Lengthening contraction-induced inflammation is linked to secondary damage but devoid of neutrophil invasion

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Lengthening contraction-induced inflammation is linked to secondary damage but devoid of neutrophil invasion. J Appl Physiol 92: 1995–2004, 2002; 10.1152/japplphysiol.00803.2001.—Inflammation triggered by exercise-induced muscle damage (EIMD) has been postulated to influence the extent of tissue destruction. We tested the hypotheses that 1) repressing inflammation decreases secondary damage production and 2) EIMD leads to a sequential appearance of inflammatory cells in which neutrophil accumulation precedes macrophage invasion. Rat ankle dorsiflexor muscles were submitted to in situ lengthening contractions. Measurement of in vitro contractile properties, inflammatory cell concentrations, and histological staining were performed postprotocol. Rats were treated with diclofenac, a nonsteroidal anti-inflammatory drug (NSAID group) to repress inflammation or with the vehicle solution (EIMD group). Muscles from the NSAID group had smaller force deficits on days 2 and 3 postexercise. This effect was associated with significantly smaller increases in the concentration of muscle macrophage ED1⁺ and ED2⁺. Surprisingly, neutrophils did not accumulate post-EIMD. These results suggest that inflammation-induced ED1⁺ macrophage accumulation is responsible for the secondary damage observed 2–3 days post-EIMD. We further conclude that an increase in ED1⁺ macrophage concentration can occur in absence of previous neutrophil invasion.

Macrophage; prostaglandin E₂; skeletal muscle; anti-inflammatory drug

EXERCISE-INDUCED MUSCLE DAMAGE (EIMD) has been investigated with several experimental models. It is characterized by focal structural alterations at the level of Z line, A band, extracellular matrix, cytoskeleton, and sarcolemma (1, 8, 25, 33, 48). Whereas most studies favor mechanical stress per se as the factor opposed to primary damage due to the initial mechanical stress. Indeed, Faulkner and colleagues (14) have reported a second decline in strength that correlates well with the peak of the postulated inflammatory reaction, giving support to the belief that some inflammation-associated events may be involved in EIMD.

To clarify the possible role of inflammation in EIMD, the influence of several anti-inflammatory strategies on outcome measures of EIMD has been investigated. In humans, studies looking at the effect of nonsteroidal anti-inflammatory drugs (NSAID) on the recovery from EIMD have been often inconclusive mainly because these studies differed widely in terms of protocols, subject training level, and type of NSAID used (13, 31, 34, 41). Voluntary elicited maximal muscle contractions, which can be highly variable in inexperienced subjects (9), were often used in those studies. More importantly, the pain associated with EIMD can also negatively influence maximum voluntary force production. Therefore, in such protocols any beneficial influence of NSAID on muscle strength after EIMD can be associated with either the analgesic or anti-inflammatory action of the drug.

Few experiments have investigated the influence of repressing the inflammatory reaction on recovery from EIMD in animal models. Flurbiprofen was reported to be beneficial in terms of force production over the first 7 days after lengthening contractions in rabbit, suggesting that some portion of the early deficit in force was due to muscle inflammation (31). However, quantification of inflammatory cells and other indexes of inflammation were not monitored, leaving open the interpretation of how NSAID resulted in a smaller loss in force in the early days post-EIMD. In another report, a decreased leukocyte accumulation and less significant histological perturbations were observed with NSAID administration, but force or other functional measurements were not reported (41). Thus a lot remains to be learned on the nature and the biological significance of muscle inflammation in EIMD.

The tissue reaction to injury involves systemic and localized responses that lead to vascular and hemodynamic changes that favor a sequential migration of leukocytes in the injured tissue. In rat skeletal muscle, microscopic damage is linked to secondary damage but devoid of neutrophil invasion...
invading neutrophils are followed by ED1+ macrophages, which usually precede an increase in the concentration of the resident ED2+ macrophages (51, 52). This sequence of leukocyte invasion has been clearly documented when skeletal muscle injury is caused by mechanical crush (32) or an intramuscular injection of histotoxic compound (35) and in a model of modified loading (17, 51). Very surprisingly, however, there is no detailed and comprehensive demonstration that such a sequence is also valid in the case of damage caused by lengthening contractions. On the basis of the fact that EIMD-induced leukocyte invasion correlates with the production of secondary damage, our objectives were therefore to test the hypotheses that 1) repressing the inflammatory response with the NSAID diclofenac decreases secondary damage production and provides early on a protective effect on muscle function and 2) EIMD leads to a sequential appearance of inflammatory cells in which neutrophil accumulation is followed by an increase in macrophage concentration. To test these hypotheses, we compared the evolution of muscle strength, PGE2 levels, histological alterations, and inflammatory cell concentrations after EIMD in rats treated or not with a NSAID.

METHODS

Animal care. Extensor digitorum longus (EDL) muscles from female Wistar rats were used in all the experiments presented. For most experiments, rats weighing 50–70 g at the start of the protocol were used because EDL muscle mass at day 28 had to be sufficiently small to allow in vitro contractile measurements. Gain in body mass and EDL muscle mass over this period were identical in NSAID-treated and untreated rats. In a few experiments with NSAID treatment, rats weighing 100–125 g at the onset of the protocol were used; however, the extent of EIMD and the time course for recovery are identical in rats weighing 50–70 vs. 100–125 g (results not shown). The treatment and care of the animals in these studies were approved by and followed the guidelines of Laval University Hospital Research Center Animal Care and Use Committee. Rats were given food and water ad libitum.

For most experiments presented, the design involved two groups of rats run in parallel: one group was treated with diclofenac (NSAID group) whereas the comparison group received the vehicle solution (water) alone (EIMD group). In each group, the right hindlimb underwent the lengthening contraction protocol whereas the contralateral leg was used as sham. Treatment consisted in giving diclofenac sodium (Sigma Chemical), 1 mg/kg body wt, twice daily by gavage. Diclofenac is widely used in humans and is efficient for the treatment of acute musculoskeletal trauma (36, 38). Its effects are thought to be mediated mainly, but not exclusively, through inhibition of the activity of cyclooxygenase-2 (COX-2), the rate-limiting enzyme for the production of PGE2 under inflammatory conditions. This dosage corresponds exactly to the one suggested for human use.

Lengthening contraction protocol. The protocol for lengthening contractions is an adaptation of one we have used previously (18). Rats were anesthetized with an intraperitoneal injection of ketamine-xylazine (87.5 and 12.5 mg/kg body wt, respectively), and supplemental ketamine injections were given as necessary. On anesthesia, lactate-Ringer solution was given subcutaneously. After the hindlimbs were shaved, chlorhexidine gluconate was applied, followed by isopropyl alcohol and providine-iodine solution. The peroneal nerve was carefully exposed before transferring the animal in an apparatus designed to forcibly stretch the dorsiflexors as they were externally activated. With the knee fixed at 90°, the right foot of the animal was installed and solidly attached in a low-compliance custom-made metallic footwear connected to a circular motion motor. The ankle total range of motion was −70°; active muscle contractions were initiated after 30° of passive lengthening, thus leaving 40° of plantar flexion for the lengthening contraction. The incision over the nerve was covered with wet cotton, and the animal rested under a lamp to keep its body temperature around 37°C. The nerve was stimulated at a frequency of 120 Hz with a supramaximal voltage (3–6 V). Each stimulation lasted 600 ms and was given every 2 s for three 5-min periods separated by a 5-min recovery period for a total of 450 contractions. Efficiency of stimulation was verified at the end of the protocol. Rats for whom maximal muscle stimulation during the entire protocol was doubtful were discarded from the study (<5% overall). At the end of the protocol, the muscle and fascia over the peroneal nerve were closed with absorbable 4-0 sutures. The skin was closed with 4-0 silk sutures, and the incision was bathed with providine-iodine solution, except for those animals whose EDL muscles were studied at time 0, in which case muscles were immediately dissected after the lengthening contraction protocol and installed for in vitro measurement of contractile properties. On recovery from the anesthesia, animals had a normal gait with no evidence of discomfort.

For the sham-operated limb, the nerve was exposed and the electrode was placed underneath but no stimulation was provided. In a preliminary experiment, we tested a sham procedure in which EDL muscles were submitted to an isometric contraction protocol using the same stimulation parameters as the lengthening protocol; force measurements at 6, 12, and 24 h postprotocol in these muscles were not different from those of muscles submitted to the sham procedure involving nerve isolation only (results not shown), which led us to use the nerve isolation sham procedure in all EIMD protocols thereafter.

Measurement of contractile properties. Immediately post-lengthening contraction (time 0) as well as 1, 2, 3, 7, 14, 21, and 28 days after (n = 6–8 muscles per group for each time), rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and given buprenorphine (0.1 mg/kg). EDL muscles were dissected and incubated in vitro in a buffered physiological solution (Krebs-Ringer with glucose) at 25°C for measurement of isometric contractile properties as described in details previously (11, 16, 23). Muscles were mounted vertically between two platinum electrodes and attached as close to fiber ends as possible to minimize the length of free tendon. Muscle length, referred to as L0, was adjusted to obtain maximal twitch tension. A force-frequency curve was obtained by using 0.5-s train stimulations at frequencies ranging between 10 and 120 Hz, and maximum absolute tetanic tension (P0) was obtained. Maximum specific tetanic tension (specific P0), which is normalized for the muscle cross-sectional area, was calculated as the product of absolute P0, muscle fiber length (0.4 L0 for rat EDL muscle), and muscle density (1.062 g/ml) divided by muscle mass (43).

Histological and immunohistochemical analyses. Histological examination was performed on EDL muscles dissected free and immersed in embedding medium, frozen in isopentane cooled in liquid nitrogen, and then stored at −80°C until processing. Transverse sections (10 μm) of the proximal and
distal third were cut at $-20^\circ$C (Jung Frigocut 2800E, Leica) and stained with hematoxylin-eosin (H/E) to permit qualitative analysis of cellular concentration and muscle fiber morphological alterations.

For immunohistochemical determination, 10-μm-thick sections were adhered to slides coated with chromium potassium sulfate and gelatin, then stored at $-20^\circ$C and used within a week. Sections were processed with antineutrophil (Serotec, clone W3/13; mouse anti-rat), anti-ED1$^+$, or anti-ED2$^+$ antibodies, all diluted 1:100 (Serotec, Indianapolis, IN). W3/13 recognizes a 95-kDa glycoprotein (CD43) specific to neutrophil; ED2$^+$ reacts with a membrane antigen (160–175 kDa) present in tissue resident macrophages, whereas ED1$^+$ reacts with a single-chain glycoprotein (90–100 kDa) expressed predominantly in lysosomal membranes of monocytes (in blood) and macrophages. Immunostaining protocols were exactly as described previously (17, 51). For each antibody, the entire sections were examined and the concentration of inflammatory cells labeled with each antibody was measured in three triplicate sections separated by 1 mm in each EDL. Muscle sections were always taken at a site corresponding to one-third of $L_m$ from either the proximal or distal end; therefore nine sections were obtained from both the proximal and distal third. The total area of the sections was manually measured by the same investigator using a grid with a surface of $0.249001 \text{ mm}^2$ at a magnification of $\times200$. The grid was initially set in the lower right corner of the section, and all immunostained cells were counted. It was then systematically moved up one grid until the upper limit of the section was reached. It was then moved one grid to the left and so on. The total area of the section was determined and multiplied by its thickness to express the number of each cell type per cubic millimeter.

**Myeloperoxidase assay.** Myeloperoxidase activity is a standard index of tissue neutrophil content (22). Myeloperoxidase (EC 1.11.1.7) activity was assayed spectrophotometrically as previously described (37). Briefly, muscle tissue was homogenized (1 in 10 vol) in a buffer containing 50 mM phosphate, 0.5% hexadecyltrimethylammonium bromide, and 5 mM EDTA, pH 7.4. After centrifugation at 850 g for 12 min ($4^\circ$C), samples were twice frozen in liquid nitrogen and thawed and then recentrifuged. For the assay, 150 μl of supernatant was added to a reaction mixture containing 0.8 mM H$_2$O$_2$, 10 mM KH$_2$PO$_4$ (pH 6.0), and 0.4 mM o-dianisidine dihydrochloride. The reaction was performed at $37^\circ$C and measurements taken at 30-s intervals for 180 s at 480 nm in a spectrophotometer (Beckman, model DU-650). H$_2$O$_2$-dependent myeloperoxidase activity (with o-dianisidine dihydrochloride as the substrate) was expressed as absorbance per minute per gram wet weight.

**Measurement of PGE$_2$.** Serum PGE$_2$ levels were determined from blood obtained by cardiac puncture in anesthetized rats. Quantification of PGE$_2$ was performed through the use of an enzyme-immunoassay technique using acetylcholinesterase-linked PG tracers as described by Asselin et al. (3). Fully characterized rabbit anti-PGE$_2$ was used (2). The inter- and intra-assay coefficients of variation were 17 and 9%, respectively.

**Statistical analysis.** In all experiments, the effects of the two independent variables, group and time, were analyzed by using a two-way ANOVA, and paired comparisons were used when indicated. Fisher’s least-squares difference test was performed when a significant difference was obtained. The $\alpha$-level was set at $P < 0.05$.

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**RESULTS**

**Contractile properties after lengthening contractions.** Pilot experiments showed that diclofenac given to control rats for 2 or 7 days had no effect on body mass or isometric contractile properties of the EDL muscle, including specific $P_o$ (N/cm$^2$) (data not shown). On the average, body mass increased from 66 ± 3 to 194 ± 8 g over the 28 days of the protocol, whereas mean values for $P_o$ of sham and control muscles, which were not significantly different, went from 72 ± 5 to 172 ± 10 g during the same period. Similarly, sham muscles from NSAID group were not different in terms of specific $P_o$ and contractile properties from EIMD sham muscles and control untreated animals. Muscles submitted to lengthening contractions alone or combined with the NSAID treatment did not show any significant change for values of time to peak twitch tension and twitch half-relaxation time when compared with sham and NSAID groups at each separate time (data not shown).

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**Fig. 1.** Effect of diclofenac on the evolution of absolute $P_o$ after the lengthening protocol is shown in Fig. 1. Immediately postexercise, a 65% decrease was seen, followed by an initial recovery to 83 and 87% of sham values at 24 h for EIMD and NSAID groups, respectively. Then, values for $P_o$ declined again in both
groups to reach a nadir at 2–3 days post-EIMD ($P < 0.05$). Recovery of contractile force began after day 3, but absolute $P_o$ remained significantly lower than the sham value at days 7 and 14 in both groups. Complete recuperation occurred somewhere between 21 and 28 days in the EIMD group. Administration of diclofenac for 2 days postexercise offered protection against EIMD because force production at days 2 and 3 was significantly higher compared with the EIMD group but still significantly lower than the sham group at these two times. At 21 days, absolute $P_o$ for the NSAID group was still significantly lower than sham ($87 \pm 1.7\%$, $P < 0.05$), whereas complete recovery was obtained at day 28.

Evolution of force production normalized for the cross-sectional area (specific $P_o$) is shown in Fig. 2. A second significant reduction from 15.7 N/cm² at day 1 to 11.9 N/cm² at day 2 was seen in the EIMD group. A recovery was observed thereafter, and no difference between sham and EIMD groups was observed at 21 and 28 days. A protective influence of diclofenac treatment was once again observed as values from days 1 to 7 were significantly higher than the ones for the EIMD group even though a significant deficit compared with sham was still present. In a separate series of experiments, we tested the possibility that the analgesic action of diclofenac over the 2–3 days postprotocol allowed the animals to be more active than the EIMD group, which could have influenced the EIMD-induced force deficit. To do so, rats were given buprenorphine (0.1 mg/kg ip, twice daily), a narcotic analgesic with no anti-inflammatory effect, for 48 h postprotocol. These animals had exactly the same force deficit 2 days postprotocol as the untreated EIMD rats (data not shown), indicating that the smaller force deficit postprotocol in the group treated with diclofenac was not due to the analgesic effect of the drug during the 2–3 days postprotocol.

**Histological assessment.** Muscles dissected 2 days postprotocol were stained with H/E to qualitatively evaluate muscle fiber morphology and nonmuscle cell infiltration. Sections obtained with sham muscles (Fig. 3a) showed typical, densely packed myofibers, with nuclei at the cell border and few resident nonmuscle cells. In contrast, the EIMD group (Fig. 3b) presented signs of muscle damage such as invading cells, swollen fibers, and enlarged extracellular spaces. These damages were mostly prevented by diclofenac administration, but a few invaded fibers remained (Fig. 3c).

**Quantitative assessment of neutrophil concentration.** Neutrophil concentration in all groups was first evaluated by immunohistochemistry using an antibody that recognizes leukosialin (CD 43) (Fig. 4). This protein is also found on some other types of cells not present in skeletal muscle under aseptic conditions. It is therefore considered to be neutrophil specific when used in skeletal muscle and has been utilized to detect neutrophils in other studies in which its specificity was described (17, 39). Initially, neutrophil quantification was performed on muscles collected at 0, 1, and 2 days post-EIMD. A second series of experiments was then performed for neutrophil quantification at time points between 3 h and 7 days. Measurement of contractile properties of these muscles was done to confirm that the lengthening contraction protocol was effective. The concentration of neutrophils in the EIMD muscles was never significantly higher than the one found in sham or control muscles (Figs. 4 and 5). In support of this unexpected finding, leukocyte nuclear morphology was examined at high magnification ($\times 600$) on histological sections stained with H/E by a blinded expert who confirmed that neutrophil counts were basically the same as in sham muscles at all time points between 3 and 72 h. A third assessment of neutrophil concentration was performed to further support the histological and immunohistochemical data. In a separate series of experiments in which EIMD was confirmed through contractile measurements, myeloperoxidase activity was measured in whole muscle extracts. Neutrophil counts, which were low in sham and EIMD muscles 6–12 h postprotocol, translated into low and nonsignificantly different values for myeloperoxidase activity in these same muscles (Table 1). As a positive control for neutrophil invasion, we injected muscles with 30 μl of collagenase (1 mg/ml), which resulted in significant tissue damage where leukocyte invasion was intense. Immunohistochemical staining of neutrophils revealed that their concentration was increased very significantly with thousands of cells per section in contrast to a maximum of 10–15 neutrophils usually seen in sham or EIMD muscles. Figure 5 shows typical patterns of neutrophil immunohistochemical staining for sham (5a), EIMD (5b), and collagenase-injected muscles (5c). Myeloperoxidase activity in the collagenase-injected muscle also increased drastically (~25-fold) compared with sham or control muscles.

![Fig. 2. Effect of diclofenac on the time course of recovery of maximum specific $P_o$. Maximum specific $P_o$ was calculated as described in METHODS. The NSAID group received 1 mg/kg of diclofenac twice a day, whereas the EIMD group received only the vehicle solution. For clarity, sham values from the 2 groups were pooled because no significant difference between them was found. Values are means ± SE; $n = 6–8$ muscles for each time in all groups. *Significantly different from sham group, $P < 0.05$; †significantly different from NSAID group, $P < 0.05$.](image-url)
Measurement of macrophage concentration. Contrary to neutrophils, a distinct increase in the number of macrophages is observed after EIMD. The concentration of ED1+ macrophages increased very significantly by roughly eightfold 2 days posttrauma in the EIMD group (Fig. 6A). ED1+ macrophage concentration is still significantly elevated at day 7 but returned to control value by day 14. Values at days 2 and 7 were significantly higher than those of the NSAID animals. Resident ED2+ macrophages in EIMD and NSAID groups showed much less significant increases in concentration (Fig. 6B) compared with sham groups 2 and 7 days post-EIMD. However, EIMD animals had a significantly higher concentration of ED2+ cells at day 7 compared with the NSAID group. For both ED1+ and ED2+ macrophages, the statistical analysis revealed a significant group-by-time interaction (P < 0.05).

PGE2 levels. PGE2 is highly produced during inflammation and is considered one of the main mediators of inflammation. As shown in Fig. 7, the lengthening contraction protocol led to a significant increase in serum PGE2 level 2 days postexercise as the values
increased approximately fivefold compared with sham. Diclofenac almost totally prevented the EIMD-induced PGE₂ increase at day 2, but a significant difference compared with sham was still observed. Interestingly, PGE₂ levels in the EIMD group were still significantly elevated compared with the sham animals at day 7.

DISCUSSION

This is the first study to assess the influence of an anti-inflammatory drug on EIMD in which histological, immunohistochemical, and biochemical measurements along with in vitro contractile properties were obtained on whole muscle samples and in which lengthening contractions were externally elicited. Muscle damage was evaluated both functionally, through measurement of P₀, and histologically, a combination allowing qualitative and quantitative assessment of damage (7). Several investigators have shown that EIMD leads to inflammation, but the link with secondary damage has not been clearly established (14, 28, 31, 45, 47). Our first finding is that repression of EIMD-triggered inflammation leads to a decreased appearance of secondary muscle damage. A second major finding is that the neutrophilic phase of inflammation is abrogated in this model of muscle trauma, which is contrary to the general belief that sterile traumatic insult in skeletal muscle triggers an inflammatory process that first goes through a neutrophilic invasion (15, 17, 27, 28, 40, 51, 52). It has also been assumed that the presence of neutrophils in a damaged tissue was necessary to attract circulating macrophages and to modulate their activity (42). The present experiments suggest that several of these concepts cannot be generalized to all tissues.

Influence of diclofenac on inflammation and secondary damage in EIMD. The evolution of P₀ in untreated animals showed that a second significant drop in force, similar to what others have previously reported (14, 24), is observed in exercised muscles 2–3 days post-protocol. In our study, this second decline is associated with histological alterations, cellular invasion, and increased PGE₂ production, supporting the hypothesis that the inflammatory process produces secondary damage. The larger relative force deficit seen 2–3 days postexercise when using specific P₀ (N/cm²) reflects the inflammation-associated edema in EIMD muscle, which further suggests the presence of an inflammatory process at that time.

The present findings demonstrate that repression of muscle inflammation by diclofenac decreased the extent of secondary damage in EIMD at days 2 and 3, which is in agreement with other data obtained in rabbits (31) and humans (20, 34) in whom NSAID treatment decreased the loss in force postexercise and/or creatine kinase release. A significant protection was not however, observed at day 1. Explanations for this could be the following: 1) primary muscle damage

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Table 1. Myeloperoxidase activity in extracts from sham, EIMD, and collagenase-injected muscles

<table>
<thead>
<tr>
<th>Group</th>
<th>6 h</th>
<th>12 h</th>
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<tbody>
<tr>
<td>EIMD</td>
<td>2.88 ± 0.2</td>
<td>2.93 ± 0.2</td>
</tr>
<tr>
<td>Sham</td>
<td>2.69 ± 0.1</td>
<td>2.84 ± 0.4</td>
</tr>
<tr>
<td>Collagenase</td>
<td>65.72 ± 1.9</td>
<td>84.11 ± 4.0</td>
</tr>
<tr>
<td>Sham</td>
<td>2.54 ± 0.3</td>
<td>3.11 ± 0.3</td>
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</tbody>
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Values are means ± SE; n = 3–4 muscles per group at each time point. EIMD, exercise-induced muscle damage. Values for collagenase-injected muscles were significantly different from all other groups (P < 0.01).

Fig. 5. Immunolabeling of neutrophils in EDL muscles from sham, EIMD, and collagenase-treated animals. Immunolabeling with W3/13 was performed as described. a: Cross-section of EDL muscle from sham rat. b: EDL muscle from EIMD animal sampled 12 h after the lengthening contractions protocol. c: Massive neutrophil accumulation in an EDL muscle injected with collagenase and processed 12 h after the injection. Arrowheads indicate neutrophil labeling. Scale bar represents 100 μm.
against EIMD-induced loss in force. Diclofenac could also possibly exert COX-2-independent anti-inflammatory actions because it can interact with leukocyte-endothelial attachment by interfering with the expression or function of adhesion proteins (12). PGE₂ is a key mediator of inflammation rapidly produced on trauma or sepsis. Because of its upstream location in the inflammatory cascade, PGE₂ has several proinflammatory actions that could exacerbate the initial injury and/or create new damage; these include chemoattractive influences on inflammatory cells, modulation of proinflammatory cytokines and inducible nitric oxide synthase expression, vasodilation, and increased vascular permeability. In addition, its role as a mediator of pain is well established. Activated macrophages can release proinflammatory cytokines, collagenases, eicosanoids, and numerous potentially cytotoxic compounds such as nitric oxide and oxygen radical species, leading to the formation of peroxynitrite (4, 53). Several of these molecules can induce EC coupling impairment, protein degradation, and cell necrosis within the healthy tissue surrounding the traumatized area. Thus EC coupling failure, which has been identified as an important factor contributing to the decreased force production in the early days after EIMD, should not be seen mechanistically as totally unrelated to inflammation. It is believed that ED1⁺ macrophages are mainly involved in the removal of cellular debris, but their contribution to muscle cell injury is still a debated topic. Although it remains possible that the neutrophils present in the injured muscles caused tissue damage, it is nevertheless most likely that ED1⁺ macrophages are responsible for the majority of the inflammation-associated injury in this model, suggesting that macrophages, like neutrophils, can cause nonspecific tissue damage.

is generally thought to be due to mechanical factors and excitation-contraction coupling (EC) failure, processes on which diclofenac had no effect (21); 2) it is also plausible that the 6-h delay between the protocol and the first dose of NSAID was long enough to allow partial establishment of an inflammatory reaction; and 3) the main attributed influence of diclofenac is through inhibition of COX-2, but several other inflammatory mediators, which do not depend on COX-2 activity, can contribute to the establishment of inflammation.

The observed protective influence of diclofenac on force production appears to be related to the anti-inflammatory action of the drug because its effect is associated in time with reduced PGE₂ level and ED1⁺ macrophage concentration. Further support for this conclusion is provided by the fact that a potent analgesic like buprenorphine did not provide any protection against EIMD-induced loss in force. Diclofenac could

![Image](71x408 to 269x724)

Fig. 6. Macrophage subpopulation concentrations in EDL muscles from EIMD and diclofenac-treated animals. Transverse sections (10 µm) of the proximal and distal third from EDL muscles were immunostained with specific antibodies against ED1⁺ (A) and ED2⁺ (B) macrophages. Sampling and counting procedures were as described in METHODS. The increase in ED1⁺ and ED2⁺ macrophages induced by the lengthening contraction protocol was largely abolished by the treatment with diclofenac. Data at time 0 are from sham muscles whose value did not change over the 14-day period and were not different from those of control animals. Values are means ± SE; n = 3 muscles per group at each time point. *Significantly different from sham group, P < 0.05; †significantly different from NSAID group, P < 0.05.

![Image](323x142 to 545x314)

Fig. 7. Effect of diclofenac on serum levels of PGE₂ after the lengthening contraction protocol. Blood was collected 2 and 7 days post-protocol by cardiac puncture in sham, EIMD, and NSAID rats. PGE₂ was measured in duplicate in serum as described in METHODS. The lengthening contraction protocol induced a large increase in PGE₂ level which was largely prevented in rats treated with diclofenac. Values are means ± SE; n = 3–4 rats per group at each time point. *Significantly different from sham group, P < 0.05; †significantly different from NSAID group, P < 0.05.
Does EIMD-induced inflammation involve a neutrophilic phase? Evidence supporting a sequential leukocyte invasion in which neutrophils are the first cells to invade damaged tissue can be found for several types of injuries (17, 32, 35, 51). However, an exhaustive review of the literature reveals that an irrefutable demonstration of neutrophil infiltration in EIMD has yet to be published. In humans, evidence that EIMD leads to an increased concentration of neutrophils is not compelling. In one study, muscle biopsies were obtained pre- and post-EIMD and leukocytes were classified as neutrophils by use of histological staining (15). In another study performed with human subjects, neutrophils were localized after lengthening contractions by use of radionuclide imaging, and a small but significant increase in neutrophil signal density was seen 2 and 4 h postexercise (27). However, this type of imaging does not clearly tell whether neutrophils are still in blood vessels, in the interstitial space, or invading muscle cells. In line with the present data, Stupka et al. (50) recently showed, using an antibody against human myeloperoxidase, that no increase in PMN content could be seen 24 h after a first bout of damaging lengthening contractions in both men and women. Similar findings were also obtained in a study performed in human subjects in which lengthening contractions did not produce any significant increase above the sham group in neutrophil concentration between 0 and 48 h post-EIMD (29). In animal studies, Armstrong et al. (1), using histological cellular identification, observed that polymorphonuclear cells did not invade the muscle between 24 and 48 h in muscles of rats that ran downhill. No data were obtained between 0 and 24 h post-EIMD, a critical time for neutrophil invasion. They also found that neutropenia had no effect on the evolution of muscle damage after lengthening contractions (26). The present experiments constitute the first demonstration in an animal model that EIMD leads to an inflammatory response in which neutrophils do not appear to play a major role. One could speculate that such observation may be rodent or species specific and irrelevant for humans. However, the two studies cited above in which no increase in indexes of PMN content could be seen after EIMD do not support the concept of a species-specific phenomenon (29, 50). So it appears that lengthening contractions lead to a neutrophil-independent tissue injury process. A similar phenomenon has been previously documented after ischemia-reperfusion in other tissues (44, 49).

Why neutrophils do not invade damaged muscle area in EIMD remains an open question. One can often observe that vascular damage appears to be less important in EIMD compared with crush or histotoxic muscle trauma. We do not know of a quantitative method to evaluate such damage, with the result that no quantification of vascular insult was performed. It should nevertheless be pointed out that vascular trauma is an important factor for triggering the sequentially organized inflammatory cascades leading to the local appearance of inflammatory mediators and specific adhesion molecules. Various combinations of cytokines and chemokines must sequentially appear to attract specific leukocyte populations. In rodents, the two best defined chemokines capable of recruiting neutrophils are the cytokine-induced neutrophil chemotactant and the macrophage inflammatory protein-2 (5, 6, 19). Activation of the complement system also contributes to neutrophil accumulation, and if its vascular activation is not of sufficient intensity with EIMD, triggering of the expression of adhesion molecules, cytokines and/or chemokines appropriate for neutrophil recruitment may not occur.

The ED2+ macrophages are resident cells that do not invade or phagocyte necrotic muscle fibers (30, 46); they are believed to produce early signals that stimulate muscle cell proliferation and differentiation (10). It is therefore noteworthy that differences were observed in terms of ED2+ cell concentration between the EIMD and NSAID groups. In our model, ED2+ macrophages increased significantly in EIMD muscle between days 2 and 7 whereas a much smaller increase was observed in the NSAID group, implying that these muscles would have a decreased secretion of important regenerative factors. One can thus hypothesize that, even with the short diclofenac treatment used in the present study, muscle regeneration is possibly slower compared with untreated animals, but no functional impairment could be observed at 28 days. As such, these data support the findings of Mishra et al. (31), who observed that a 7-day treatment with flurbiprofen could be beneficial in terms of Po at day 7 but detrimental later on because values for Po at day 28 were lower in treated animals compared with control groups. These data also support the view that the extent of the increase in ED2+ concentration postinflammation is possibly related to the number and/or activity of ED1+ macrophage that previously invaded the injured area.

In summary, the present data support the hypothesis that the inflammatory reaction is involved in the production of injury after lengthening contractions. However, these secondary damages are not due to the autolytic activity of neutrophils but are rather associated with the presence of a high number of ED1+ macrophages. Repression of inflammation induced a significant positive change in EIMD evolution on a short-term basis because the second phase of damage production was largely abolished, resulting in a better preservation of the muscle’s ability to produce isometric force during the first few days after the insult. However, although the extent of the functional deficit was decreased by repressing inflammation, the time needed for total recovery was not accelerated.

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