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Sensitization of Group III and IV Muscle Afferents in the Mouse After Ischemia and Reperfusion Injury

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Abstract: **Ischemic myalgia is a unique type of muscle pain in the patient population. The role that discrete muscle afferent subpopulations play in the generation of pain during ischemic events, however, has yet to be determined. Using 2 brachial artery occlusion models to compare prolonged ischemia or transient ischemia with reperfusion of the muscles, we found that both injuries caused behavioral decrements in grip strength, as well as increased spontaneous pain behaviors. Using our ex vivo forepaw muscles, median and ulnar nerves, dorsal root ganglion, and spinal cord recording preparation, we found after both prolonged and transient ischemia that there was a significant increase in the number of afferents that responded to both noxious and non-noxious chemical (lactate, adenosine triphosphate, varying pH) stimulation of the muscles compared to uninjured controls. However, we found an increase in firing to heat stimuli specifically in muscle afferents during prolonged ischemia, but a distinct increase in afferent firing to non-noxious chemicals and decreased mechanical thresholds after transient ischemia. The unique changes in afferent function observed also corresponded with distinct patterns of gene expression in the dorsal root ganglia. Thus, the development of ischemic myalgia may be generated by unique afferent-based mechanisms during prolonged and transient ischemia.**

Perspective: ***This study analyzed the response properties of thinly myelinated group III and unmyelinated group IV muscle afferents during prolonged and transient ischemia in addition to pain behaviors and alterations in*** **DRG** ***gene expression in the mouse. Results suggest that mechanisms of pain generation during prolonged ischemia may be different from ischemia/reperfusion.***

Key words: *Ischemic myalgia, electrophysiology, gene expression, artery occlusion, plasticity.*

Myalgia is one of the most common complaints of patients seeking treatment for pain; however, multiple disorders can cause muscle pain.[42] One vexing, but quite prevalent,[5] set of muscle pain disorders in the population include those that originate from issues of peripheral circulation. Patients with peripheral vascular disease, for example, have small occlusions of the vessels supplying the limb muscles that cause them to experience intermittent claudication.[2] In addition, many patients with sickle cell anemia have chronic pain as a result of successive ischemia/reperfusion events during and after a crisis.[64] Recently, complex regional pain syndrome has also been linked to issues of peripheral perfusion where both hyper- and hypoperfusion have been documented, and this is thought to be a major underlying cause of subsequent muscle pain.[9] A unique feature of disorders of peripheral perfusion is that transient ischemia with reperfusion (I/R) causes muscle atrophy and microvasculature changes distinct from prolonged occlusion,[7] which may suggest that muscle pain in these distinct injury states may be generated through different mechanisms.

It has been well documented that primary muscle afferents are crucial in generating muscle pain after

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inflammation[16,41,55] or incision[61]; however, the contribution of specific populations of muscle afferents to ischemic myalgia is not understood to the same degree. In uninjured animals, it is known that the thinly myelinated group III and unmyelinated group IV muscle afferents respond to mechanical and thermal stimuli similar to cutaneous afferent subpopulations; however, roughly half of group III and group IV muscle afferents are chemosensitive.[23] The chemical responsiveness of these afferents can be subdivided into 2 physiologically relevant classes based on their sensitivity to distinct metabolite mixtures.[6,23,36] Metaboreceptors, which are thought to regulate sympathetic reflexes and possibly the sensations of fatigue, have recently been defined as cells that respond to a non-noxious metabolite mixture of lactate and adenosine triphosphate (ATP, pH 7.0) that is typically found to be produced in the muscles during moderate exercise.[23,27,28,50,53] Conversely, metabonociceptors, which are thought to regulate pain responses, are stimulated by higher concentrations of these same metabolites (pH 6.6, increased lactic acid and ATP), a mixture known to be produced in the muscles during ischemic contractions.[1,23,29,34-36,41]

Because ischemic conditions produce these mixtures in injured muscles, it is reasonable to suggest that the afferents that respond to these metabolites and modifications of gene expression in the dorsal root ganglia (DRGs) are crucial in the generation of ischemic myalgia.[14,20,37,40] Therefore, in this study, we investigated whether muscle afferent response characteristics uniquely changed in response to prolonged or transient peripheral ischemia using an ex vivo forepaw muscle/median and ulnar nerves/DRG/spinal cord recording preparation and compared the results to behavioral changes after injury to determine how the observed changes in afferents could lead to muscle nociception. Prolonged ischemia was induced by a total brachial artery occlusion (BAO) in one forelimb, whereas transient I/R was established by removing the BAO after several hours. Finally, we surveyed DRG receptor expression and muscle-derived signaling pathways to explore potential mechanisms involved in the hypothesized differential muscle afferent plasticity.

Methods

Animals

Adult male Swiss Webster mice (Charles River, Wilmington, MA), between 3 and 6 weeks of age, were used in all experimental analyses. Mice were held in a climate-controlled barrier facility with 12-hour light/dark housing and ad libitum access to food and water. All procedures were approved by the Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee and adhered to NIH Standards of Animal Care and Use under Association for Assessment and Accreditation of Laboratory Animal Care International–approved practices.

Induction of Prolonged and Transient Peripheral Ischemia

One day prior to all analyses, mice were anesthetized with 3% isoflurane. The right brachial artery was exposed proximal to the ulnar artery/radial artery split. The vessels were gently loosened from adjacent connective tissue and then the brachial artery was occluded with a 7-0 silk suture. Incisions were closed with 6-0 silk sutures. For the prolonged ischemia condition (BAO), the occlusion was left intact for 24 hours or up to 3 days for behavioral analyses. For transient I/R, a modified version of Coderre et al[9] was employed. Briefly, animals were again anesthetized with 3% isoflurane 6 hours postocclusion for removal of the brachial artery suture. I/R mice were allowed to recover for 18 hours after the second surgery to induce reperfusion injury. Again for behavioral analyses only, a 3-day time point post initial occlusion was also analyzed. To account for possible effects of sutures around the nerves for ex vivo or molecular experiments (below), a sham surgery was also utilized in which a suture was placed around the artery but was not tied, such that the artery was not occluded. In addition, another group of mice received a 6-hour BAO only to aid in the determination of how the occlusive aspect of these injuries may be different in addition to determination of how prolonged ischemia may differ from the reperfusion phase.

Nerve Crush Injuries

Mice were anesthetized as described using 3% isoflurane. For grip strength positive control experiments (see below), the right median and ulnar nerves were exposed just above the elbow and crushed with #5 fine-tip forceps for approximately 3 to 4 seconds. For ATF3 immunostaining–positive control experiments (see below), only the right ulnar nerve was exposed just above the elbow and crushed with the forceps. The nerve(s) was then visually inspected to confirm the injury and qualitatively verify a translucent appearance of the nerve that is prevalent after these types of peripheral injuries.[25] The wounds were then closed using 7-0 silk sutures. One day after unilateral median and/or ulnar nerve crush, animals (n = 8) underwent grip strength testing (below) for comparisons to mice that received BAO or I/R. In other experiments, C8 and T1 DRGs were isolated and processed immunocytochemically as described below 1 day after ulnar crush injury.

Behavioral Assays

Separate groups of sham, I/R, and BAO mice were used for behavioral analysis (n = 7–9 per group). Mice were first tested at baseline, approximately 2 hours prior to injury (described above), then again on days 1 and 3 post injury. All behavioral testing was performed in the morning during light hours, and each testing day included 3 behavioral tasks: forelimb guarding, von Frey filament stimulation of the plantar surface of the forepaws, and forepaw grip strength, in this order. Mice were placed in a raised acrylic glass chamber with a steel mesh bottom and allowed to habituate for at least