell lines in vitro is increased apoptosis (9), which suggests that SC-236 and COX-2 deficiency may also adversely affect myoblast survival during muscle regeneration. To clarify the role of COX-2-derived PGs in muscle regeneration, we are currently examining the muscle-intrinsic roles of this pathway.

COX-2-selective NSAIDs are widely prescribed for the treatment of pain and inflammation associated with acute tissue damage due to injury or surgery, as well as chronic inflammatory conditions such as arthritis. The data presented here have important clinical implications in that administration of COX-2 inhibitors soon after muscle injury, while providing desired analgesic effects, may adversely affect muscle regeneration. Evidence also suggests that COX-2-selective NSAIDs attenuate wound healing in many other tissues (2, 12, 47, 49, 52). COX-2-selective NSAIDs are typically more tolerable than nonselective NSAIDs because they lack many of the side effects associated with COX-1 inhibition. These drugs are, therefore, often used chronically and at higher doses, especially in the treatment of rheumatoid and osteoarthritis. Given that the growth and regeneration capacity of muscle declines with age (29), elderly patients undergoing chronic COX-2 inhibitor treatment may be particularly susceptible to the adverse effects of COX-2 inhibitors on skeletal muscle. Whereas our studies suggest that COX-2-dependent PG synthesis is required during muscle regeneration, they do not identify the specific PGs that are involved. Both PGE2 and PGF2α have been implicated in various aspects of myogenesis, both in vivo and in vitro. Furthermore, decreased PGE2 has been associated with a decrease in the number of macrophages within injured muscle (25), consistent with its role as a potentiator of inflammation. Pharmacological inhibition or genetic deletion of specific PG synthases or PG receptors could be used to define the roles of individual PGs during regeneration. Drugs that block the synthesis or action(s) of specific PGs may allow pain to be ameliorated without adversely affecting muscle repair.

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