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THE PATHOGENESIS OF

INFLAMMATORY MUSCLE PAIN

Lisa Carole Loram

A thesis submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2007

DECLARATION

This thesis is submitted in the format, approved by the Faculty of Health Science, of published work with encompassing introduction and conclusion.

I declare that the work contained in this thesis is my own. For Chapter 2, 3 and 4, the co-authors assisted with data analysis, interpretation and writing of the manuscript. However, I was responsible for the conceptualization of each project, the overall project design, data collection and writing of the manuscripts for each of the chapters. This work has not been submitted before for any degree or examination at any other university.



ABSTRACT

The aim of my thesis is to further investigate the mechanisms underlying inflammatory muscle pain. Despite numerous studies investigating the mechanisms of inflammatory hyperalgesia, little is known of the mechanisms underlying inflammatory muscle hyperalgesia. Using rats as experimental animals, I investigated inflammatory hyperalgesia in muscle and compared it to that of inflamed cutaneous tissue. I injected carrageenan, a plant-origin polysaccharide, into leg muscle and into the hind paw of rats, and measured the behavioural response, as well as cytokine changes, in both plasma and inflamed tissue. Carrageenan induced inflammatory hyperalgesia but the cytokine cascade was not the same in muscle and cutaneous tissue. At no time following carrageenan injection was muscle tumour necrosis factor alpha (TNF-α) concentration elevated above that of muscle injected with saline. TNF-α is a key inflammatory mediator in cutaneous tissue, but apparently not in muscle. Interleukin-1β (IL-1β) and interleukin-6 concentrations also were different during muscle inflammation compared to those of cutaneous inflammation. IL-1β and IL-6 concentrations, following carrageenan injection, were elevated later in muscle compared to in cutaneous tissue. IL-1β is a potent sensitizer of nociceptors in cutaneous tissue, and also may play an important role in sustaining muscle pain, but it is unlikely to be an initiator of the inflammatory muscle hyperalgesia. In the course of comparing muscle hyperalgesia and cutaneous hyperalgesia, I aimed to identify whether these differences in cytokine concentrations were unique to muscle tissue or if similar differences in cytokine concentrations existed between the hind paw and other cutaneous sites. To explore an alternative cutaneous tissue site, I injected carrageenan into the rat tail and measured the behavioural response, changes in cytokine concentrations and histological changes. Elevations of pro-inflammatory cytokines

occurred concurrently with the infiltration of leukocytes into the inflamed tail tissue with the thermal and mechanical hyperalgesia similar to that found in the hind paw. Different mechanisms therefore appear to underlie muscle and cutaneous inflammatory hyperalgesia, regardless of the site used to investigate cutaneous inflammation. One of the consequences of the poor understanding of muscle pain is the lack of a reliable regimen for treating human muscle pain, including delayed-onset muscle soreness (DOMS). DOMS, which has a partial inflammatory pathogenesis, is not relieved by non-selective cyclo-oxygenase inhibitors. This phenomenon may be that prostaglandins are not produced peripherally or centrally, when muscle tissue is damaged. I investigated the effect of inhibiting cyclo-oxygenase-2, the isoform released during inflammation, on DOMS in human volunteers. I found that rofecoxib, a cyclo-oxygenase-2 inhibitor, did not attenuate DOMS and nor did tramadol, a central-acting analgesic. The neurochemical pathway underlying DOMS therefore appears to be distinct from the pathways which underlie pain and hyperalgesia in other syndromes. Future research should include investigations into the central mechanisms of muscle pain and blocking the action of IL-1β and CINC-1 both peripherally and centrally may prove a beneficial target for the treatment of clinical muscle pain.

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With the support of my supervisors, I have employed the option offered by the University for submission of my thesis by published papers, with the over-arching introduction and conclusion. Papers 1 and 2 above appear as chapter 2 and 4 of my thesis.

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LIST OF ABBREVIATIONS

ASIC	acid-sensing ion channels
ATP	adenosine triphosphate
B_1 receptor	bradykinin receptor type one
B_2 receptor	bradykinin receptor type two
BD	administered twice daily
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
CINC-1	cytokine-induced neutrophil chemoattractant one
CK	creatine kinase
COX	cyclo-oxygenase
CXC	cysteine-amino acid-cysteine chemokine receptor
DOMS	delayed-onset muscle soreness
GLUT-1	glucose transporter one
GLUT-4	glucose transporter four
gp120	glycoprotein Mr120
gp80	glycoprotein Mr80
i.pl.	intraplantar
IL-1α	interleukin-one alpha
IL-1β	interleukin-one beta
IL-1βRI	interleukin-one beta receptor type one
IL-1βRII	interleukin-one beta receptor type two
IL-6	interleukin-six
IL-8	interleukin-eight

kDa	kiloDalton
LPS	lipopolysaccharide
MIP-1	macrophage-inflammatory protein type one
mRNA	messenger ribonucleic acid
NGF	neurotrophic growth factor
NMDA	n-methyl-D-aspartate
PGE_2	prostaglandin E_2
QID	administered four times per day
SD	standard deviation
TDS	administered three times per day
TNFRI	tumour necrosis factor alpha receptor type one
TNFRII	tumour necrosis factor alpha receptor type two
TNF-α	tumour necrosis factor alpha
TrkA	tyrosine kinase A receptor
TRPV1	transient receptor potential vanilloid one

CHAPTER 1

INTRODUCTION

Muscle pain is a common morbidity affecting all people at some stage of their lives with a prevalence of at least 20% within the western world (Brooks, 2005). The prevalence of musculoskeletal pain also is increasing as the age of the general population increases (Siebens, 2007). Because there is a common comorbidity of musculoskeletal pain with other diseases, for example muscle pain in cancer patients, fibromyalgia associated with posttraumatic stress disorder, and rotator cuff injury after a cerebrovascular accident (Siebens, 2007), it is vital that optimal treatment is found for musculoskeletal pain while minimising the side-effects of the treatment. The mechanisms underlying muscle pain were first investigated by Kellgren, in 1938, who injected hypertonic saline into muscle and identified characteristic patterns of pain from each muscle (Kellgren, 1938). Siegfried Mense, a pioneer in the field of muscle pain, in the late 1970's investigated the electrophysiological pathways involved in muscle hyperalgesia (Mense, 1977). Although some studies have been conducted on muscle pain, the most current information about muscle pain has been inferred from studies investigating the mechanisms of cutaneous pain. Over the last few decades, it has become apparent that the mechanisms of muscle pain are distinct from that of cutaneous pain, and therefore many inferences may be inaccurate.

Although the mechanism of clinical muscle pain is not yet clear, there is an emergence of information implying that the origin of most clinical muscle pain may lie in inflammatory pathways activating peripheral muscle nociceptors, leading to peripheral sensitization, and finally progressing to central sensitization (Inanici & Yunus, 2004; Murata *et al.*, 2005). The aim of my introduction is to survey the literature on the role that pro-inflammatory cytokines and related inflammatory mediators play in primary hyperalgesia and, more specifically, in experimental models of primary muscle hyperalgesia. A confounding factor in examining the mechanisms of inflammatory

muscle pain is that exercise alone is able to induce a cytokine response similar to that of an inflammatory response, but with the absence of pain. Therefore, in order to understand whether inflammatory mediators are likely to contribute to muscle pain, I must account for the role that cytokines play during exercise. Finally, I will investigate the role of inflammatory mediators in clinical muscle pain.

1. Inflammation

Inflammation is a common cause of pain, and more often hyperalgesia, and the treatment of inflammation contributes to a large proportion of the global pharmaceutical sales. Inflammatory pain and the treatment of inflammatory pain have been investigated extensively, but most treatment regimens are based on findings from studies conducted on cutaneous tissue. Although a similar process to that in cutaneous tissue occurs in other tissues and organs during inflammation, the sequence of events and severity of inflammation differ depending on the tissue involved. To identify whether the inflammatory process in muscle is similar to that of cutaneous tissue, it is necessary to understand the inflammatory mediators that contribute to cutaneous inflammation and the associated pain.

Inflammation can occur as a result of chemical, thermal or mechanical injury. The purpose of inflammation is to alert the organism to the injury and promote healing and repair of the damaged tissue (Smith, 1991). The inflammatory process involves both vascular changes and a cellular response. Inflammatory mediators are released from the injured tissue, stimulating an increased blood flow to the area and an increased capillary permeability leading to local oedema, increased temperature and redness.

The local vasodilation of the arterioles during inflammation allows for the influx of leukocytes and chemical mediators into the injured and surrounding area. Neutrophils are the first to invade the injured area, followed by monocytes and lymphocytes. The neutrophil invasion, aiding the digestion of the injured cells, is rapid and brief (Smith, 1991). Hours later, monocytes migrate to the area and remain there for several days. Once monocytes enter the tissue they mature into macrophages and aid in the removal of foreign debris and necrotic tissue. The leukocytes release growth factors and cytokines which degrade the injured tissue, but also are important mediators in the proliferative phase of wound repair (Smith, 1991). In addition, the growth factors and cytokines contribute in producing hyperalgesia, or an increased sensitivity to a painful stimulus. Cytokines are soluble proteins that act at very low concentrations in an autocrine, paracrine or endocrine function (Moldoveanu *et al.*, 2001). The pro-inflammatory cytokines that are present during inflammation and that contribute towards inflammatory hyperalgesia are tumour necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6) and, cytokine-induced neutrophil chemoattractant (CINC-1) in rats or interleukin-8 (IL-8) in humans (Poole et al., 1999a).

1.1 Inflammatory hyperalgesia

Various chemical mediators, released from immune cells and resident cells, are involved in sensitizing or directly activating nociceptors by acting on receptors and ion channels on the nociceptors. Peripheral sensitization of the nociceptors occurs by lowering the threshold to a stimulus, usually of a thermal or mechanical quality, leading to hyperalgesia, an increased sensitivity to a noxious stimulus, and allodynia, an increased sensitivity to a non-noxious stimulus (Mitchell, 1999). The nociceptor

threshold is lowered by gene translation, transcription and post-translation changes in the receptors and ion channels, for example increasing the TRPV1 receptor expression via mitogen-activated protein kinases and therefore making the nociceptors more sensitive to stimuli (Costigan & Woolf, 2000; Meyer *et al.*, 2006). This peripheral sensitization results in phenotypic changes in sensory neurones from inflamed tissue and changes within the spinal cord such as altered transduction sensitivity of high-threshold nociceptors and a change in excitability of spinal neurones (Woolf *et al.*, 1997). Some mediators affect transduction directly through changes within ion channels, while others, such as cytokines, act on gene transcription of nociceptors creating peripheral hypersensitivity. Cytokines may act on cytokine receptors found on the nociceptors, for example TNF-α, or stimulate the release of other cytokines or chemical mediators, which in turn activate or sensitize nociceptors, leading to nociceptor transduction or neuronal transcription (Woolf *et al.*, 1997).

In addition to peripheral sensitization of nociceptors during inflammation, antidromic afferent nerve activity causes the release of neurotransmitters in other branches of the afferent nerve resulting in neurogenic inflammation. (Meyer et al., 2006). Neurogenic inflammation results in further local vasodilation and increased vascular permeability, perpetuating the inflammatory process. The nociceptors in the injured tissue release neuropeptides, such as calcitonin growth-related peptide (CGRP) and substance P, into the interstitium promoting a further increase in vascular permeability and sensitizing and activating surrounding nociceptors (Opree & Kress, 2000; Bianchi *et al.*, 2004). The neurotransmitters released exacerbate the influx of leukocytes and the release of pro-inflammatory mediators, including cytokines, and other direct activators of peripheral nociceptors, such as bradykinin.

1.1.1 Bradykinin

Bradykinin is released into the interstitium as a result of injury to tissue. Bradykinin is a short-acting, but potent, vasodilator and is formed from the enzymatic action on kallikrein, and broken down quickly by carboxypeptidase. Bradykinin is able to stimulate nociceptors directly by acting on B_1 and B_2 receptors found on nociceptors, but B_1 receptors requires the presence of cytokines to be activated by bradykinin during inflammation (Poole *et al.*, 1999b). Once inflammation is established, blocking both B_1 and B_2 receptors does not attenuate mechanical hyperalgesia (Poole et al., 1999a). Also, blocking B_1 receptors does not attenuate the mechanical hyperalgesia induced by other inflammatory mediators, such as TNF-α, IL-8 or prostaglandin E_2 (Poole et al., 1999a). Therefore, although bradykinin is able to activate nociceptors directly through bradykinin receptors, other mediators are required to sustain the inflammatory hyperalgesia.

1.1.2 Tumour necrosis factor alpha

Tumour necrosis factor alpha (TNF-α) is a pleiotropic cytokine, a potent pyrogen, and initially was called cachectin for its catabolic effects during disease. TNF-α is a 17kDa protein that acts in cytolysis, mitogenesis, recruiting leukocytes to an injured area by inducing the expression of surface adhesion molecules allowing for adhesion of leukocytes, and stimulating the release of IL-1β, IL-6 and IL-8 (Woolf *et al.*, 1997). It has potent paracrine and endocrine functions and often acts in combination with other cytokines. TNF-α is secreted by most cells, including monocytes, macrophages and myocytes, and plays a critical role in the acute phase response of fever and cutaneous inflammation. Most cells within the body express receptors for TNF-α and therefore are able to respond to TNF-α (Moldoveanu et al., 2001).

TNF-α binds to two receptors, namely TNF-receptor one and TNF-receptor two. These two receptors have different intracellular domains and therefore exert different cellular responses (Zhang *et al.*, 2000). TNF-α is cleared from the circulation by binding to a TNF-α binding protein, thus inactivating the TNF-α but also making it more biologically stable (Moldoveanu et al., 2001). The complex of TNF-α and binding protein are then metabolised mainly by the liver but also by the kidneys (Moldoveanu et al., 2001).

TNF-α mediates hyperalgesia by decreasing the nociceptor threshold and triggering the release of other pro-inflammatory cytokines which further contribute to peripheral sensitization (Bianchi *et al.*, 2004). Injecting inflammatory agents such as carrageenan and complete Freund's adjuvant (CFA) into the hind paw of rats induces inflammatory hyperalgesia with an associated release of TNF-α in the hind paw, remaining elevated for at least 15 days (Woolf *et al.*, 1997). Neutralizing TNF-α with antibodies co-administered with CFA into the hind paw decreases the initial hyperalgesia, but by 24h the hyperalgesia has returned (Woolf *et al.*, 1997). A possible explanation for this transient attenuation of the hyperalgesia is the brief activity of the TNF-α within the inflamed hind paw, or it may be that TNF-α is important for the initiation of inflammatory hyperalgesia while other mediators sustain the inflammatory hyperalgesia. Table 1 summarizes research using exogenous TNF-α to induce experimental hyperalgesia in the hind paw of rodents.

Table 1. Summary of research investigating TNF-α injected into the hind paw of rodents to produce hyperalgesia

Dose of TNF-α	Response to noxious stimulus	Intervention	Response to intervention	Reference
1-500ng (rat)	Dose dependent intensity of thermal and mechanical hyperalgesia lasting 6h	Block NGF	Decrease in mechanical and thermal hyperalgesia	Woolf et al, 1997
0.0025-2.5pg (rat)	1-3h mechanical hyperalgesia Dose dependent intensity	Block TNF-α Block IL-1 Block IL-6 Block IL-8 Block IL-1 and IL-6 Block IL-1 and IL-8 Block IL-6 and IL-8	Abolish hyperalgesia Attenuate hyperalgesia Attenuate hyperalgesia Attenuate hyperalgesia Attenuate hyperalgesia Abolish hyperalgesia Abolish hyperalgesia	Cunha et al, 1992
2.5pg (rat)	Mechanical hyperalgesia 1h after injection	IL-1ra	Attenuate hyperalgesia	Cunha et al, 2000
2.5pg (rat)	Mechanical hyperalgesia 3h after injection	Block TNF-α Block B_1 receptors	Abolish hyperalgesia No effect	Poole et al, 1999a
1-1000pg (mouse)	Dose and time dependent mechanical hyperalgesia			Cunha et al, 2005

B_1 receptor – bradykinin 1 receptor, IL-1ra – interleukin one receptor antagonist,
TNF-α – tumour necrosis factor alpha, species in parentheses indicate the rodent used for each experiment.

In peripheral inflammation, no TNF-α is detected in the circulation. However, TNF-α has been detected in other tissues, such as the contralateral paw, which were not directly affected by the inflammation and did not present with hyperalgesia (Woolf *et al.*, 1997; Bianchi *et al.*, 2004). Also, injecting TNF-α into the hind paw, without the stimulation of a noxious challenge, does not stimulate the release of the neurotransmitter calcitonin growth-related peptide (CGRP) within the injected paw, inferring that no nociceptor activation or development of neurogenic inflammation occurred. In the presence of a noxious thermal challenge, TNF-α administration significantly increases the CGRP concentration above that observed with a noxious thermal challenge alone (Opree & Kress, 2000). Therefore, TNF-α requires a concurrent noxious stimulus in order to sensitize nociceptors.

TNF-α may contribute to the induced hyperalgesia by stimulating prostaglandin synthesis via IL-1β and IL-6, and activate adrenergic receptors via IL-8 (Cunha *et al.*, 1992). Hyperalgesia induced by local injection of TNF-α into the hind paw of rats was attenuated by neutralizing IL-1β, IL-6 and IL-8 individually, and abolished by neutralizing a combination of IL-1β and IL-6, or IL-6 and IL-8 or IL-1β with IL-8 (Cunha *et al.*, 1992), implying that the cytokines released down-stream of TNF-α play a greater role in maintaining the hyperalgesia than the TNF-α itself.

Therefore, TNF-α alone does not activate nociceptors producing spontaneous pain, but sensitizes nociceptors to noxious stimuli and so produces hyperalgesia. Also, TNF-α stimulates the release of other inflammatory mediators, in particular other cytokines, which may sensitize nociceptors or stimulate the release of other chemical mediators that directly activate nociceptors.

1.1.3 Interleukin-1 beta

Interleukin-1β is a 17kDa protein that is cleaved from the precursor pro-IL-1β by IL-1β converting enzyme, an intracellular protease also called caspase-1 (Authier *et al.*, 1999; Zhang *et al.*, 2000). Interleukin-1β is mostly secreted by macrophages and monocytes (Rothwell & Luheshi, 2000), and is important for attracting and coordinating the responses of immune cells during infection or inflammation. Interleukin-1β is detected only in pathological states, is a pleiotropic cytokine, and is involved in inducing many of the sickness behaviours, such as fever, lethargy and also hyperalgesia (Watkins et al., 1995). Interleukin-1β also inhibits vascular smooth muscle contraction via the stimulation of nitric oxide, which may further exacerbate the symptoms associated with vasodilation that is induced during inflammation (Moldoveanu et al., 2001).

IL-1β binds to one of two membrane-bound receptors, namely receptor type I and type II, using IL-1R accessory protein. IL-1βRI transduces an intracellular signal while IL-1βRII does not transduce an intracellular signal, but likely acts as a decoy receptor. Interleukin receptor antagonist is an endogenous antagonist to IL-1β and acts by competing for receptor sites thus blocking the action of IL-1β.

Interleukin-1β release, stimulated by TNF-α, is a well described sensitizer of nociceptors that ultimately produces hyperalgesia in cutaneous tissue (Ferreira et al., 1988). An injection of IL-1β into the hind paw of rats is able to induce mechanical hyperalgesia that is dose and time dependent (Cunha *et al.*, 2000; Cunha *et al.*, 2005). However, regardless of the dose of IL-1β administered, the hyperalgesia resolves within 24h. IL-1β is released during inflammation as an injection of CFA into the hind

paw produced an increase in hind paw tissue IL-1β concentration 48h after injection, which is concomitant with the hyperalgesia induced (Safieh-Garabedian *et al.*, 1995). Although IL-1β is produced at the site of inflammation and IL-1β given systemically induces hyperalgesia, IL-1β is not detected in the circulation when inflammation is induced in the hind paw by inflammatory agents such as carrageenan or CFA (Ferreira *et al.*, 1988; Safieh-Garabedian *et al.*, 1995). Table 2 summarizes research using exogenous IL-1β to induce experimental hyperalgesia in the hind paw of rodents.

Interleukin-1β does not directly activate nociceptors, as evident by the lack of CGRP release into tissue injected with IL-1β (Opree & Kress, 2000). However, IL-1β sensitizes nociceptors and produces hyperalgesia by stimulating the release of cyclo-oxygenase enzyme (COX) which converts arachidonic acid into prostaglandins (Cunha *et al.*, 1992). Interleukin-1β induces hyperalgesia through local peripheral sensitization, and is mediated via prostaglandin synthesis or nerve-growth factor (NGF) release stimulating trkA receptors (Ferreira et al., 1988). IL-1β also has central effects acting either via the vagal afferents (Watkins et al., 1995) or via central release of IL-1β (Samad *et al.*, 2001) and, therefore, COX-2 centrally.

Although IL-1β stimulates the release of other cytokines and cyclo-oxygenase, IL-8 and IL-6 alone do not mediate its action, and blocking TNF-α does not attenuate IL-1β-induced hyperalgesia (Cunha *et al.*, 1992). A combination of TNF-α and IL-1β injected into the hind paw of rats produced a hyperalgesia comparable to that found after 50μg carrageenan injected into the hind paw (Ferreira *et al.*, 1988; Cunha *et al.*, 2000). Therefore, the hyperalgesia induced by inflammatory agents may be produced by TNF-α and IL-1β, with minor involvement by IL-6 and IL-8.

Table 2. Summary of research investigating il-1β injected into the hind paw of rodents to produce hyperalgesia

Dose of IL-1β per paw	Response to noxious stimulus	Intervention	Response to intervention	Reference
0.25-2.5pg (rat)	Bilateral mechanical hyperalgesia except with low doses	Analogue to IL-1β (Lys-D-Pro-Thr)	Dose-dependent decrease in mechanical hyperalgesia induced by IL-1β	Ferreira et al (1988)
0.05pg and 0.5pg (rat)	Mechanical hyperalgesia 3h after injection	IL-1ra Block IL-1ra	Decreases intensity of hyperalgesia Increase intensity of hyperalgesia	Cunha et al (2000)
10-1000pg (mouse)	Dose and time dependent mechanical hyperalgesia			Cunha et al (2005)
1pg, 100pg, 1000pg (rat)	Thermal but not mechanical hyperalgesia	IL-1ra	Abolish hyperalgesia	Safieh-Garabedien et al (1995)
0.5pg (daily for 10-18days, rat)		Indomethacin Atenolol and indomethacin	Decrease in mechanical hyperalgesia induced by IL-1β	Sachs et al (2002)

IL-1β – interleukin-one beta, IL-1ra – interleukin one receptor antagonist, species in parentheses indicate the rodent used for each experiment

Like TNF-α, IL-1β is not able to directly activate nociceptors producing spontaneous pain, but IL-1β is a potent hyperalgesic agent acting by sensitizing nociceptors, stimulating the release of prostaglandins and acting centrally within the spinal cord. In addition, IL-1β stimulates the release of other pro-inflammatory cytokines, such as IL-6, and growth factors such as nerve growth factor NGF (Woolf *et al.*, 1997).

1.1.4 Interleukin-6

Interleukin-6 is a 26kDa protein secreted by most cells in the body including myocytes. Interleukin-6, during inflammation, is a potent mediator for the release of acute phase proteins from the liver and plays a role as an inflammatory mediator in autoimmune diseases such as systemic lupus erythematosis and rheumatoid arthritis (Moldoveanu et al., 2001). IL-6 has only one receptor (Zhang *et al.*, 2000; De Jongh *et al.*, 2003) but two IL-6 receptor signalling proteins, gp80 and gp130, are upregulated during pain.

Both TNF-α and IL-1β can stimulate the release of IL-6, and IL-6 influences the synthesis of substance P, NGF and prostaglandins (Poole *et al.*, 1999a). IL-6 when injected into the hind paw is able to produce an intensity of mechanical hyperalgesia comparable to that produced by intraplantar TNF-α administration. However, the dose of IL-6 required (10-1000pg) is much greater than that of TNF-α (Cunha *et al.*, 1992). Also, blocking IL-6 only attenuated carrageenan-induced hyperalgesia, whereas blocking TNF-α abolished carrageenan-induced hyperalgesia (Cunha *et al.*, 1992).

IL-6 is the least potent hyperalgesic agent of the pro-inflammatory cytokines (Poole *et al.*, 1999a), but IL-6 does partly drive the production of prostaglandins, via cyclo-oxygenase, which then activate or sensitize nociceptors. IL-6 does contribute to

inflammatory hyperalgesia since after inducing inflammation in IL-6 knockout mice, the mice are less responsive to a noxious thermal stimulus (Xu *et al.*, 1997). IL-6, like TNF-α and IL-1β, is unable to activate nociceptors, as evident by a lack of CGRP release into tissue injected with IL-6. However, unlike TNF-α and IL-1β it also does not sensitize nociceptors unless there is the presence of soluble IL-6 receptors (Opree & Kress, 2000). Although gp130 signalling protein is constitutively expressed in normal tissue, the soluble IL-6 receptor is not (Opree & Kress, 2000). It is not clear what triggers the release of the soluble IL-6 receptors or if all tissues are able to express it and further work is required to investigate the interaction between IL-6 and the associated receptors. Nonetheless, IL-6 is biologically active only in the presence of both the soluble receptor and the signalling protein gp130 (Song & Kellum, 2005).

Although IL-6 is present during inflammation, it requires the presence of a soluble IL-6 receptor and gp130 signaling protein to produce hyperalgesia, and therefore, may contribute to sensitizing nociceptors during inflammation in combination with other mediators such as IL-1β.

1.1.5 Cytokine-induced neutrophil chemoattractant-1 and interleukin-8

Cytokine-induced neutrophil chemoattractant (CINC-1) is a small 8kDa protein, originally identified in isolated rat epithelial cell lines (Yamamoto *et al.*, 1998), and is categorised as a chemokine. Subfamilies of chemokines exist, depending on the site of one or two cysteine residues, with each group attracting a certain type of leukocyte (Lorenzetti *et al.*, 2002). Interleukin-8 and CINC-1 belong to the same cysteine-amino acid-cysteine (CXC) family with CINC-1 being the rat homologue to human IL-8. Lipopolysaccaride (LPS), the pyrogenic cell wall of Gram positive cocci, TNF-α, and

IL-1β, all are able to induce the release of CINC-1 into circulation (Yamamoto *et al.*, 1998; Campbell *et al.*, 2003). CINC-1 induces transient neutrophil accumulation without the migration of lymphocytes or monocytes (Matsumoto *et al.*, 1997). CINC-1 also may act as an early acute phase protein during inflammation (Campbell et al., 2003), and it has potent angiogenic properties (Akerstrom et al., 2005).

The receptors for CINC-1 or IL-8 have yet to be identified, and whether any of these receptors are present on nociceptors is still to be investigated. However, IL-8 injected into the hind paw of a rat is able to produce a dose-dependent hyperalgesia peaking in intensity at 6h and resolving by 24h (Cunha et al., 1991). Table 3 summarizes research using exogenous CINC-1 or IL-8 to induce experimental hyperalgesia in the hind paw of rodents.

IL-8 may contribute to hyperalgesia, but its effects are likely to occur downstream of IL-1β, IL-6, TNF-α. (Cunha *et al.*, 1992). Blocking CINC-1 attenuated carrageenan-induced hyperalgesia and TNF-α-induced hyperalgesia in the hind paw, but if both CINC-1 and IL-1β were blocked, both types of hyperalgesia were abolished (Lorenzetti *et al.*, 2002). Therefore, the release of CINC-1 is stimulated by TNF-α, but the mechanism of hyperalgesia induced by CINC-1 is independent of IL-1β and TNF-α.

CINC-1 produces hyperalgesia via adrenergic receptor activation as evidenced by attenuation of hyperalgesia by administration of atenolol, a beta-adrenergic antagonist, but not indomethacin, a non-specific cyclo-oxygenase inhibitor (Cunha *et al.*, 1991; Cunha *et al.*, 1992; Lorenzetti *et al.*, 2002). IL-8, with or without the stimulation of a

Table 3. Summary of research investigating CINC-1 and IL-8 injected into the hind paw of rodents to produce hyperalgesia

Dose of CINC-1 or IL-8	Response to noxious stimulus	Intervention	Response to intervention	
0.1ng (rat)	mechanical hyperalgesia 3h after injection	Block B_1 receptors	No change	Poole et al (1999)
3-300pg (rat)	Mechanical hyperalgesia measured 3h and 6h after injection	Indomethacin Atenolol Block IL-1 Block CINC-1	No change Attenuate hyperalgesia No change Abolish hyperalgesia	Lorenzetti et al (2002)
10pg and 100pg (rat)	Mechanical hyperalgesia 3h after injection	IL-1ra	No change	Cunha et al (2000)
100pg (daily for 10-18 days, rat)		Atenolol	Decrease in mechanical hyperalgesia induced by CINC-1	Sachs et al (2002)
1-10ng (mouse)	Dose and time dependent mechanical hyperalgesia			Cunha et al (2005)
1pg, 10pg and 100pg (rat)	Dose dependent mechanical hyperalgesia (3h after injection)	Atenolol Propanolol Guanethidine	Attenuate hyperalgesia Attenuate hyperalgesia Attenuate hyperalgesia	Cunha et al (1991)

B_1 receptor – bradykinin one receptor, IL-1ra – interleukin one receptor antagonist, CINC-1 – cytokine-induced neutrophil chemoattractant, IL-1 – interleukin 1, species in parentheses indicate the rodent used for each experiment.

noxious challenge, does not activate or sensitize nociceptors (Opree & Kress, 2000), suggesting that IL-8 may produce hyperalgesia through central effects, acting on the spinal cord or the brain, but not through direct nociceptor activation. CINC-1 injected into one hind paw produces a dose-dependent mechanical hyperalgesia in both hind paws further implicating a central, rather than peripheral role for CINC-1 (Lorenzetti *et al.*, 2002).

CINC-1 may play a role centrally by acting on the anterior pituitary and hypothalamus (Matsumoto *et al.*, 1997). A painful stimulus, such as a formalin injection into the hind paw, increases the serum CINC-1 concentration and increases the CINC-1 concentration in the hypothalamus. Whether CINC-1 is released from the hypothalamus and anterior and posterior pituitary, or is released elsewhere and acts on these target organs, is still to be determined. However, CINC-1, injected intracerebroventricularly, induces mechanical hyperalgesia (Yamamoto *et al.*, 1998). Interestingly, macrophage-inflammatory protein (MIP-1), a chemokine for monocytes, does not induce hyperalgesia, suggesting that either CINC-1 itself is able to bind to receptors within the pain pathway and induce hyperalgesia, or the neutrophils that are attracted to the inflamed area release mediators that are able to induce hyperalgesia.

Therefore, CINC-1 or IL-8 is able to produce hyperalgesia, independent of TNF-α and IL-1β, with the most likely mechanism being through central mediation. Further work into the role of CINC-1 and IL-8, such as the receptors and the mechanism of inducing hyperalgesia, is required.

1.1.6 Prostaglandins

Prostaglandins and prostacyclins are potent peripheral vasodilators and act synergistically in producing oedema and inflammatory pain (Baldwin Lanier, 2003). Prostaglandins are able to sensitise nociceptors, but also increase vascular permeability and allow other mediators into the injured tissue, which may further sensitize nociceptors (Baldwin Lanier, 2003). Prostaglandins are synthesized from membrane lipids via cyclo-oxygenase enzyme (Samad *et al.*, 2001). Cyclo-oxygenase (COX) is the rate-limiting enzyme that converts arachidonic acid into prostaglandins. Figure 1 below, taken from Hinz and Brune (2002), shows the process by which prostaglandins are synthesized from arachidonic acid.

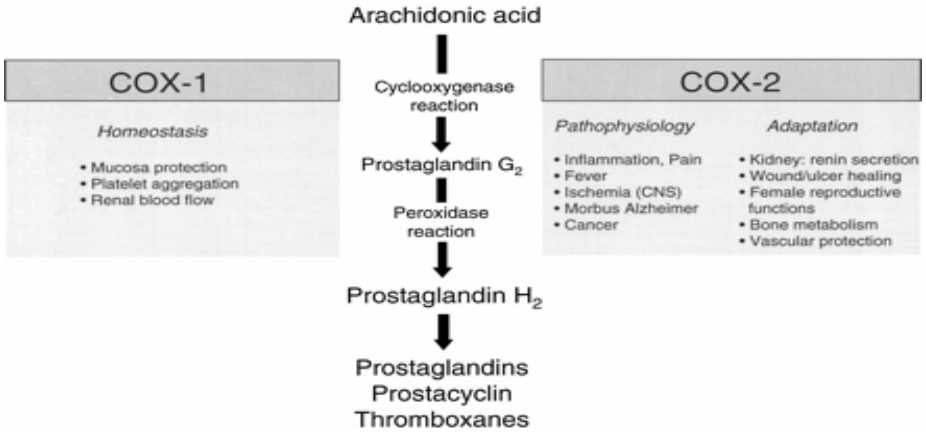

Figure 1. The prostanoid synthesis cascade, and the physiological and pathophysiological effects of the COX isoenzymes (Hinz & Brune, 2002).

Three isoforms of the cyclo-oxygenase enzyme have been identified: COX-1, COX-2 and COX-3. Cyclo-oxygenase-1 is the housekeeping enzyme, responsible for regulating homeostatic function such as platelet function and gastric mucosa (Hinz & Brune,

2002), but may also be involved centrally in incisional pain (Zhu *et al.*, 2003). Cyclo-oxygenase-3 isoform, found in the central nervous system, behaves in a similar manner to COX-2, and both COX-2 and COX-3 have been identified in the central nervous system (Graham & Scott, 2005). Cyclo-oxygenase-2 is the adaptive COX, which is released peripherally during tissue injury and inflammation resulting in prostaglandin synthesis, and not found in normal non-injured tissue (Baldwin Lanier, 2003).

Prostaglandins released during inflammation and injuries sensitize nociceptors. Although it has been known that prostaglandins contribute towards peripheral sensitization, the mechanism has been only recently elucidated. Prostaglandins bind to prostaglandin receptors, found on primary afferent nerves, decreasing the threshold of voltage-gated sodium channels, tetradoxin-resistent sodium channels (Gold *et al.*, 1996). In addition to direct activation of prostaglandins receptors, indirect activation of the peripheral nociceptor occurs making the nociceptor more sensitive to other mediators such as bradykinin and heat. These actions of prostaglandins are thought to occur via inhibition of voltage-gated potassium currents via activation of protein kinase-A and altering the sensitivity of TRPV1 receptors (Gold *et al.*, 1996; Lopshire & Nicol, 1998; Burian & Geisslinger, 2005).

In summary, numerous chemical mediators, including cytokines, contribute to inflammatory hyperalgesia either via direct activation of receptors, such as bradykinin, or sensitization of nociceptors such as prostaglandin. Much research still needs to be conducted regarding the mechanisms behind cytokines sensitizing nociceptors. Although a clear pro-inflammatory cytokine cascade has been described (Poole *et al.*, 1999a), identifying whether the cytokine, such as IL-1β, sensitizes nociceptors itself, or via mediators released downstream of IL-1β has not been clearly investigated.

Inflammatory hyperalgesia is common in all tissues, and further research is required to fully understand the mechanisms underlying this common ailment in all tissues, especially muscle.

2. Muscle pain

2.1 Muscle hyperalgesia

Muscle pain is different to cutaneous pain in that muscle pain is a poorly localized, dull aching pain, while cutaneous pain is sharp and well localized (Mense, 1991). Muscle pain can be spontaneous, such as an active trigger point and myofascial pain. The pain associated with muscle injury can spread to adjacent muscles and other deep structures, with common patterns of referred pain (Mense, 1991; Simons *et al.*, 1999). Muscle hyperalgesia, usually mechanical hyperalgesia, is identified by applying a blunt pressure to the muscle (Treede *et al.*, 2002). The presence of mechanical hyperalgesia is used to diagnose muscle conditions such as fibromyalgia and myofascial pain (Nampiaparampil & Shmerling, 2004; Ferna´ndez-de-las-Pen˜as *et al.*, 2007).

Muscle nociceptors respond to numerous chemical stimuli and to high-intensity mechanical stimuli. Muscle nociceptors are high-threshold receptors predominantly located in the wall of arterioles and in the surrounding connective tissue (Mense, 1993; Mense, 2003). They are free nerve endings on group III (thinly myelinated) and group IV (unmyelinated) afferent fibres (Mense, 1993). Although the mechanism by which mechanical stimuli lead to signal transduction is not entirely clear, muscle nociceptors are slow-adapting, high-threshold mechanoreceptors that respond to noxious blunt pressure (Treede et al., 2002).

Various receptors are found on muscle nociceptors, including purinergic P_2X_3 receptors that respond to adenosine triphosphate (ATP) and vanilloid $TRPV_1$ receptors that respond to hydrogen ions and capsaicin (Sluka, 2002; Rukwied *et al.*, 2007). Acid-sensitive ion channels (ASIC) have been identified in primary muscle afferents and the associated dorsal horn cells, with ASIC3 channels mediating the development of secondary mechanical hyperalgesia after repeated injection of acidic saline (Sluka et al., 2003). Other substances such as serotonin, glutamate, bradykinin and cytokines induce muscle hyperalgesia, but which receptors are activated and where they are situated is still to be elucidated (Babenko *et al.*, 1999; Skyba *et al.*, 2002; Mense, 2003; Schafers *et al.*, 2003; Svensson *et al.*, 2003; Fernandez-De-Las-Penas *et al.*, 2007; Rukwied *et al.*, 2007; Svensson, 2007).

Some substances are able to produce more intense pain than are other substances. Bradykinin, for example, is able to induce muscle pain of a greater intensity than that induced by serotonin or substance P (Babenko et al., 1999). Although chemical mediators, such as bradykinin and serotonin, are able to induce muscle pain through peripheral sensitization, central sensitization develops very effectively in muscle pain, (Mense, 1993). It is not clear whether the time for central sensitization to develop differs between cutaneous tissue and muscle tissue but hyperalgesia in muscle tissue is usually associated with central sensitization. Allodynia is defined by the International Association for the Study of Pain as pain due to a stimulus which does not normally provoke pain. Excitation of primary muscle nociceptors results in an increased release of substance P and glutamate from the presynaptic terminal of primary muscle afferents. Glutamate and substance P act on the post-synaptic n-methyl d-aspartate (NMDA) and neurokinin-1 receptors, respectively, increasing the excitability of the

dorsal horn neurones (Mense, 2003), and so produce neuronal changes leading to central sensitization and symptoms such as referred pain.

Referred pain arising from muscle is thought to arise from an increased receptive field of the dorsal horn neurones that occurs with central sensitization (Mense, 1993). Therefore, adjacent neurones innervating agonist muscles and adjacent muscles may be stimulated with the result that pain is perceived in non-injured muscle or tissue. Afferent muscle fibres converge on second order neurones in the dorsal horn with numerous other inputs. It is possible that there is increased substance P and CGRP release, resulting in the presence of referred pain. However, this evidence only is present in referred pain with pancreatitis and not muscle pain (Wick *et al.*, 2006). Further studies are required to identify whether the same neurotransmitter release occurs with referred muscle pain.

The mechanisms underlying the quality and area of referred and local pain associated with muscle injury still need to be investigated. Various models in both humans and animals are used to understand the mechanisms of peripheral and central sensitization during muscle pain since no ideal comparative model to clinical muscle pain has arisen. Muscle hyperalgesia in animal models such as the cat or the rat, exhibits characteristics comparable to those found in human experiments and therefore may be a reliable model to investigate the mechanism of human muscle pain (Mense, 1993; Kehl & Fairbank, 2003).

2.2 Clinical muscle pain

It is possible that inflammation is an initiator of clinical muscle pain syndromes, including fibromyalgia, lower back pain and chronic whiplash (Koelbaek Johansen *et al.*, 1999; Wallace *et al.*, 2001; Blanco *et al.*, 2005; Murata *et al.*, 2005). Although the disease process progresses to central sensitization and pathological pain, it is possible that treating the initial inflammation, if it is present, may prevent the development of chronic pain and produce better clinical outcomes. Unfortunately, current treatment for inflammation is ineffective for muscle inflammation and therefore novel treatments targeting the mechanisms of muscle pain should be sought. Although the most frequent treatment given for muscle pain is non-steroidal anti-inflammatory drugs, which target prostaglandin synthesis, very little evidence exists that prostaglandins are produced during experimental and clinical muscle pain.

Fibromyalgia is a chronic diffuse musculoskeletal pain, defined by the American College of Rheumatology as a decreased pain threshold in a minimum of 11 out of a possible 18 tender muscle sites (Wolfe *et al.*, 1990). Although the exact mechanism of fibromyalgia is not clear, it is thought that there is central sensitization and changes in pain modulatory pathways (Treede et al., 2002; Petzke et al., 2003), since patients are sensitive not only to blunt pressure at the tender sites, but also have increased sensitivity to electrical stimulation, radiant and cutaneous heat, and cold stimulation (Petzke et al., 2003). Nonetheless, it does appear that peripheral mechanisms are not involved in the maintenance of the pain, although peripheral activation and possibly inflammatory mediators must be involved in the initiation of the pathology.

Researchers have found elevated circulating concentrations of IL-6 and IL-8 in patients with fibromyalgia compared to those of age and gender matched control subjects (Wallace et al., 2001). Although these cytokines are pleiotropic and fibromyalgia presents with multiple symptoms, such as fatigue and insomnia, further investigations into the possible role that cytokines may play in the musculoskeletal pain associated with fibromyalgia are warranted. Although muscle biopsy samples from patients diagnosed with fibromyalgia show no overt inflammatory response, there is necrosis and atrophy in the painful muscle with associated sarcolemmal disruption (Kalyan-Raman et al., 1984; Yunus et al., 1989). Also, these muscle biopsy samples were taken in patients with well established fibromyalgia and by the time the biopsies were taken, evidence of inflammation may have subsided.

Another common musculoskeletal complaint is myofascial pain which occurs in a regional site, as opposed to fibromyalgia which is generalised muscle pain. Myofascial pain can produce local spontaneous pain, referred pain, and pain elicited by mechanical pressure on trigger points, or points of tenderness (Simons *et al.*, 1999; Treede *et al.*, 2002). The trigger points are tight bands of muscle fibres that possess a local twitch response when pressure is applied and follow a characteristic referral pattern (Simons *et al.*, 1999; Rivner, 2001). Again, the mechanism of myofascial pain is not clear, but proposed mechanisms include muscle spindle hyperactivity, neuronal hyperexcitability at the motor-end plate and hypoperfusion of a localised area in the muscle leading to ischaemia (Rivner, 2001; Borg-Stein & Simons, 2002). To date, muscle damage and inflammation have not been identified, yet there is increased presence of inflammatory cytokines, IL-1β and TNF-α in active trigger points. In addition bradykinin, CGRP and substance P are elevated in active muscle trigger points, as identified by microdialysis but not seen in latent trigger points or normal muscle (Shah *et al.*, 2005).

Therefore, it is possible that muscle pain may emanate from inflammatory mediators released into the injured muscle.

Numerous other musculoskeletal diseases, such as rheumatoid arthritis, tension-headaches and low back pain, include a muscle pain component. The mechanisms of such complex musculoskeletal pains range from abnormal autoimmune responses to abnormal mechanical loading, leading to injury. Although part of the mechanism behind these diseases is clear, the mechanism behind the muscle pain is not. Indeed, unless we can understand the mechanisms behind experimental muscle pain, it is unlikely we will be able to identify the mechanisms behind the muscular component of more complex-mediated musculoskeletal pain.

In summary, muscle pain is different to that of cutaneous pain. Experimental models of clinical muscle pain, such as exercise-induced muscle pain and pain generated by carrageenan injected into the muscle are useful to investigate the mechanisms of inflammatory muscle pain. Much is still unknown regarding the mechanisms of muscle pain, but it is likely that inflammatory mediators, such as cytokines, initiate the muscle pain, and that central sensitization occurs quickly and maintains the muscle pain.

2.3 Experimental models of muscle pain

Various models of muscle pain have been employed to investigate the mechanism of muscle pain, including exercise-induced muscle pain, muscle ischaemia, intramuscular injection of hypertonic saline, and injection of inflammatory agents into the muscle of animals. In human and rat experimental models, muscle pain is commonly induced by

an injection of hypertonic saline or other algogenic agents, such as bradykinin, serotonin, substance P, glutamate, NGF (Babenko *et al.*, 1999; Svensson *et al.*, 2003).

Intramuscular hypertonic saline injection produces pain that mimics clinical muscle pain and is short-lasting if injected once into subjects, but longer-lasting if injected repeatedly into rats, or infused chronically into humans (Graven-Nielsen & Mense, 2001; Sluka *et al.*, 2001). Intramuscular hypertonic saline injection was first used in 1938 by Kellgren to produce a short-acting muscle pain, where the intensity of pain was dependent on the volume of saline injected (Kellgren, 1938; Capra & Ro, 2004). Pain induced by injection of intramuscular hypertonic saline mimics clinical pain in its quality, and leads to the development of referred pain. However, injecting hypertonic saline does not allow for the investigation of peripheral mechanisms underlying muscle pain since the pain induced by 5% hypertonic saline exceeds pain induced by bradykinin, serotonin and substance P, all of which are known to be released during inflammation (Babenko *et al.*, 1999). Hypertonic saline may directly activate nociceptors by rapidly changing the concentration of sodium or potassium in the muscle following injection (Graven-Nielsen *et al.*, 1997a), which is unlikely to be the only change, or indeed even a primary event, that occurs in clinical muscle pain. Five-percent hypertonic saline is thought to activate muscle nociceptors synapsing on wide dynamic range neurones within the dorsal horn, which may account for the referred pain (Capra & Ro, 2004). Therefore, intramuscular hypertonic saline injection is an ideal technique for activating muscle nociceptors and associated pathways, but will not mimic mechanisms of clinical inflammatory muscle pain.

Muscle ischaemia is another technique used to induce the qualities of muscle pain with good effect (Graven-Nielsen & Arendt-Nielsen, 2003). The mechanisms of muscle

pain following ischaemia are complex and involve numerous tissues including skin, fascia and muscle. The mechanism of this muscle pain is not fully understood. A reduction in blood supply alone does not evoke pain, but a decrease in blood supply in conjunction with muscle contractions evokes pain rapidly (Mense, 1993). It is thought that the nociceptor activation is as a result of changes in pH (Graven-Nielsen *et al.*, 1997b; Graven-Nielsen *et al.*, 2003; Hoheisel *et al.*, 2004), and a release of bradykinin and adenosine (Graven-Nielsen & Arendt-Nielsen, 2003; Segerdahl & Karelov, 2004). These chemical mediators would then activate ASIC receptors on muscle nociceptors, comparable to those found to be activated by intramuscular injection of hypertonic saline (Sluka *et al.*, 2003; Sluka *et al.*, 2007).

Another experimental pain that may provide further insight into the mechanism underlying clinical muscle pain is exercise-induced pain. Although pain can be experienced immediately after exercise, the most researched exercise-induced muscle pain is delayed-onset muscle soreness (DOMS). The mechanism of DOMS is not entirely clear. However, there do seem to be some similarities in the pathogenesis of DOMS compared to that of clinical muscle pain, especially the inflammatory component and the associated decrease in muscle function.

A direct method for studying inflammatory muscle pain is through intramuscular injection of inflammatory agents. Carrageenan and CFA, in particular, have been used in rats and cats to induce inflammatory muscle pain (Berberich *et al.*, 1988; Radhakrishnan *et al.*, 2003). These models mimic myositis and allow us to investigate the mechanism and treatment of inflammatory-induced muscle pain. In addition, these inflammatory agents are used regularly to investigate the mechanisms of cutaneous

inflammatory pain, allowing comparisons between cutaneous and muscle inflammatory hyperalgesia to be made.

Therefore, the most appropriate model to induce inflammatory muscle pain is injecting inflammatory agents into muscle. However, it is unethical to inject inflammatory agents such as carrageenan and CFA into humans. Therefore, an alternative needs to be sought for experimental inflammatory muscle pain in humans. Since DOMS may have an inflammatory component, it is currently the best experimental model to study inflammatory muscle pain in humans. Both injecting inflammatory agents into animals and DOMS will be discussed in more detail below.

2.2.1 Delayed-onset muscle soreness

Eliciting delayed-onset muscle soreness (DOMS) is an ideal method of inducing muscle pain in a controlled, experimental fashion. However, DOMS, is not only induced in the laboratory, but also afflicts people who participate in unaccustomed exercise. The muscle pain is experienced as a dull ache with associated tenderness and stiffness occurring 24-48h after unaccustomed and usually eccentric exercise, and resolves within 4-7 days after exercise (MacIntyre *et al.*, 1995). The muscle pain or soreness experienced after exercise occurs on muscle contraction and on passive stretch of the muscle.

Delayed-onset muscle soreness also can be induced in an animal model using forced lengthening contractions, simulating eccentric muscle contractions. The mechanical hyperalgesia that develops after forced lengthening contractions peaks 48h after the completion of muscle contractions, with complete resolution by four days after the

muscle contractions (Taguchi *et al.*, 2005). Thus, the hyperalgesia in this model mimics the temporal pattern of muscle hyperalgesia found with DOMS in humans.

There are three theories proposed to explain the mechanism of DOMS: myofibrillar damage, enzyme efflux, and inflammation (Cheung *et al.*, 2003). Eccentric exercise produces muscle damage with an associated loss of dynamic and static strength (Willoughby et al., 2003). Eccentric exercise produces a higher force per active cross-sectional area of muscle fibres compared to concentric exercise (Armstrong et al., 1983). The high mechanical load results in myofibrillar damage, releasing calcium and adenosine triphosphate (ATP) amongst other substances from the myotubes. During muscle injury resulting from eccentric exercise, z-lines are damaged producing z-line streaming, and a disruption of sarcomeric organization (Friden & Lieber, 2001). The z-lines seem to be more vulnerable to injury, than other parts of the muscle fibre, since type II muscle fibres have the narrowest and weakest z-lines and are more susceptible to injury than are type I muscle fibres (Cheung *et al.*, 2003).

Muscle injury also results in proteolysis of contractile and non-contractile proteins (Baldwin Lanier, 2003). Disruption of the sarcomere leads to a loss of calcium homeostasis. An increased uptake of calcium into the cell activates calcium-dependent proteases, such as calpains which cleave proteins and cause further muscular disruption (Tidball, 1995). The increase in intracellular calcium activates lysosomal and non-lysosomal enzymes such as glucose-6 phosphate dehydrogenase, creatine kinase and lactate dehydrogenase, all of which are elevated 36-48h after eccentric exercise (Armstrong et al., 1983). These enzymes may be good markers of the extent of muscle injury. For example, creatine kinase (CK) is an intramuscular enzyme responsible for maintaining adequate ATP levels during muscle contraction and is released during

muscle damage (Armstrong, 1984). However, CK plasma concentrations only peak 48-72h after the exercise, which does not correlate with the peak in muscle soreness occurring 24-48h after exercise. Therefore, although CK may give an indication of the extent of muscle injury, CK release does not correlate with the extent or duration of pain experienced (Friden & Lieber, 2001).

Substances released during myofibrillar damage and other injuries resulting in muscle inflammation spark the initiation of an inflammatory soup. Numerous studies have shown that the initial inflammatory response is necessary for the breakdown of the muscle tissue, followed, by the reparative phase necessary for hypertrophy of the muscle. During muscle injury and inflammation, acute phase proteins (C-reactive protein, ferritin, heat shock protein 70) may be released, together with neutrophil mobilisation and activation (Cannon et al., 1991). Neutrophils are rapid invaders during inflammation (Pedersen & Hoffman-Goetz, 2000), and are involved in phagocytosis of cellular debris. Neutrophils possibly contribute to the injury after eccentric contractions of the skeletal muscle by producing adhesion molecules and reactive oxygen species (Pizza *et al.*, 2005b). Neutrophil infiltration is elevated significantly 6h to 3 days after eccentric muscle contractions, while macrophages are elevated only 3 days after eccentric muscle contractions (Pizza *et al.*, 2005b). This increase in both neutrophil and macrophage count corresponded with the amount of myofibre damage, but has not been correlated to the extent of muscle pain experienced. In contrast, no neutrophil infiltration was found following forced lengthening contractions in rats (Lapointe *et al.*, 2002). After forced lengthening contractions in mice (Pizza *et al.*, 2005b), downhill running in rats (Tsivitse *et al.*, 2003), and after eccentric exercise in humans (Pizza et al., 2005a), there is early migration of neutrophils to the exercised

muscle, suggestive of a degenerative state following muscle injury, regardless of species investigated.

Since inflammation is thought to occur during DOMS, signs and symptoms of inflammation, such as swelling, pain, increased temperature and loss of function, should be apparent. Numerous studies have shown that swelling, loss of function and pain do exist in DOMS (for review see Smith 1991). However, increased muscle temperature is difficult to measure in human studies and there is a lack of biochemical evidence of inflammation, although the techniques currently available may not be sensitive enough to identify the presence of mild muscle inflammation. Even if inflammation is present, it is still not clear which of the mediators or mechanisms contribute to the soreness associated with the muscle damage.

If the muscle pain during DOMS was attributed to inflammation, prostaglandins (PG) would be produced and the time of increased production would correlate to the muscle pain. An increase in muscle PGE_2 has been detected 24h after eccentric exercise, as measured by microdialysis, but only during a muscle contraction and not at rest (Tegeder *et al.*, 2002). In another study, both prostaglandin F_2 and PGE_2 were increased in non-contracting skeletal muscle after eccentric exercise (Trappe et al., 2001). If PGE_2 indeed is released from skeletal muscle after muscle-damaging eccentric exercise and contributed to the pain, then inhibiting prostaglandin release should be beneficial in treating DOMS.

As mentioned previously, cyclo-oxygenase (COX) is one of the key enzymes that convert arachidonic acid into prostaglandins, and inhibiting COX is the most common treatment for decreasing inflammation and inflammatory pain. Numerous studies have

been conducted to investigate the efficacy of non-selective COX-inhibitors on DOMS (see Table 5 for a summary). However, most of these agents were ineffective in reducing pain associated with DOMS. Ibuprofen, for example, attenuated PGF_2 concentrations in muscle, but it did not attenuate the muscle soreness or the PGE_2 concentration (Trappe et al., 2001). Another non-selective COX-inhibitor, diclofenac, administered to rats after forced-lengthening contractions, produced an impairment to the repair process (Lapointe et al., 2002), without alleviating the hyperalgesia. COX-2 gene expression in muscle peaks five days after a freeze injury, so it appears that COX-2 may be important for muscle regeneration following traumatic freeze injury (Bondesen et al., 2004), and blocking prostaglandin synthesis may be detrimental to the repair process after injury, without alleviating the muscle soreness.

An effective way to alleviate muscle pain experienced after eccentric exercise was through prophylactic COX-inhibitor administration (Hasson *et al.*, 1993; O'Grady *et al.*, 2000). It is possible that administration of COX-inhibitors is effective in blocking the production of DOMS, but once inflammation has been established and other mediators have been released, and central sensitization has occurred, the administration of COX-inhibitors is ineffective in reversing DOMS. Further research is required to identify the mechanism underlying the role of prostaglandins in muscle pain, in particular DOMS.

Administration of a non-specific COX-inhibitor after exercise, regardless of the dose or drug administered, appears to be ineffective, or of limited effectiveness, in treating DOMS. A criticism of studies using COX-inhibitors in treating DOMS may be that the dose of the drug was inappropriate. However, high doses of aspirin and ibuprofen also have been used with little success (Donnelly *et al.*, 1990; Barlas *et al.*, 2000a). These

results show that either the wrong cyclo-oxygenase enzyme has been targeted or that a complex mechanism mediates DOMS. No studies have investigated the effect of a COX-2 inhibitor on DOMS, and therefore, the ineffectiveness of COX-inhibitors may be that the wrong isoform is being targeted rather than inflammation not playing a role in DOMS.

Not only have antihyperalgesics, such as COX-2 inhibitors not been investigated in attenuating DOMS, but also little work has been done using analgesics. Central sensitization occurs more rapidly in muscle pain (Wall & Woolf, 1984) and therefore may contribute to the muscle pain in DOMS. Morphine administered after inducing DOMS in humans, and after forced lengthening contractions in rats attenuated the hyperalgesia in the muscle (Tegeder *et al.*, 2003). However, codeine administration after eccentric exercise in humans did not attenuate DOMS (Barlas *et al.*, 2000a). Further work is required investigating the role of central-acting analgesics in DOMS to identify which receptors and pathways are important in DOMS.

Table 4. Summary of research on the use of pharmacological treatment of delayed-onset muscle soreness in human subjects

Exercise	Drug intervention	Muscle soreness	Class of drug	Reference
Eccentric elbow flexion (n=12 per group)	Codeine (60mg) Paracetamol (1000mg) Aspirin (900mg)	No change No change No change	Opioid COX-3 inhibitor COX-1 & 2 inhibitor	Barlas et al (2000a)
Isometric concentric and eccentric quadriceps contractions (n=32)	Transdermal 10% ketoprofen	Change in VAS from 55mm (placebo) to 30mm (ketoprofen)	COX-1 & 2 inhibitor	Cannavino et al (2003)
Downhill running (n=16 per group)	Ibuprofen (1200mg before and 600mg QID)	No change	COX-1 & 2 inhibitor	Donnelly et al (1990)
Downhill running (n=20)	Diclofenac 50mg TDS)	No change	COX-1 & 2 inhibitor	Donnelly et al (1988)
Bench stepping (n= 5 per group)	Ibuprofen (400mg)	Prophylactic ibuprofen decreased muscle soreness 24h and 48h after exercise Therapeutic ibuprofen decreased muscle soreness 48h after exercise	COX-1 & 2 inhibitor	Hasson et al (1993)
Eccentric leg extensions (n=20)	Naproxen (500mg)	VAS decreased from 53mm to 39mm	COX-1&2 inhibitor	Lecomte et al (1998)

Exercise	Drug intervention	Muscle soreness	Class of drug	Reference
Bench stepping (n=54)	Diclofenac (75mg BD)	Decrease in pain rating with prophylactic treatment	COX-1&2 inhibitor	O'Grady et al (2000)
Eccentric arm curls (n=10)	Ibuprofen (800mg 5d before and after, TDS)	No change	COX-1&2 inhibitor	Pizza et al (1999)
Eccentric elbow flexors (n=48)	Ketoprofen (25mg, 100mg)	19% decrease in VAS after 100mg ketoprofen	COX-1&2 inhibitor	Sayers et al (2001)
Eccentric leg curls (n=19)	Ibuprofen (400mg) TDS	VAS decreased from 55mm to 38mm	COX-1&2 inhibitor	Tokmakidis et al (2003)
Eccentric knee extension (n=24)	Ibuprofen (400mg) TDS Paracetamol (1500mg) TDS	No change	COX-1&2 inhibitor COX-3 inhibitor	Trappe et al (2001, 2002)
Concentric and eccentric calf exercise (n=10)	Morphine-6-β-glucuronide (M6G, 500ng.ml^{-1} of plasma) Morphine (100ng.ml^{-1} of plasma)	A decrease in VAS by 37% with M6G and 71% decrease with morphine	Opioid	Tegeder et al (2003)
Eccentric elbow flexor exercise (n=15 per group))	Flurbiprofen (100mg TDS) started pre-exercise	No change	COX 1&2 inhibitor	Howell et all (1998a)
Eccentric elbow flexor exercise (n=20 per group)	Ibuprofen (400mg QID, 800mg QID) started pre-exercise	No change	COX 1&2 inhibitor	Howell et al (1998b)

COX- cyclo-oxygenase, BD, administered twice daily, QID, administered four times per day, TDS- administered three times per day, VAS-visual analogue scale

2.2.2 Injection of carrageenan and complete Freund's adjuvant

Carrageenan is a plant-based polysaccharide that induces neurogenic inflammation regardless of the site of administration. When injected into the muscle, carrageenan excites group III and group IV muscle afferent fibres, lowering the threshold to mechanical stimuli (Berberich et al., 1988). Carrageenan injected into the forelimb muscle of rats reduces the grip strength of the forelimb (Kehl *et al.*, 2000) and increases the sensitivity to mechanical stimuli (Diehl et al., 1988; Radhakrishnan et al., 2003). Both decreased muscle strength and mechanical hyperalgesia, present in the carrageenan model, also are present in clinical muscle pain conditions.

An intramuscular injection of carrageenan evokes leukocyte infiltration into the perivascular space 2h after administration (Diehl et al., 1988). The types of leukocytes, were not identified, in the study, but were concentrated around the capillaries and small arterioles, where nociceptors are present (Mense, 1993). Treatment of carrageenan-induced muscle inflammation with acetylsalicylic acid, a non-selective COX-inhibitor, showed that some muscle nociceptors still were sensitized after the COX-inhibitor, and therefore, likely to be sensitized by mediators other than prostaglandins (Diehl et al., 1988). These results confirm that various receptors are present on muscle nociceptors, with only a few responsive to prostaglandins.

Complete Freund's adjuvant (CFA), another inflammatory agent, produces a longer-lasting hyperalgesia compared to that of carrageenan, regardless of the tissue injected. When injected into the masseter muscle, CFA produced mechanical hyperalgesia and allodynia within 30min of injection, and TNF-α, IL-1β, IL-6 and CINC-2 and CINC-3 tissue concentrations (CINC-1 was not measured) were elevated 5h after injection (Watanabe et

al., 2005). Because the cytokines were detected only long after hyperalgesia and allodynia appeared, it is possible that the prominent pro-inflammatory cytokines measured may not have initiated the inflammatory muscle hyperalgesia, but may have sustained the hyperalgesia, while other mediators such as bradykinin, hydrogen ions and ATP may initiate the hyperalgesia (Babenko et al., 1999; Babenko et al., 2000). Clinical muscle pains also are characterized by sustained hyperalgesia and production of central sensitization but the pro-inflammatory cytokine release in the muscle has not been measured.

3. The role of cytokines in muscle

3.1 Cytokines in muscle

If inflammation is the key initiator behind muscle pain, it is necessary that we understand the role that key mediators, such as pro-inflammatory cytokines, play in muscle homeostasis. Cytokines have numerous functions other than contributing towards hyperalgesia. For instance, TNF-α may play a direct role in regulating muscle function and homeostasis, but also may induce insulin resistance and suppress protein synthesis (Zhang *et al.*, 2000). TNF-α, expressed by myocytes, is able to downregulate the expression of the glucose transporter GLUT-4, therefore increasing insulin resistance and increasing lipolysis (Saghizadeh et al., 1996). The proposed hypothesis is that TNF-α release is suppressed in normal muscle. However, during certain diseases such as diabetes and rheumatoid arthritis, TNF-α suppression is downregulated such that TNF-α is able to exert catabolic effects on muscle (Petersen & Pedersen, 2005).

In skeletal muscle, TNF-α and IL-1β stimulate the synthesis of nitric oxide, a potent vasodilator (Zhang *et al.*, 2000). The expression of IL-1β, mainly observed in the

postsynaptic domain of the motor endplate, may be involved in proteolysis, since muscle exposed to IL-1β have less myogenic cells (Authier *et al.*, 1999).

Human myoblasts, in vitro, constitutively express IL-6. However, IL-1β and TNF-α release in muscle requires stimulation by TNF-α itself or interferon (De Rossi *et al.*, 2000). Most research into the role of IL-6 in muscle has concerned the role of IL-6 during exercise. Although IL-6 is released by numerous other cells, during exercise the predominant source of IL-6 in plasma is muscle (Febbraio & Pedersen, 2002). An important function of IL-6 in muscle is to regulate glucose homeostasis, either by affecting glucose production by the liver, or regulating the uptake of glucose by the muscle (Febbraio & Pedersen, 2002).

3.2 Cytokines during exercise

Cytokines are released during muscle contraction regardless of the type, intensity or duration of the exercise. However, the type, intensity and duration of the exercise determine the degree and, to some extent, which cytokines are released. TNF-α has been detected after exhaustive endurance exercise. After a marathon run of 42.5km, plasma TNF-α was elevated. However, there was no increase in TNF-α gene expression in muscle (Ostrowski et al., 1998b). Another study showed no increase in circulating TNF-α after marathon running, but the ambient temperature during that marathon was about 7 ºC (Suzuki et al., 2000). It is likely that the increases in circulating TNF-α seen after marathon running arise from the combination of heat stress and exhaustive exercise leading to increased gut permeability, rather than as a result of TNF-α released from contracting muscle or leukocytes.

Since TNF-α impairs glucose uptake by the cell, it would be counter-adaptive to express or release TNF-α from muscle during exercise. Also, TNF-α does not appear in serum for up to 5 days after eccentric exercise or exercise that induces muscle damage (Toft *et al.*, 2002). Although myocytes, when stimulated in vitro, are able to release and express TNF-α, TNF-α is not expressed nor released in normal healthy muscle at rest or after exercise (Saghizadeh *et al.*, 1996; Steensberg *et al.*, 2002). It is possible though, that the method of measuring circulating TNF-α, in some of the studies, was not sensitive enough to detect small concentrations of TNF-α after eccentric exercise (Smith et al., 2000). Peterson and Pederson (2005) propose that a negative feedback mechanism, possibly IL-10 or IL-1ra, occurs in muscle to prevent the release of TNF-α. If this hypothesis is true, it would explain why TNF-α is not elevated after eccentric exercise but is increased in other autoimmune muscle diseases. One of the proposed mediators contributing to the negative-feedback on TNF-α is IL-6.

Interleukin-6, released downstream of TNF-α during inflammation, is released from muscle into the plasma, during and after isometric, concentric and eccentric contraction (Febbraio & Pedersen, 2002). Increases in plasma IL-6 concentrations are dependent on the duration and intensity of the exercise (Ostrowski et al., 1998a). IL-6 plays an important role in substrate utilization. It was initially thought that the release of IL-6 was related to the extent of muscle damage. However, the release of IL-6 is independent of muscle damage since there is no correlation between the creatine kinase concentrations and the IL-6 plasma concentrations (Ostrowski et al., 1998a).

Although TNF-α stimulates IL-6 production from monocytes and macrophages during fever and inflammation, monocytes do not contribute to the plasma IL-6 or TNF-α concentrations during prolonged exercise, but rather the circulating IL-6 emanates from

contracting muscle cells (Steensberg et al., 2000; Starkie et al., 2001). IL-6 most likely plays an endocrine function since it is released into the circulation, targeting the liver, leading to increased substrate availablitity. Also, IL-6 release and expression by glycogen-depleted muscle is significantly higher than that of glycogen-replete muscle (Steensberg *et al.*, 2001; Steensberg *et al.*, 2002), and it may act on the GLUT-1 transporter to increase glucose uptake into the cell (Steensberg et al., 2001). Therefore, unlike in fever and inflammation, there does not appear to be a clear link between TNF-α and IL-6 in exercising muscle, unless IL-6 acts as a negative feedback to TNF-α.

Interleukin-8, like IL-6, plays a role in substrate utilization in skeletal muscle during exercise. IL-8 is produced by monocytes and macrophages and other tissues including skeletal muscle (Akerstrom et al., 2005). Plasma IL-8 concentration, after marathon running, increased by 2.5 times over that of pre-marathon values (Suzuki et al., 2000). However, in other studies there was no change in plasma IL-8 after a 3h treadmill run (Nieman *et al.*, 2003). IL-8 expression in skeletal muscle is increased after exhaustive exercise where both concentric and eccentric muscle contraction are done, but an increase in muscle IL-8 also occurs with concentric exercise alone (Akerstrom et al., 2005). Therefore, IL-8 expression and release in muscle is induced by concentric muscle contraction and is influenced by glycogen availability (Chan *et al.*, 2004).

One cytokine not involved in glucose metabolism but more likely to be related to muscle injury is IL-1β (Chan *et al.*, 2004). It is produced by unstimulated myogenic cells and induces disorganization of the actin cytoskeleton in vitro (Authier *et al.*, 1999). IL-1β appears to be released most prominently in monocytes and myocytes after eccentric exercise. IL-1β is expressed in post-synaptic domain of motor-end plates in normal muscle and in myofibres showing myofibrillar breakdown in diseased muscle (Authier *et al.*, 1999).

Lipopolysaccharide-stimulated mononuclear cells taken from subjects 24h after a downhill run had increased concentration of IL-1β and TNF-α (Cannon et al., 1991). This increase in IL-1β and TNF-α correlated to an increase in protein breakdown as evident by urinary 3-methylhistadine (Cannon et al., 1991). Not only was IL-1β release increased in monocytes but muscle biopsy samples taken from subjects completing 45min of downhill running showed an increase in IL-1β immunostaining around the capillaries and along plasma membranes 45min after the exercise and 5 days after the exercise. No IL-1β staining was detected within the myocytes and no TNF-α was detected within or around the muscle at either time interval (Cannon *et al.*, 1989; Fielding *et al.*, 1993).

In another study where muscle biopsies were taken in 23 healthy male subjects after leg exercises that induced voluntary muscle failure, IL-1β was increased in some subjects more than other subjects (Dennis *et al.*, 2004). There was a correlation between the IL-1 genotype and the amount of IL-1β expressed. If IL-1β does contribute to muscle pain, this finding may explain why certain people experience more muscle pain than others, even after taking the muscle strength and fitness into account. Although IL-1β concentration in muscle is increased after eccentric exercise, immediately after and 24h after a 42km marathon run, there was no significant change in myocyte IL-1β expression (Ostrowski et al., 1998b). Therefore, the IL-1β may be synthesized by residents cells other than myocytes. It appears that IL-1β may be released in muscle only during muscle damage and that it has a local function since, regardless of the type of exercise, circulating IL-1β does not appear to be elevated after any type of exercise, regardless of intensity or duration (Fielding *et al.*, 1993; Ostrowski *et al.*, 1998b; Suzuki *et al.*, 2000). Therefore, the main function of IL-1β in muscle is proteolysis and it may be a key mediator in muscle pain, but no studies have correlated muscle IL-1β concentration and the subject's perceived pain.

Since some cytokines are pleiotropic, they may produce different effects depending on the type of muscle contraction, intensity and severity of the exercise. However, it does appear that IL-6 and IL-8 play a more prominent role in muscle metabolism, while IL-1β is involved in muscle degeneration. TNF-α release is inhibited in most instances including after both concentric and eccentric exercise and possibly even endurance exhaustive exercise. TNF-α is likely to be increased only in instances where homeostasis is disturbed or marked injury and inflammation occurs.

3.3 Role of cytokines in muscle pain

A primary problem in identifying the pro-inflammatory cytokines contributing to muscle pain, as mentioned in section 3.2, is that muscle releases and produces some of the same cytokines during normal muscle contraction. Since most muscle pains are associated with movement and muscle contraction, it is difficult to establish whether the pro-inflammatory cytokines released in the muscle and in the circulation contribute to normal muscle homeostasis or if they indeed contribute to the pain experienced. In order to investigate the contribution of pro-inflammatory cytokines to muscle pain, it is best then, to use experimental models that do not incorporate exercise, or to compare the concentration of cytokines in clinical disease states to those of normal healthy controls. In this section I have discussed the literature on the role that cytokines play in muscle pain, independent of exercise.

Exogenous TNF-α injected into the gastrocnemius muscle of a rat produced a dose-dependent reduction in force grip strength and a decrease in pressure threshold on the muscle (Schafers et al., 2003). The mechanical hyperalgesia was produced 6h after injection and lasted until 28h after injection, and the intensity of hyperalgesia was comparable to that

found after formalin was injected into the gastrocnemius muscle. There was no development of secondary hyperalgesia, as measured by pressure and heat applied to the contralateral paw, implying a local effect (Schafers et al., 2003). It is likely that TNF-α stimulated the release of other inflammatory mediators such as prostaglandin E_2 and calcitonin-gene related peptide, which were found to be elevated in the muscle tissue (Schafers et al., 2003). Whether TNF-α stimulates the release of other cytokines was not investigated and would be worthy of further investigation. In a conflicting study, TNF-α intramuscular injection desensitized group IV afferents to pressure (Hoheisel et al., 2005). However, these measurements were taken within the first ten minutes of injection and it is possible that a different response may have been obtained 5h after injection when the hyperalgesia of the previous study was most apparent (Schafers et al., 2003).

Even though TNF-α injected into muscle may produce hyperalgesia, it is not clear whether TNF-α is endogenously produced during muscle injury. Five hours after a freeze injury to rat muscle, another model used to induce muscle injury, TNF-α concentration in the muscle increases, peaking 24h after injury, with TNF-α concentrated in the invading inflammatory cells (Warren et al., 2002). Therefore, TNF-α may contribute towards muscle pain, but a freeze injury is a severe muscle injury and the release of TNF-α may be dependent on the extent of the muscle injury.

Unlike TNF-α, IL-6 injected into muscle resulted in an increased response in two out of seven low-threshold mechanoreceptors, but not to high-threshold nociceptors (Hoheisel et al., 2005). Also, IL-6 serum concentration measured in patients with fibromyalgia was not different compared to that of age and gender matched controls (Wallace *et al.*, 2001). Therefore, IL-6 may not play a role in clinical muscle pain, although only a handful of studies have looked at the role of IL-6 in muscle pain.

In the same group of patients diagnosed with fibromyalgia, in which IL-6 was not elevated, there was an elevation in serum IL-8 (Wallace et al., 2001). Although IL-8 plays a role in muscle homeostasis, it also is a chemokine. Since fibromyalgia includes other symptoms such as insomnia and fatigue, IL-8 may play a role in fibromyalgia other than that of the associated muscle pain. Little work has been done investigating the role of IL-8 in muscle pain and therefore may be worthy of future studies.

Few studies have investigated the role that IL-1β may play in muscle pain. Muscle biopsies, from patients with various myopathies, showed an increased expression for both TNF-α and IL-β, with a more intense staining for TNF-α around muscle spindles. Also, IL-1β was found in the vascular walls and in some inflammatory cells located in the endomysium and perimysium (Mackiewicz *et al.*, 2003). However, circulating IL-1β was not increased in patients with fibromyalgia compared to that of age and gender matched controls. As mentioned in section 3.2, IL-1β is increased in muscle in exercise-induced muscle injury, without an increase in plasma IL-1β. Therefore, if IL-1β was to play a role in clinical muscle pain, it is likely that there would be no increase in circulating IL-1β, but rather an increase in the muscle itself, or in the central nervous system.

Although some studies have begun to investigate the role that cytokines play in animal models of muscle pain and in clinical muscle pain conditions, the field of research requires substantial progress before a full understanding and optimal treatment regimens are obtained.

Thesis aims

Our understanding of inflammatory muscle pain has been based mainly either on studies using cutaneous pain models in animals or on human models where exercise induces pain. Although the mechanisms of inflammatory muscle pain are thought to be similar to those of cutaneous inflammatory pain, it is not known if the profile of mediator release indeed is similar. In Chapter 2 I wished to identify whether differences were apparent in cytokine profiles and hyperalgesia between inflamed hind paw tissue and another inflamed cutaneous tissue such as the rat tail. Many studies have investigated the pro-inflammatory cytokine release during inflammation and compared the concentrations measured to that of the behavioural response of the rats, yet few have investigated the temporal profile of the four prominent pro-inflammatory cytokines concurrently, and compared the cytokine response to both the behavioural response of the rats and to histological changes. Therefore, the aim of Chapter 2 was to fully characterize the inflammatory response to a carrageenan injection into cutaneous tissue. I found that the inflammatory process in the rat tail and the associated hyperalgesia were comparable to that of the hind paw. Because injection into the tail, unlike that in the paw, does not impair animal movement, carrageenan injection into the rat tail may be a useful model to investigate the relationship between inflammatory hyperalgesia and voluntary activity.

Once I had investigated the pro-inflammatory cytokine cascade in a unique cutaneous tissue of the rat tail, and found comparable cytokine and behavioural changes to those found in the hind paw of rats, I wanted to identify if the responses in muscle tissue were different to that in cutaneous tissue. In Chapter 3 I set out to identify the pro-inflammatory cytokine concentrations, in both the muscle tissue and the plasma, after carrageenan injection into the gastrocnemius muscle of the rat. I also compared the concentrations of

cytokines to the behavioural response of the rats after applying noxious mechanical pressure to the inflamed muscle. In order to better understand the mechanism behind clinical inflammatory muscle pain, I needed to identify a model of inflammatory muscle pain that is independent of exercise. Since painless muscle contraction itself causes the local release of cytokines, a model of inflammatory muscle pain that releases cytokines in resting muscles may provide a better method to elucidate the cytokines that contribute to the hyperalgesia. In Chapter 3 I showed that cytokines are released after carrageenan injection into the muscle of rats and found that the cytokine release and hyperalgesia followed a different temporal profile to that found in cutaneous carrageenan-induced inflammation.

Although carrageenan-induced muscle inflammation may provide answers to the mechanisms underlying muscle inflammation in animal models, we do not have a comparable model in humans and cannot inject carrageenan into humans. Possibly the best human model of inflammatory muscle hyperalgesia, where the variables can be controlled in a laboratory setting, is exercise-induced muscle pain or DOMS. Although our understanding of how pain arises from exercise-induced muscle damage is incomplete, the damage is thought to have an inflammatory component. If so, blocking some part in the inflammatory process should attenuate the muscle pain. At the time that I conducted this research, drugs were not available to block the release of any of the pro-inflammatory substances measured thus far in this thesis. Interleukin-1β and IL-6 both stimulate the release of cyclo-oxygenase, increasing the production of prostaglandins, so it also may be possible to attenuate muscle pain by blocking prostaglandin synthesis. Many studies have attempted to attenuate DOMS using non-selective COX-inhibitors, but the results are contradictory and tentative. Therefore, the aim of Chapter 4 was to inhibit the activity of COX-2, the COX isoform released during inflammation, after inducing delayed-onset

muscle soreness by downhill running of human volunteers. Although inflammation is thought to play a role in DOMS by leading to peripheral sensitization of the muscle nociceptors, it also is thought that central sensitization occurs. If so, a central-acting analgesic may alleviate the pain experienced in DOMS. Tramadol binds to mu-opioid receptors. It also prevents the reuptake of serotonin and adrenaline within the central nervous system, therefore stimulating the descending inhibitory pathway involved in analgesia. The second aim of Chapter 4 was to identify whether a central-acting analgesic was able to attenuate DOMS.

CHAPTER 2

Histological, cytokine and behavioural response characterisation of carrageenan-induced hyperalgesia in the rat tail.

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BEHAVIOURAL, HISTOLOGICAL AND CYTOKINE RESPONSES DURING HYPERALGESIA INDUCED BY CARRAGEENAN INJECTION IN THE RAT TAIL

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ABSTRACT

We produced experimental inflammatory hyperalgesia by injecting carrageenan into the tail of Sprague-Dawley rats. We compared the rats' voluntary running wheel activity following carrageenan injection into the tail to that after carrageenan injection into the hind paw, the conventional site of inflammation, to identify whether the site of inflammatory-induced hyperalgesia altered voluntary activity. We also measured voluntary running before and after injection of carrageenan or saline into the tail or hind paw, and in separate groups of rats we measured the nociceptive response and the associated pro-inflammatory cytokine profiles following a carrageenan injection into the tail. Female rats were injected intradermally with either 2mg carrageenan or saline into the dorsal surface of the tail. Withdrawal responses to noxious heat (49°C water), and punctate mechanical (electronic anaesthesiometer) challenges were recorded in 12 rats for three days before and 1h to 48h after injection. In a separate group of rats, interleukin (IL)-1β, IL-6, tumour necrosis factor-alpha (TNF-α) and cytokine-induced neutrophil chemoattractant (CINC-1) concentrations were measured in plasma and tail tissue samples taken at the site of injection, 3h, 6h and 24h after injections. Voluntary wheel running was reduced significantly following carrageenan injection into the hind paw compared to that after saline injection into the hind paw. Carrageenan injection into the tail did not result in significant reduction in wheel running compared to that after saline injection into the tail. Both thermal and mechanical hyperalgesia were present after carrageenan injection into the tail ($P<0.01$, ANOVA). The hyperalgesia at the site coincided with significant increases in TNF-α, IL-1β, IL-6 and CINC-1 tissue concentrations, peaking 6h after carrageenan injection ($P<0.01$, ANOVA). We conclude that carrageenan injection into the tail produces inflammatory hyperalgesia with underlying pro-inflammatory cytokine release, but does not affect voluntary running wheel activity in rats.

1. INTRODUCTION

Inflammatory pain often has been investigated experimentally by injecting noxious agents such as carrageenan, complete Freund's adjuvant and formalin into the hind paw of a rat (Vinegar *et al.*, 1987; Hargreaves *et al.*, 1988; Woolf *et al.*, 1997; Bianchi *et al.*, 2004) These studies have advanced substantially our understanding of the mechanisms of cutaneous inflammatory pain. However, pain in the cutaneous tissue of the hind paw affects the weight-bearing ability of the animals and disrupts their normal activities. Therefore, the hind paw model may not be suitable for use in studies that wish to investigate the effects of pain on exercise, or any other activities in rats.

Clinically, exercise regimens are prescribed routinely to patients with various chronic pains (Sculco *et al.*, 2001). However, there is no mechanistic evidence to support the notion that exercise positively alters pain perception. To investigate the therapeutic effects of exercise on pain it would be useful to identify an alternate site of inflammation, in a non-weight bearing site, that produces the same inflammatory response and behavioural response to that seen in the hind paw of the rat. Additionally, it is important to understand the interaction between activity and pain in nociceptive testing. Voluntary running, at least in rats, is known to alter the antinociceptive effects of opioids (Kanarek *et al.*, 1998; D'Anci *et al.*, 2000; Mathes & Kanarek, 2006). Rats engage in high noctural activity, even in small cages (Ottenweller *et al.*, 1998), so patterns of activity may be important in some nociceptive tests.

The tail of the rat, like the paw, is accessible to noxious thermal and mechanical challenges with the animal loosely restrained but not handled (Vidulich & Mitchell, 2000). The tail has a thick keratinised epithelium and is the site used conventionally for many other nociceptive assays, including the tail flick test (D'Amour & Smith, 1941), our reperfusion

hyperalgesia model (Gelgor *et al.*, 1986), and incisional (Weber *et al.*, 2005). We therefore investigated whether procedures developed to generate carrageenan-induced inflammation in the foot also could be used in cutaneous tissue of the tail. The first aim of our study was to compare the voluntary activity of rats following carrageenan injection into the hind paw to that after carrageenan injection into the tail

We also investigated the inflammatory cytokine cascade, which contributes to the development and maintenance of hyperalgesia in cutaneous inflammation (Poole *et al.*, 1999a). Inflammatory mediators, like bradykinin, which are released from injured tissue, sensitize and directly stimulate nociceptors, and stimulate tumour necrosis factor alpha (TNF-α) release (Basbaum & Woolf, 1999). This TNF-α in turn, stimulates the release of interleukin-1beta (IL-1β) and interleukin-6 (IL-6). Interleukin-1β and IL-6 promote the induction of cycloxygenase enzymes, which convert arachidonic acid to prostaglandins that sensitize nociceptors. Tumour necrosis factor-alpha also stimulates the release of cytokine-induced neutrophil chemoattractant (CINC-1) in rats or interleukin-8 (IL-8) in humans (Shibata *et al.*, 2000). In addition to attracting inflammatory cells to the injured site, CINC-1 stimulates the release of sympathetic amines (Poole *et al.*, 1999a; Lorenzetti *et al.*, 2002). Cytokines, like IL-1β, TNF-α and IL-6, contribute directly to the development of hyperalgesia by sensitizing peripheral nociceptors, decreasing the peripheral nociceptor threshold (Ferreira *et al.*, 1988; Cunha *et al.*, 1992; Lorenzetti *et al.*, 2002). We therefore measured pro-inflammatory cytokine concentrations in tissue at the site of injection, and in the blood of rats to which we had given tail injections of carrageenan, to determine if a similar cytokine response occurred in the tail tissue to that found in the hind paw.

2. MATERIALS AND METHODS

2.1 Animals

For tests of voluntary running wheel activity, female Sprague-Dawley rats, weighing 200-220g at the time of injection, were housed individually in cages, at an ambient temperature of 25-26°C, with attached running wheels. For the nociceptive testing, female Sprague-Dawley rats, weighing 200-220g at the start of the experiment, were housed in groups of four per cage, and were allowed standard rat chow and tap water *ad libitum*. The rats were housed in a temperature-controlled environment at 21-22°C on a 12:12 hour light dark cycle (lights on at 07:00). A group of animals different to that which underwent behavioural nociceptive testing, randomly selected from the same breeding stock, was used to obtain blood and tissue samples and housed in the same experimental conditions. The experimental procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, South Africa (2003/99/3 and 2004/73/3), and were conducted in accordance with the International Association for the Study of Pain requirements for research on animals (Zimmerman, 1983).

2.2 Experimental procedures

Carrageenan (lambda carrageenan, 2mg, Sigma-Aldrich, Johannesburg, South Africa) was suspended in 100µl sterile isotonic saline (0.9%) before injections. We injected 100µl of carrageenan solution or 100µl 0.9% saline into the dermis of the rat's tail using a 0.5mm diameter needle, at the midpoint of the dorsal surface of the tail, adjacent to the vein. For the running wheel measurements, each rat received one injection at 17:00 so that the peak of the inflammation occurred in the dark phase of the 24h-day, when rats are most active.
For the behavioural, cytokine and histological testing, each animal received one injection only, which was administered between 08:00 and 10:00.

2.3 Voluntary running wheel activity

Each rat was placed individually in a cage with an attached running wheel (circumference of 1.06m). Each wheel was equipped with a magnet and a magnetic switch connected to a computer where voluntary running wheel activity, in five-minute intervals, was measured using a behavioural monitoring system (VitalView, Minimitter, Sunriver, OR, USA). Rats with a voluntary daily running distance of at least 1 km, over 14 days, were selected for the study. Since day-time running is negligible, only night-time running distance was analyzed (19:00-07:00). Running distance was determined from the number of wheel turns for the 12-hour night-time interval. The total number of wheel turns per night was expressed as a percentage change from pre-injection (average of 5 nights) running distance. Voluntary activity was recorded for three nights after injection. Since voluntary activity is affected by oestrus cycle, we performed two vaginal lavages (100µl sterile saline) on each rat, at least three days apart. The live cell samples were analysed under a microscope and classified, using the system used by Montes and Luque (Montes & Luque, 1988) into the four phases of the oestrus cycle, namely oestrus, proestrus, metoestrus and dioestrus. The rats were divided randomly into four groups, each rats received either saline or carrageenan into either the hind paw or the tail (n=8-9 per group).

2.4 Behavioural nociceptive measurements

For behavioural measurements, the rats (n=12 per group) were placed in clear plastic restrainers that allowed free movement of the tail while limiting movement of the rest of the body. Rats were familiarized with the restrainers for two hours per day on three consecutive days before measurements started. Behavioural measurements were taken daily for 4d before injection, and 1h, 3h, 6h, 24h and 48h after injection. Withdrawal response latency to a noxious thermal challenge was assessed before the noxious mechanical

challenge was applied, to eliminate any risk of hyperalgesia developing as a result of the application of a noxious mechanical stimulus (Vidulich & Mitchell, 2000).

2.4.1 Withdrawal response to a noxious thermal challenge

The rat's tail was immersed in a water bath controlled at 29 °C, to maintain a constant tail temperature, 30min before measurements and between measurements (Berge *et al.*, 1988; Tjolsen *et al.*, 1989). One of us (LL) measured the withdrawal response latency to a noxious thermal challenge following rapid immersion of the entire tail in an unstirred water bath controlled at 49 °C, as the time from submerging the tail in the water to the first coordinated response. The mean of three consecutive measurements, taken at one-minute intervals, was recorded. Any rat that did not respond within 30s had its tail removed from the water bath, to avoid tissue damage to the tail.

2.4.2 Withdrawal response to a noxious mechanical challenge.

We assessed withdrawal response threshold to a noxious mechanical pressure by applying punctate pressure, using an electronic von Frey anaesthesiometer (ITTC, Life Sciences, USA), at a consistently increasing rate, to the dorsal surface of the tail, at the site of injection. We recorded the minimum force (g) which elicited a brisk withdrawal or escape behaviour. A mean of three measurements at the injection site, and 30mm above the injection site, one minute apart, was recorded.

2.5 Histology

We injected an additional nine animals with 2mg carrageenan in 100µl saline at the midpoint of the dorsal surface of the tail. Three animals were killed, with intraperitoneal injection of sodium pentobarbital (Euthanase, Kyron, Johannesburg, South Africa), at 3h, 6h and 24h after injection and sections from the site of injection were removed and

processed for later histological analysis. A further three animals were killed 24h after 100µl saline injection, and tissue sections of the tail were removed for histological analysis as control tissue. Sections of tail tissue, including dermis and underlying fascia, tendon and muscle were fixed in 10% formalin and embedded in paraffin wax. Paraffin sections of all tissues were stained with haematoxylin and eosin and examined by light microscopy. Descriptive histological analysis of the sections was performed by one of us (BM), a pathologist, blinded to the treatment.

2.6 Cytokine concentrations

In separate groups of rats, under isofluorane anaesthesia (Safeline Pharmaceuticals, Johannesburg, South Africa), we collected blood and tail tissue 3h, 6h and 24h after injection of carrageenan or saline (n=6 per treatment group and time interval). Blood first was collected by cardiac puncture, into sterile tubes containing EDTA, and centrifuged at 2 000rpm for 10min at 4°C. The plasma was stored at -70 °C until assayed. The rats then were killed by intracardiac injection of 1ml sodium pentobarbital (Euthanase, Kyron, Johannesburg, South Africa), and tail tissue from the site of injection was removed, weighed, frozen in liquid nitrogen and stored at -70 °C for cytokine analysis. Tissue samples were homogenised in phosphate buffered saline (PBS, pH = 7.4) containing 0.4M NaCl, 0.05% Tween-20, 0.5% bovine serum albumin, 0.1mM benzethonium chloride, 10mM EDTA and 20KI/ml aprotinin. The homogenates were centrifuged at 12 000g for 60 min at 4 °C. The supernatant was removed and analysed, as was the plasma, for IL-1β, IL-6, CINC-1 and TNF-α.

2.7 Rat-specific ELISA for cytokines

Cytokine concentrations were determined using an enzyme-linked immunosorbent assay (ELISA, National Institute of Biological Standards and Control, South Mimms, UK), as

previously described (Safieh-Garabedian *et al.*, 1995). Microtitre plates were coated overnight with sheep anti-rat polyclonal antibody. 100µl standard recombinant rat cytokine, or sample, was added to each well and left overnight at 4 °C. Each sample was measured in duplicate. Sheep anti-rat biotinylated polyclonal antibodies were added at a 1:2000 dilution, and the sample incubated at room temperature (21-22 ºC) for 1h. Finally, 100µl of streptavidin-polyHRP (1:10000 dilution, Euroimmun, Cape Town, South Africa) was added to each well, at room temperature. After 30 min the plates were washed and the colour reagent *o*-phenylenediamine dihydrochloride (40µg, 100µl.well^{-1}, Sigma-Aldrich, Johannesburg, South Africa) added. The reaction was terminated with H_2SO_4 (1M, 150µl.well^{-1}) and the optical density measured at 490nm. The results were expressed as pg.ml^{-1} for the plasma, and pg.mg^{-1} for tail tissue. TNF-α, IL-1β, IL-6 and CINC-1 concentrations were measured with the appropriate sheep anti-rat polyclonal and biotinylated antibodies for each cytokine. The ELISA for IL-β detected both precursor and mature IL-1β but did not cross-react with IL-1α or IL-1ra. The ELISAs for TNF-α, IL-6 and CINC-1 did not cross react with each other or with the ELISA for IL-1β. As a result of the high concentrations of cytokines in some samples from sites of inflammation, the samples required large dilutions. The detection limit of each assay, which allowed for the dilution factor of the sample, varied between tissue and plasma samples, and between treatment groups, and is reported with the results of the assay.

2.8 Data analysis

All data are expressed as mean ± SD. Voluntary activity, response latency to noxious heat and response threshold to noxious pressure were expressed as the percentage change from pre-injection value for each rat. Voluntary activity, behavioural responses and cytokine concentrations were compared between animals receiving carrageenan and saline by means of two-way analysis of variance (main effects, intervention and time) with Student

Newman-Keuls (SNK) *post hoc* test when appropriate. When cytokine concentrations were undetectable, samples were assigned the detection limit of the assay. Statistical significance was taken at $P < 0.05$.

3. RESULTS

3.1 Voluntary running wheel activity

The rats' voluntary running wheel activity, averaged over four days before injection, was significantly higher in the oestrus phase (11232 ± 5228counts/12h) compared to proestrus (5149 ± 5662 counts/12h), metoestrus (4960 ± 7166counts/12h) and dioestrus (5632 ±4767 counts/12h, $P<0.001$, $F_{3,132}=9.36$). Therefore, the pre-injection running wheel data excluded running activity when the rats were in oestrus, and injections were not given to rats in the oestrus phase. Figure 1 shows the percentage change in running wheel activity from pre-injection running, following saline or carrageenan injection into the hind paw or the tail. The percentage change in running wheel activity following an injection into the hind paw showed a significant difference between rats injected with carrageenan compared to those injected with saline ($P<0.001$, $F_{1,45}=40.90$) on the night of injection and one night after injection ($P<0.05$, $F_{1,45}=4.80$), but there was no significant interaction ($P=0.48$, $F_{2,45}=0.74$). The percentage change in running wheel activity following an injection into the tail showed no significant difference between rats injected with carrageenan or saline ($P=0.09$, $F_{1,45}=3.08$), at any time ($P=0.19$, $F_{1,45}=1.70$) and there was no significant interaction ($P=0.69$, $F_{2,45}=0.37$).

3.2 Behavioural nociceptive responses

Figure 2 shows the response latency to noxious heat and the response threshold to noxious pressure at the site of injection. The response latency to noxious heat showed a significant agent effect ($P<0.01$, $F_{1,22}=13.47$) and time effect ($P<0.001$, $F_{4,88}=6.65$), but no significant interaction ($P=0.08$, $F_{4,88}=2.16$). Pressure threshold showed an agent effect ($P<0.01$, $F_{1,22}=9.04$), but no significant time effect ($P=0.67$, $F_{4,88}=0.59$) or interaction ($P=0.06$, $F_{4,88}=2.31$).

3.3 Histology

The animals that received a saline injection showed a trivial influx of inflammatory cells at the site of injection, and then in only one out of the three animals (Fig. 3D). Samples taken at the site of injection, three hours after carrageenan injection showed mild inflammatory infiltrate in the subcutaneous tissue, as evident by the presence of neutrophils, lymphocytes, plasma cells, and occasional eosinophils (Fig. 3A). Samples taken six hours after carrageenan injection (Fig. 3B) showed moderate to severe inflammatory cell infiltrate in the dermis and subcutaneous tissue. The severe inflammation was evident by the presence of neutrophils, eosinophils, lymphocytes, and plasma cells, but no macrophages. Twenty-four hours after carrageenan injection, the tail tissue at the site of carrageenan injection (Fig. 3C) showed a moderate inflammatory cell infiltrate containing mainly lymphocytes and neutrophils with associated oedema at both sites. No fibrosis or granulomas appeared within 24h of the carrageenan injection.

3.4 Cytokine concentration

TNF-a: Six hours, but not 3h or 24h, after carrageenan injection, TNF-α concentration in the tail tissue was elevated significantly compared to the concentration in animals injected with saline ($P<0.001$, SNK, Fig. 4), at the site of injection. Plasma TNF-α concentration, after carrageenan or saline injection, was below the detection limit of the assay at all times.

IL-1β: Six hours after carrageenan injection, IL-1β tail tissue concentration was elevated significantly compared to the concentration found in animals injected with saline ($P<0.001$, SNK, Fig. 4), and had dropped, but still was elevated significantly 24h after injection ($P<0.01$, SNK, Fig. 4). IL-1β tail tissue concentration of animals injected with carrageenan was significantly higher at 6h compared to the concentration found 3h after carrageenan injection ($P<0.001$, SNK), and that found 24h after carrageenan injection ($P<0.001$, SNK).

Tail IL-1β concentration still was elevated significantly 24h after carrageenan injection compared to 3h after carrageenan injection ($P<0.001$, SNK, Fig. 4). Following carrageenan injection into the tail, there was no significant increase in plasma IL-1β ($P=0.42$, ANOVA) concentration compared to that of the animals injected with saline, and concentrations were below the detection limit of the assay (7.1pg.ml^{-1}, data not shown) at all times.

IL-6: Six hours after carrageenan injection, IL-6 concentration at the site of injection was elevated significantly compared to the concentration found in animals injected with saline ($P<0.001$, SNK, Fig. 4), and had dropped markedly, but still was elevated significantly ($P<0.05$, SNK, Fig. 4) 24h after injection. IL-6 tail tissue concentration of animals injected with carrageenan was significantly higher at 6h compared to the concentration found 3h after carrageenan injection ($P<0.001$, SNK). Following a carrageenan injection into the tail, there was no significant increase in plasma IL-6 concentration ($P=0.56$, ANOVA) compared to the animals injected with saline, and in most cases IL-6 concentration was below detection limit of the assay (6.6pg.ml^{-1}, data not shown).

CINC-1: Figure 5 shows the CINC-1 concentration in both tail tissue and plasma after saline or carrageenan injection into the tail. There was a significant elevation in tail CINC-1 concentration, at the site of injection, after carrageenan injection compared to that after saline injection, 3h ($P<0.0001$, SNK, Fig. 5) and more so 6h ($P<0.001$, SNK) after injection. CINC-1 concentration in the tail of animals injected with carrageenan had dropped to the same as that following saline injection by 24h after injection. The plasma CINC-1 concentration after carrageenan injection was significantly higher than that of saline-injected animals ($P<0.01$, SNK, Fig. 5), 6h after injection, but was not elevated either 3h or 24h after injection.

4. DISCUSSION

We determined that the tail is a suitable site, comparable to the hind paw, for producing inflammatory-induced hyperalgesia. We assessed the behavioural responses of rats to two modalities of noxious challenge, applied to the site of an intradermal injection of the irritant carrageenan into the tail, and also investigated the concomitant changes in histology and in tail tissue and plasma cytokine concentrations. The change in withdrawal response to both noxious heat and noxious pressure showed the animals to be hyperalgesic after carrageenan injection into the tail. The peak in hyperalgesia, at the site of the carrageenan injection, which occurred 6h after carrageenan injection, coincided with moderate to severe inflammatory cell infiltration. Our results also showed substantial increase in pro-inflammatory cytokine concentrations at the site of injection with the concentrations of all four pro-inflammatory cytokines, TNF-α, IL-1β, IL-6 and CINC-1, also peaking 6h after injection. However, only CINC-1 concentration was elevated at the site of injection at 3h after injection, by which time the hyperalgesia was established. Twenty-four hours after carrageenan injection tail TNF-α and CINC-1 concentrations were no longer significantly elevated; the interleukins still were elevated, but their concentrations had dropped significantly below the 6h peak. We found no difference in running wheel activity between rats injected with carrageenan into the tail compared to rats injected with saline into the tail. In contrast, we found a significant reduction in voluntary running wheel activity in rats injected with carrageenan into the hind paw compared to that of rats injected with saline into the hind paw.

No previous studies have investigated the effects of hyperalgesia on voluntary activity or exercise in experimental animals. Although a previous study found a reduction reduction in cage activity in rats injected intramuscularly into the thigh with turpentine, an hyperalgesic agent, the researchers did not measure the presence of hyperalgesia (Saha *et al.*, 2005). A

reduction in voluntary running wheel activity occurs during infection and fever, which are often associated with inflammation (Katafuchi *et al.*, 2003; Harden *et al.*, 2006). Our finding that an injection of carrageenan into the hind paw, but not the tail, reduces voluntary running wheel activity reveals the impoprtance of the site of inflammation in altering activity. Injection into the tail, which results in comparable cytokine and behavioural responses after injection into the hind paw, therefore offers a useful alternative site for producing hyperalgesia without altered voluntary activity.

Regardless of the site of carrageenan-induced inflammation, TNF-α and IL-1β concentrations increase early, with peak IL-1β concentration, according to previous reports, occurring before a peak in TNF-α concentration (Woolf *et al.*, 1997; Loram *et al.*, 2007). In our hands, peak TNF-α concentration coincided with the elevation in other cytokines, but we believe that our initial sampling intervals may have been too long to reveal accurately the temporal sequence of TNF-α, IL-1β, IL-6 and CINC-1 secretion. In addition, although we produced significant thermal hyperalgesia and mechanical hyperalgesia, our statistical analysis could not resolve when hyperalgesia first appeared, or disappeared. We also could not analyse the resolution of cytokine profiles following carrageenan injection, because the concentrations of some cytokines in tail tissue still were elevated at 24h, when we took our last tissue samples.

Numerous studies investigating inflammatory hyperalgesia have used carrageenan as an inducer of acute inflammation by injecting carrageenan into the hind paw of a rat (Winter *et al.*, 1962; Vinegar *et al.*, 1987; Hargreaves *et al.*, 1988). The responses to noxious heat and noxious pressure, which we observed, are comparable to those described after carrageenan injection into a rat's hind paw, with peak hyperalgesia occurring within a few hours and resolving by 24h (Winter *et al.*, 1962; Vinegar *et al.*, 1987; Hargreaves *et al.*, 1988; Tabo *et al.*,

1998; Yamamoto *et al.*, 1998). Similar behavioural changes to noxious heat and noxious pressure, with the same temporal pattern, were evident after a carrageenan injection, in spite of the tail having thick keratinised skin, and the hind paw having thin glabrous tissue.

Our hyperalgesia to both noxious heat and noxious pressure reached its peak 6h after injection, coinciding with the peak inflammatory cell infiltrate at the site of injection. In our study, comparable to a previous study injecting carrageenan into the hind paw (Vinegar *et al.*, 1987), neutrophils, lymphocytes and plasma cells, with occasional eosinophils, invaded the site by 3h after carrageenan injection into the rat's tail. Although we found oedema only at 24h after injection, following injection of carrageenan into hind paw Vinegar et al. (1987) found oedema from 1h after injection, and lasting for 48h, perhaps indicating a greater susceptibility of the hind paw to oedema during inflammation.

TNF-α, which is produced by inflammatory cells and other tissue cells such as keratinocytes (Zhang *et al.*, 1995), produces hyperalgesia to noxious pressure within 1h of injection, peaking 3-5h after injection, and resolving within 24h (Cunha *et al.*, 1992; Woolf *et al.*, 1997). Also, neutralising TNF-α attenuated thermal hyperalgesia at 3h and prevented mechanical hyperalgesia occurring 3h to 6h after carrageenan injection (Cunha *et al.*, 2000). In the rat tail, like in the rat hind paw, we found coincidence in the time course of peak local TNF-α concentration and hyperalgesia (Woolf *et al.*, 1997) where thermal hyperalgesia appeared before elevated TNF-α concentration could be detected in the hyperalgesic tissue, and mechanical hyperalgesia persisted after TNF-α concentration had returned to normal.

As with TNF-α, IL-1β and IL-6 concentrations in tail tissue were not elevated before or 6h after carrageenan injection, but remained elevated 24h after injection. IL-1β, a well-described activator of nociceptors, (Ferreira *et al.*, 1988), when injected into the hind paw

produces mechanical hyperalgesia in a dose and time-dependent manner (Cunha *et al.*, 2000; Cunha *et al.*, 2005), but the hyperalgesia resolves within 24h (Ferreira *et al.*, 1988; Cunha *et al.*, 2000; Cunha *et al.*, 2005). Elevation of local IL-1β and IL-6 concentrations are likely to play a role in sensitizing peripheral nociceptors following a carrageenan injection, though, just as with TNF-α, they are unlikely to be the sole cytokines responsible for sensitizing the nociceptors, particularly because hyperalgesia was established before a detectable elevation of IL-1β or IL-6 concentration occurred, in tail tissue.

Of the cytokines we measured, the time course of CINC-1 best matched the onset of hyperalgesia. The chemokine, CINC-1, stimulates the release of sympathetic amines involved in hyperalgesia (Lorenzetti *et al.*, 2002), and attracts neutrophils to the injured area (Shibata *et al.*, 2000). CINC-1 has been implicated not only in peripheral nociceptor sensitization but also in central nervous system activation during inflammation, thereby contributing to mechanical hyperalgesia (Yamamoto *et al.*, 1998). In our study, tissue CINC-1 concentration had increased by 3h, and both tissue and plasma concentration peaked 6h after carrageenan injection. Recombinant rat CINC-1 injected into the hind paw of rats produced a mechanical hyperalgesia similar to the hyperalgesia found after carrageenan injection (Lorenzetti *et al.*, 2002). In our study, CINC-1 was the only cytokine with elevated plasma concentration after tail carrageenan injection, but we do not know whether that circulating CINC-1 had any functional role.

In conclusion, we have identified a site of carrageenan-induced inflammation that results in hyperalgesia with concurrent changes in local tissue histology and cytokine production of the thick keratinized tissue of the tail comparable to those characterized for the well established models of inflammation in the rat hind paw. We also have developed a method

for generating hyperalgesia in the rat tail without decreasing voluntary running activity, as occurs following inflammation in the hind paw of rats.

FIGURE LEGENDS

Figure 1 Change in voluntary running wheel activity after 100µl saline (□) or 2mg/100µl carrageenan (■) into the tail or the hind paw (n=8-9 per group). Values are expressed as a percentage change from pre-injection values for each rat (mean ± SD). Injections were given at 17:00 and running activity was recorded as the sum of the wheels counts from 19:00 to 07:00 on the night of injection and for 2 nights after injection. Pre-injection running wheel counts were 5795±4139counts/12h. There was a significant reduction in the voluntary activity of rats injected with carrageenan into the hind paw compared to rats injected with saline into the hind paw (**P<0.001, *P<0.05). Carrageenan injection into the tail did not significantly affect running wheel activity compared to rats injected with saline into the tail, at any time after injection (P=0.09).

Figure 2 Change in withdrawal response latency to noxious heat, and change in response threshold to noxious pressure at the site of injection after 100µl saline (○) or 2mg/100µl carrageenan (●) into the tail 1h, 3h, 6h, 24h and 48h (n=12 per group). Values are expressed as a percentage change from pre-injection values for each rat (mean ± SD). Pre-injection withdrawal latency to noxious heat was 5.9±1.3s and pre-injection to noxious pressure threshold was 181±54g. Carrageenan injection induced significant thermal (P<0.01, ANOVA) and mechanical hyperalgesia (P<0.01, ANOVA) at the site of injection compared to animals injected with saline.

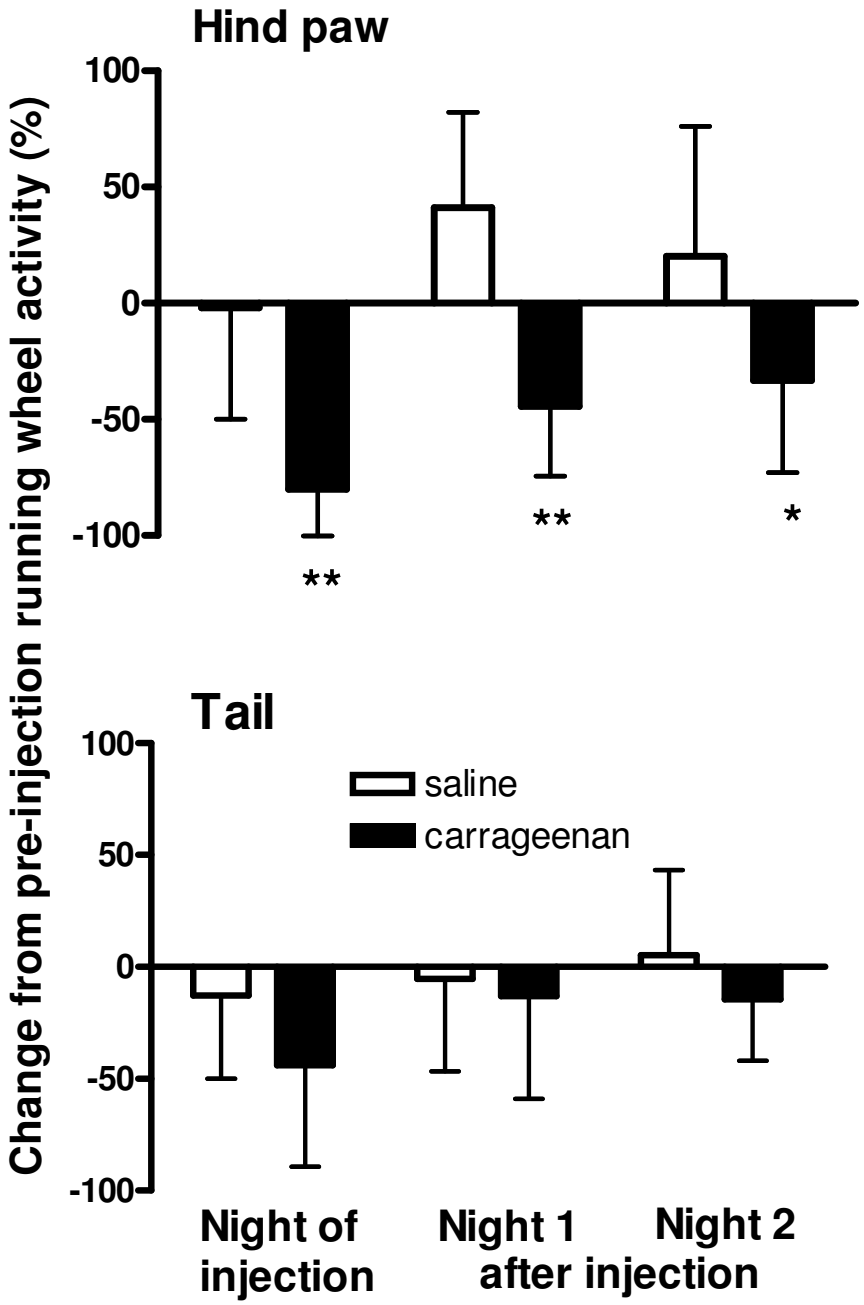
Figure 3 Histological changes in the superficial tissue of a rat's tail, at the site of injection, 3h (A), 6h (B) and 24h (C) after 2mg/100µl carrageenan injection into the dorsal surface of the tail. (D) shows histology, at the injection site, of a rat's tail 24h after saline injection. All sections were stained with haemotoxylin and eosin and photographed at 100x magnification with epidermis orientated to the top. Arrows indicate: (A), the presence of

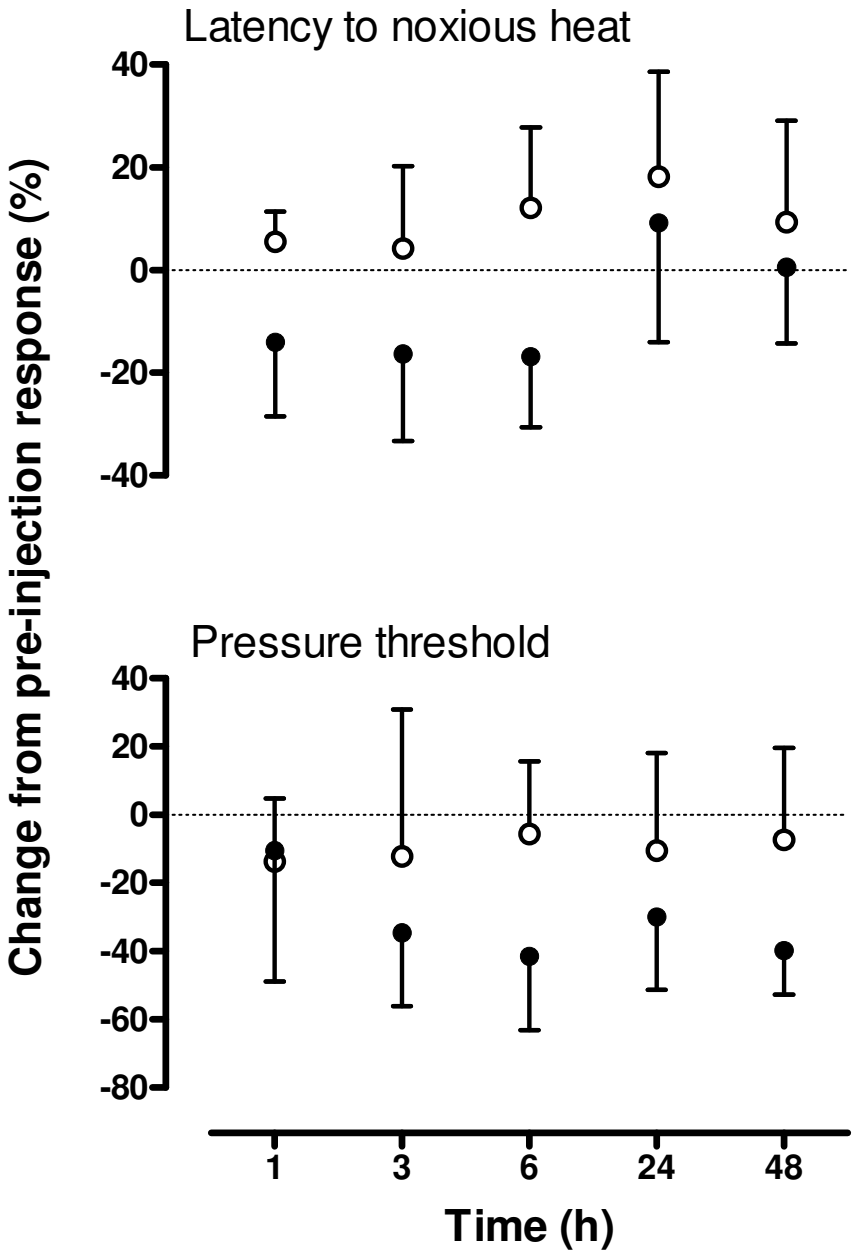
neutrophils, lymphocytes, plasma cells, and occasional eosinophils; (B), the presence of neutrophils, eosinophils, lymphocytes, and plasma cells; and (C), a moderate inflammatory cell infiltrate containing mainly lymphocytes and neutrophils with associated oedema .

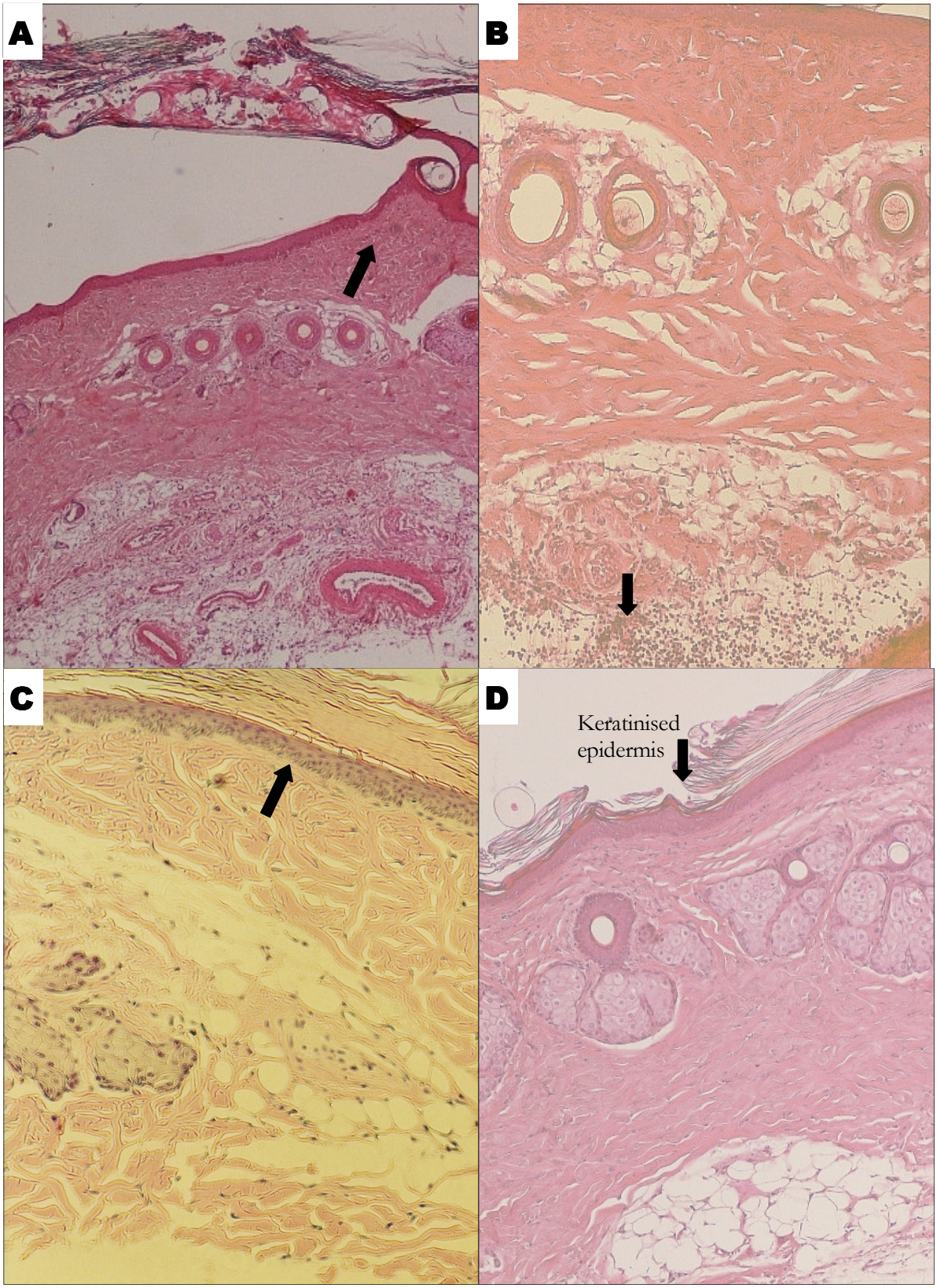
Figure 4 TNF-α, IL-1β and IL-6 concentration (mean ± SD, n=6 per group) in the tail tissue at the site of intradermal injection of carrageenan (2mg/100ul saline,■) or saline (□, 100μl). There was a significant difference in TNF-α concentration in the tail tissue after carrageenan injection compared to saline injection 6h after injection, but not at 3h or 24h after injection. IL-1β concentration in tail tissue was significantly higher following carrageenan injection than following saline injection, 6h and 24h after injection. IL-1β concentration following carrageenan injection was higher 6h than 24h after injection. Tissue IL-6 concentration was significantly higher following carrageenan injection than following saline injection, 6h and 24h after injection. IL-6 concentration following carrageenan injection was higher at 6h than at 24h following injection. ---- indicates level of detection of assay. * indicates $P<0.05$ and *** indicates $P<0.001$ between saline and carrageenan injected animals at the same time interval. $^{\#\#\#}$ indicates $P<0.001$ between time intervals after carrageenan injection.

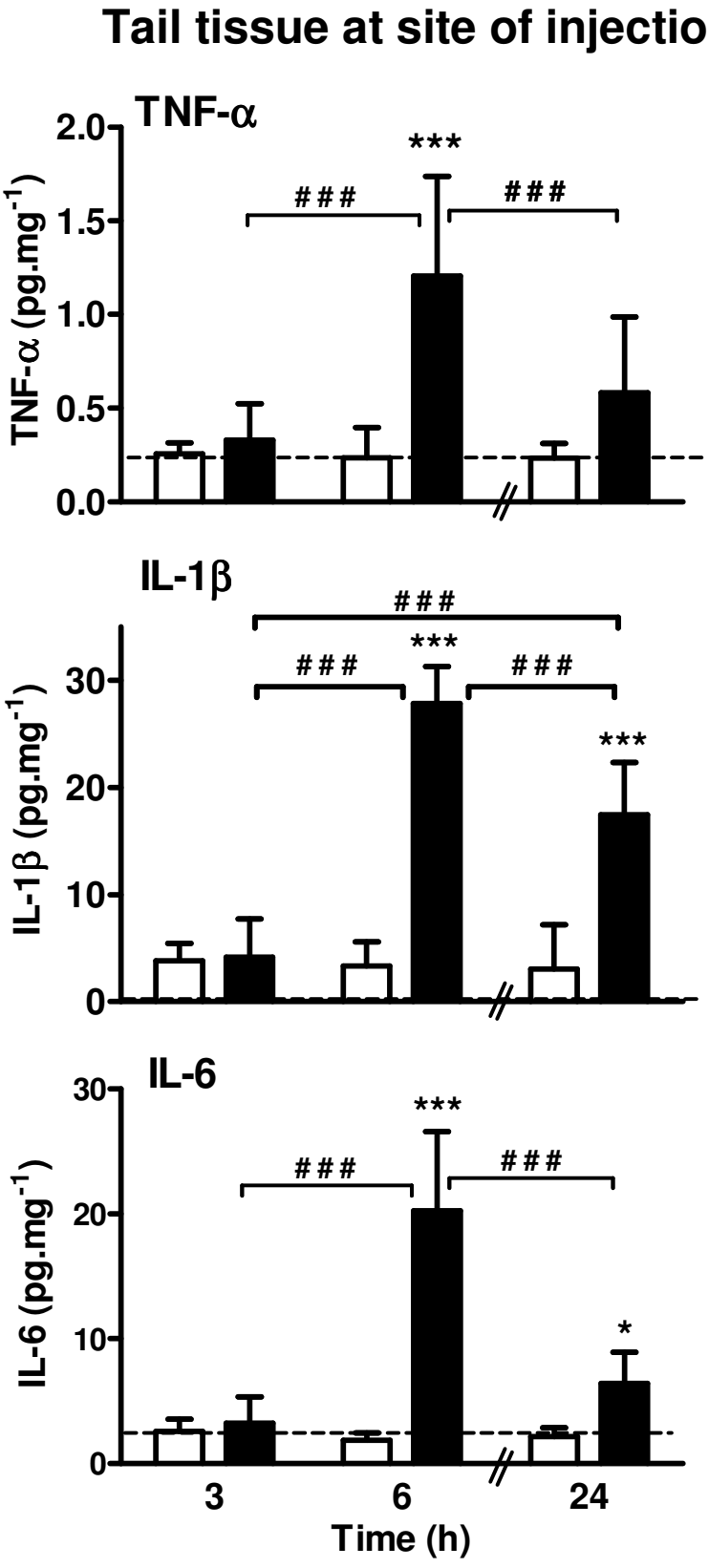
Figure 5 CINC-1 concentration (mean ± SD, n=6 per group) in the plasma and homogenized tail tissue at the site of injection after intradermal injection of carrageenan (2mg/100ul saline, ■) or saline (100μl, □). Tissue CINC-1 concentration was significantly higher 3h following carrageenan injection, and even more so 6h afterwards, than following saline injection. Plasma CINC-1 concentration was significantly higher 6h after carrageenan injection into the tail than after saline injection into the tail. ---- indicates level of detection of assay. * indicates $P<0.05$ and ** indicates $P<0.01$ between saline and carrageenan

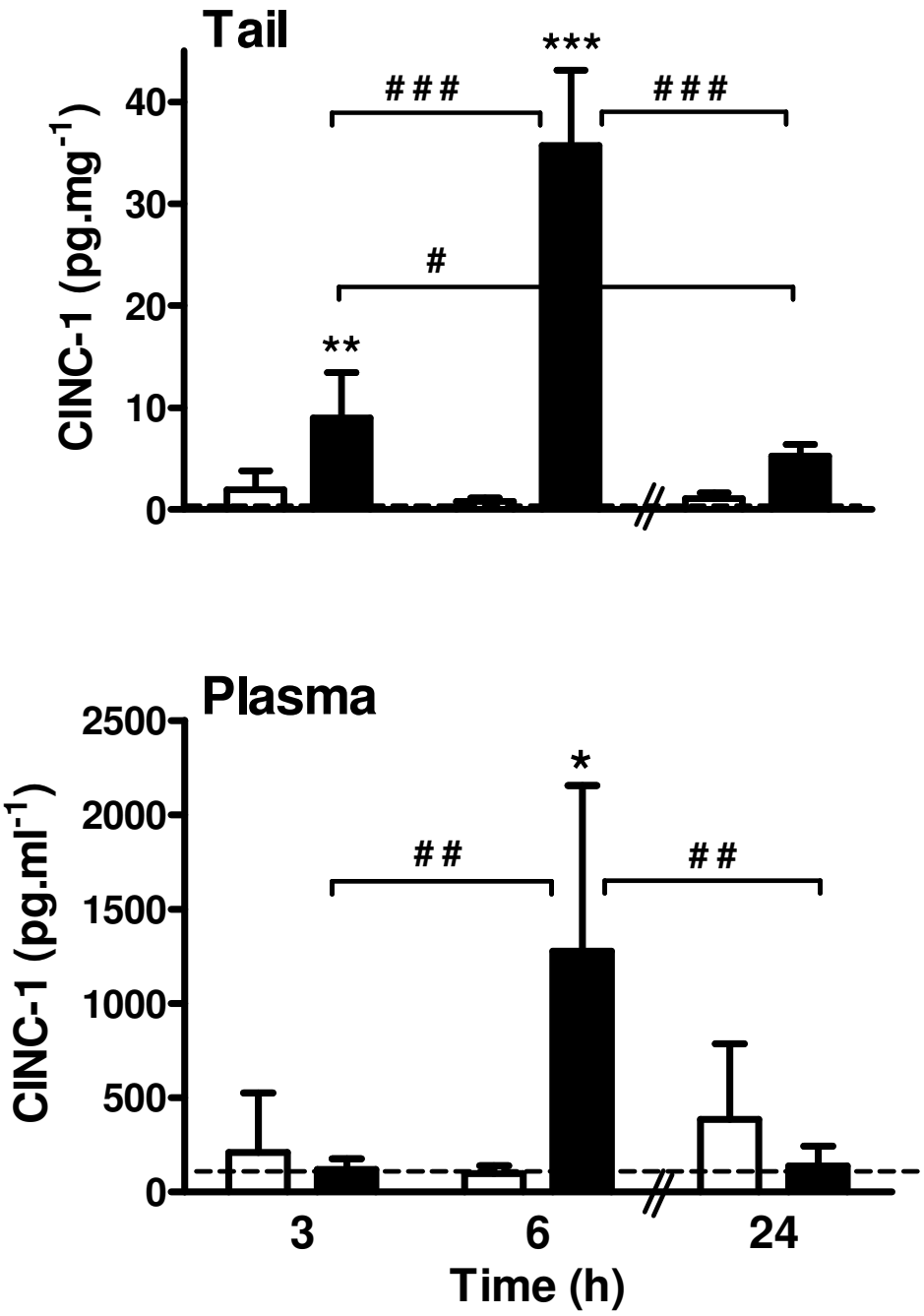
injected animals at the same time interval. $^{\#}$ indicates $P<0.05$, $^{\#\#}$ indicates $P<0.01$ and $^{\#\#\#}$ indicates $P<0.001$ between time intervals after carrageenan injection.


Hind paw
Change from pre-injection running wheel activity (%)
100
50
0
-50
-100
**
**
*
Tail
saline
carrageenan
Night of injection
Night 1 after injection
Night 2


Latency to noxious heat
Pressure threshold
Change from pre-injection response (%)
40
20
0
-20
-40
-60
-80
1
3
6
24
48
Time (h)


A
B
C
D
Keratinised
epidermis


Tail tissue at site of injection
TNF-α
TNF-α (pg.mg-1)
2.0
1.5
1.0
0.5
0.0
***
###
###
IL-1β
IL-1β (pg.mg-1)
30
20
10
0
###
***
###
###
***
IL-6
IL-6 (pg.mg-1)
30
20
10
0
***
###
###
*
3
6
24
Time (h)


Tail
CINC-1 (pg.mg-1)
40
30
20
10
0
***
###
###
#
**
Plasma
CINC-1 (pg.ml-1)
2500
2000
1500
1000
500
0
*
##
##
3
6
24
Time (h)

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CHAPTER 3

Cytokine profiles during carrageenan-induced inflammatory hyperalgesia in rat muscle and hind paw.

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Cytokine Profiles During Carrageenan-Induced Inflammatory Hyperalgesia in Rat Muscle and Hind Paw

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Abstract: It is not known if a cytokine cascade develops during muscle inflammation and whether cytokines contribute to muscle inflammatory pain. We measured plasma and tissue cytokine concentrations, and behavioral responses to noxious mechanical stimuli, after inducing inflammation in the gastrocnemius muscle and the hind paw of rats. Tissue and plasma samples were taken 3, 6, or 24 h after carrageenan or saline injection into one of the 2 sites. Tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and cytokine-induced neutrophil chemoattractant 1 (CINC-1) concentrations were measured. Hyperalgesia was present 3 h after carrageenan injection into the hind paw and muscle. The TNF-α was elevated significantly in the inflamed hind paw tissue ($P < .001$) but not in inflamed muscle tissue. IL-1β was elevated 6 h after carrageenan injection in the hind paw tissue but only 24 h in the muscle tissue ($P < .001$). The IL-6 was elevated 3 h after injection in the hind paw tissue but only after 6 h in the muscle tissue ($P < .01$). The CINC-1 in plasma, muscle, and hind paw was elevated from 3 h to 24 h after carrageenan injection ($P < .01$). The release of IL-1β and IL-6, known to mediate hyperalgesia elsewhere, is delayed in muscle inflammation compared with cutaneous inflammation, whereas TNF-α is not elevated during muscle inflammation.

Perspective: The quality and mechanisms of muscle pain are different from that of cutaneous pain. So too is the pattern of cytokine release during inflammation. Inhibiting TNF-α is unlikely to be effective in managing inflammatory muscle pain, but other cytokines, notably IL-1β and CINC-1, may prove useful therapeutic targets.

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Key words: *Interleukin, tumor necrosis factor, inflammation, pain.*

The subjective perception and processing within the nervous system of muscle pain, a common cause of morbidity,[5,16] differs from the perception and processing of cutaneous pain.[25,26] A complex neural mechanism, involving muscle spindles, tendon receptors, and cutaneous receptors, as well as central sensitization, underlies muscle pain.[18] Common symptoms of muscle pain include local tenderness on palpation and pain on movement of the affected muscle.[25,26] Mechanical hyperalgesia, the increased sensitivity to a noxious mechanical stimulus, is evident in muscle diseases such as fibromyalgia and myositis.

Many muscle pains, including exercise-induced muscle pain, appear to have their origin in muscle inflammation.[1,17,27] In other tissues, cytokines, growth factors, and other inflammatory mediators that are released during inflammation sensitize nociceptive pathways.[3] At least in cutaneous tissue, a distinct cytokine cascade unfolds during inflammation. Agents derived from injured tissue, such as bradykinin, stimulate the release of tumor necrosis factor alpha (TNF-α), which in turn stimulates the release of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6).[28] Interleukin-1β and IL-6 promote the release of the cycloxygenase enzymes, which convert arachidonic acid to prostaglandins.[28] Tumor necrosis factor α also stimulates the release of cytokine-induced neutrophil chemoattractant 1 (CINC-1) in rats and its homolog IL-8 in humans.[33] Cytokine-induced neutrophil chemoattractant 1 and

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IL-8 mediate sympathetic pain by stimulating the release of sympathetic amines.[21,28]

Whether a specific cytokine cascade also underlies muscle inflammatory pain, and indeed whether cytokines predominantly play a role in muscle function unrelated to pain, is not known. Cytokines are released in exercise-induced muscle pain, but they are also released during nonpainful muscle contractions.[23,35] Cytokines and chemokines released during muscle inflammation play a vital role in healing and repair of muscle tissue.[37] Cytokines indeed are released during muscle inflammation, but whether the cytokines are able to sensitize muscle nociceptors as they do cutaneous nociceptors is not known. Although injections of recombinant TNF-α produce hyperalgesia in the gastrocnemius muscle of rats,[32] that TNF-α is released endogenously during muscle inflammation has not been established. Interleukin-6 does not activate noxious mechanosensitive pathways in rat muscle,[15] but whether IL-1β does so is not known. The temporal profile of IL-6 and IL-1β release in muscle inflammation also is not known, and may be independent of prior TNF-α release. Therefore, although cytokines are released from muscle during exercise and exercise can induce muscle pain, and although muscle pain often is associated with muscle inflammation, it is not clear whether endogenous proinflammatory cytokines contribute to muscle pain, or play an alternative role, perhaps contributing to the homeostatic function of muscle.

It is difficult to distinguish the roles of these cytokines in situations in which muscle inflammation is the consequence of exercise or muscle contraction. We therefore set out to induce muscle inflammation without muscle contraction or exercise, to identify the proinflammatory cytokine profile of contraction-independent muscle inflammation. We did so experimentally by injection of carrageenan, a plant-based polysaccharide, into the gastrocnemius muscle of rats. Injection of carrageenan is a well established procedure for inducing both muscle and hind paw inflammation and the associated hyperalgesia.[14,19,25,29,40] We measured the profile of release of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and CINC-1) in plasma and at the site of inflammation. We compared those profiles with the equivalent profiles after carrageenan was injected into the hind paw, the conventional site used experimentally for inducing cutaneous inflammation. We assessed whether the cytokines might play a functional role in nociception by also measuring the time courses of hyperalgesias that followed carrageenan injection in muscle and hind paw.

Materials and Methods

Animals

Female Sprague-Dawley rats, weighing 200-220 g at the start of the experiments, were housed in groups of 4 per cage and were allowed standard rat chow and tap water ad libitum. We kept the rats in a temperature-controlled environment at 21-22°C on a 12:12-h light: dark cycle (lights on at 07:00). The Animal Ethics Screening Committee of the University of the Witwatersrand, South Africa, approved the experimental procedures (2003/99/3, 2004/73/3). Different groups of animals, randomly selected from the same breeding stock, were used to obtain blood and tissue samples and to undergo behavioral nociceptive testing.

Behavioral Measurements

Because primary muscle hyperalgesia is best measured with a blunt noxious stimulus and cutaneous hyperalgesia with a sharp noxious stimulus,[39] the hyperalgesia in the hind paw was measured using a sharp stimulus. Primary muscle hyperalgesia was measured with a blunt noxious stimulus and secondary hyperalgesia was measured with a sharp noxious pressure in the cutaneous tissue overlying the gastrocnemius muscle.

Mechanical hyperalgesia in the hind paw was assessed with an electronic von Frey anesthesiometer (IITC; Life Sciences, Woodland Hills, CA). We recorded the minimum mass (g), applied to the hind paw or muscle required to produce a flexor withdrawal response in the injected leg. For measurement of potential mechanical hyperalgesia in the hind paw, the animals were placed in clear plastic restrainers that allowed access to the hind paws. The animals were habituated to the restrainers for 2 h per day on 3 consecutive days before experimentation.

Primary mechanical hyperalgesia in the gastrocnemius muscle was assessed with a pressure algometer with a 50-mm^2 rubber probe (Somedic, AB, Horby, Sweden). We recorded the minimum pressure (kPa) applied to the gastrocnemius muscle that produced a flexor withdrawal response in the injected leg. In addition, secondary hyperalgesia was assessed by applying the von Frey anesthesiometer to the skin overlying the gastrocnemius muscle at increasing pressure until a flexor withdrawal response of the injected leg was elicited. For measurement of potential hyperalgesia in the muscle, each rat was gently restrained and blindfolded while the hind limb was exposed and the pressure algometer or anesthesiometer applied over the belly of the muscle. The electronic von Frey anesthesiometer and the pressure algometer were applied in different groups of rats.

One of the researchers, who was blinded to the treatment, measured all animals' behavioral responses. A mean of 3 measurements taken about 1 min apart was recorded as the pressure threshold. Behavioral responses were recorded in groups of 8 animals for each type of injection, once each day for 3 days before injection, and 3, 6, 24, 48, and 72 h after injection.

Inflammatory Stimulus

We injected carrageenan (2 mg/100 μL saline, lambda carrageenan; Sigma, Johannesburg, South Africa) subcutaneously into the plantar surface of the left hind paw and the belly of the left gastrocnemius muscle. Control injections consisted of 100 μL saline injected into the same site in different animals. Each animal was injected once only, between 08:00 and 10:00.

Tissue and Plasma Samples

At 3, 6, and 24 h after injection, rats (n = 6 per treatment and per time interval) were anesthetized in a chamber perfused with 2% isofluorane (SafeLine Pharmaceuticals, Johannesburg, South Africa). Blood was collected by cardiac puncture and placed into sterile tubes containing EDTA, and then centrifuged at 2,000*g* for 10 min at 4°C. The plasma was stored at −70°C until assayed. The rats were killed by intracardiac injection of 1 mL sodium pentobarbital (Euthanase; Kyron, Johannesburg, South Africa). In animals that received paw injections, the entire hind paw skin of the injected paw was removed. In animals that received muscle injections, gastrocnemius muscle sections (about 200 mg) were removed from the injected leg. All tissue samples were weighed, frozen in liquid nitrogen, and stored at −70°C. For cytokine assays, samples were homogenized in phosphate-buffered saline (PBS, pH 7.4) containing 0.4 mol/L NaCl, 0.05% Tween-20, 0.5% bovine serum albumin, 0.1 mmol/L benzethonium chloride, 10 mmol/L EDTA, and 20 KI/mL aprotinin. The homogenates were centrifuged at 12,000*g* for 60 min at 4°C. The supernatant was removed and analyzed, as was plasma, for IL-1β, IL-6, CINC-1 and TNF-α.

Rat-Specific ELISA for Cytokines

Cytokine concentrations were determined using an enzyme-linked immunosorbent assay (ELISA; National Institute of Biological Standards and Control, South Mimms, UK), as previously described.[30] Microtiter plates were coated overnight with sheep polyclonal antirat antibody. One hundre microliters standard recombinant rat cytokine or sample was added to each well and left overnight at 4°C. Each sample was measured in duplicate. Sheep polyclonal antirat biotinylated antibodies were added at a 1:2,000 dilution and the sample incubated at room temperature (21-22°C) for 1 h. Finally, 100 μL streptavidin-polyHRP (1:10,000 dilution; Euroimmun, Cape Town, South Africa) was added to each well at room temperature. After 30 min, the plates were washed and the color reagent *o*-phenylenediamine dihydrochloride (40 μg per 100-μL.well; Sigma-Aldrich, Johannesburg, South Africa) added. The reaction was terminated with H_2SO_4 (1 mol/L per 150 μL.well) and the optical density measured at 490 nm. The results were expressed as pg/mL for the plasma, and pg/mg for muscle. We compared hind paw cytokine concentrations after carrageenan and saline injection by expressing concentrations as pg/hind paw, because the mass of the inflamed tissue after carrageenan injection was at least 3 times that of the noninflamed tissue. We also have converted the hind paw tissue cytokine concentrations into pg/mg for the animals receiving carrageenan, so that direct comparisons can be made between the inflamed muscle and hind paw tissue, recognizing the inaccuracies caused by the swelling. We were unable to establish the degree to which the inflamed muscle tissue was heavier than the noninflamed tissue, but inflamed muscle tissue did contain more exudates than the noninflamed muscle tissue.

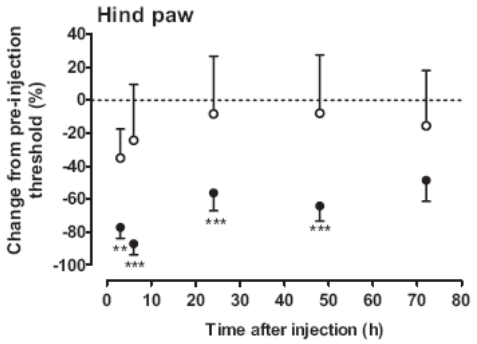

Figure 1. Change in noxious pressure threshold at the site of injection after a carrageenan (●, 2 mg/100 μL) or saline (○, 100 μL) injection into the hind paw. Responses are expressed as mean ± SD percentage change from preinjection values, n = 6. Preinjection noxious pressure threshold was 77 ± 26 g. $^{**}P < .01$; $^{***}P < .001$, carrageenan vs saline groups.

The TNF-α, IL-1β, IL-6, and CINC-1 cytokines were analyzed using the appropriate sheep polyclonal antirat and biotinylated antibodies for each cytokine. The ELISA for IL-β detected both precursor and mature IL-1β but not IL-1α or IL-1ra. The ELISAs for TNF-α, IL-6, and CINC-1 did not cross-react with each other or with the ELISA for IL-1β. As a result of the high concentrations of cytokines from some samples at the sites of inflammation, the samples required large dilutions. The detection limit of each assay, which allowed for the dilution factor of the sample, varied between tissue and plasma samples and between treatment groups and is reported with the results of the assay. Where the values obtained were below the detection limit, the detection limit of the assay was assigned.

Statistical Analysis

All data are expressed as mean ± SD. Response latencies to noxious pressure thresholds were expressed as the percentage change from preinjection values. The differences in percentage changes in behavioral responses and in cytokine concentrations between animals receiving carrageenan and those receiving saline were compared by means of 2-way analysis of variance (main effects agent and time), with Student-Newman-Keuls (SNK) post hoc test when appropriate. When cytokine concentrations were undetectable, samples were assigned the detection limit of the assay. Statistical significance was taken at $P < .05$.

Results

Behavioral Responses

Figure 1 shows the change in response to a noxious mechanical pressure after either saline or carrageenan was injected into the hind paw (n = 6 per group). There was a significant agent effect ($P < .001$, $F_{1,10} = 29.05$),

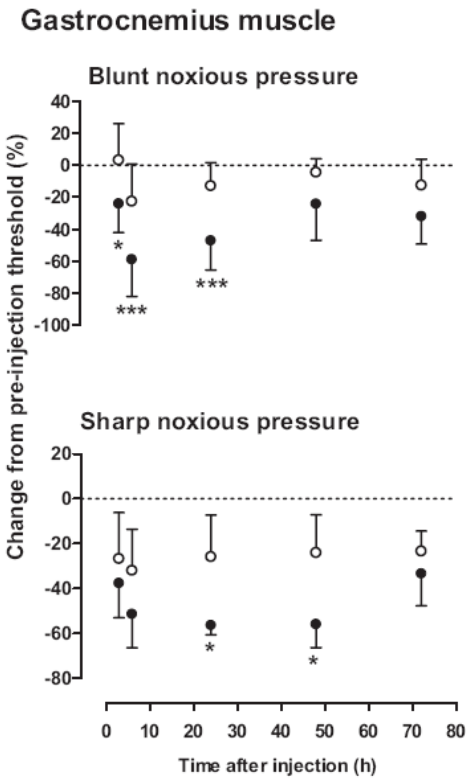

Figure 2. Change in pressure threshold, to a noxious blunt pressure and noxious sharp pressure, at the site of injection after a carrageenan (●, 2 mg/100 μL) or saline (○, 100 μL) injection into the gastrocnemius muscle. Responses are expressed as mean ± SD percentage change from preinjection thresholds, n = 8. Pre-injection noxious threshold to blunt pressure was 36 ± 4kPa, n = 8. Preinjection noxious threshold to a sharp pressure was 112 ± 24 g, n = 6. *$P < .05$; ***$P < .001$, carrageenan vs saline groups.

time effect ($P < .0001$, $F_{5,50} = 17.26$), and interaction ($P < .001$, $F_{5,50} = 5.08$). Carrageenan into the hind paw induced mechanical hyperalgesia from 3 to 48 h after injection, compared with that of animals injected with saline and compared with preinjection values ($P < .01$, SNK; Fig 1). Saline injection produced hyperalgesia in rats 3 h after injection compared with preinjection values, but the hyperalgesia had resolved by 6 h ($P < .05$, SNK). Figure 2 shows the change in response to noxious blunt and sharp mechanical pressure after either saline or carrageenan was injected into the gastrocnemius muscle. With the blunt pressure to the muscle (primary hyperalgesia) there was a significant agent effect ($P < .01$, $F_{1,13} = 17.29$), time effect ($P < .0001$, $F_{5,65} = 17.70$), and interaction ($P < .01$, $F_{5,65} = 3.89$). Saline injected into the muscle produced no hyperalgesia to blunt pressure throughout the experiment. Carrageenan injection into the muscle produced significant muscle hyperalgesia to blunt pressure 3 h ($P < .05$, SNK) until 24 h ($P < .001$, SNK) after injection compared with injection with saline. The sharp noxious pressure to the gastrocnemius muscle (secondary hyperalgesia) produced a significant agent effect ($P < .01$, $F_{1,9} = 15.56$), time effect ($P < .0001$, $F_{5,45} = 15.30$), and interaction ($P < .05$, $F_{5,45} = 2.44$). There was a significantly greater decrease in threshold to sharp pressure, ie, secondary hyperalgesia, between 24 and 48 h after carrageenan injection ($P < .05$, SNK) than after saline injection into the muscle.

Tissue TNF-α

Injection of carrageenan into the hind paw induced a significant elevation in hind paw TNF-α concentration compared with cytokine concentrations measured at the same time after injection in animals injected with saline

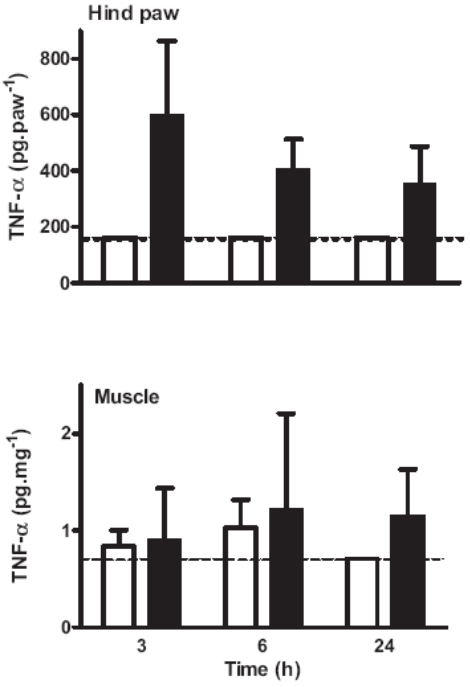

Figure 3. Concentration of TNF-α in tissue after injection of carrageenan (2 mg/100 μL saline, ■) or saline (□, 100 μL) into the hind paw and gastrocnemius muscle of the rat. There was a significant difference in the TNF-α concentration in the hind paw tissue after carrageenan injection compared with that after saline injection ($P < .001$, analysis of variance (ANOVA)). There was no significant difference in TNF-α concentration in the muscle between animals that received carrageenan and saline ($P = .24$, ANOVA) or over time ($P = .43$, ANOVA). Data expressed as mean ± SD, n = 6 per group. *Dashed line* indicates level of detection of assay.

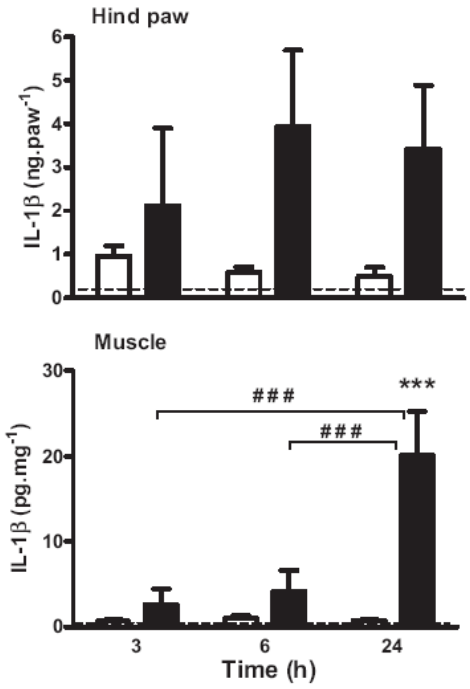

Figure 4. Concentration of IL-1β in tissue at the injection site after injection of carrageenan (2 mg/100 μL saline, ■) or saline (□, 100 μL) into the hind paw and gastrocnemius muscle of the rat. The concentration of IL-1β differed significantly overall between rats receiving carrageenan and those receiving saline both in hind paw tissue ($F_{1,10} = 36.14$, $P < .001$) and in muscle ($P < .001$, ANOVA). In addition, concentrations at 24 h differed significantly to those at 3 and 6 h after carrageenan injection into muscle ($P < .001$). Data expressed as mean ± SD, n = 6. *Dashed line* indicates level of detection of assay. ***$P < .001$, carrageenan vs saline groups. ###$P < .001$ between time intervals after carrageenan injection.

3, 6, and 24 h after injection ($P < .001$, SNK; Fig 3). There was no significant difference in the muscle TNF-α concentration between the rats that received carrageenan and and those that received saline at any time after injection (Fig 3). There was no significant difference in TNF-α concentration, expressed per mg, between the muscle tissue and hind paw tissue injected with carrageenan at any time interval, with the TNF-α concentration in the hind paw ranging from 0.9 ± 0.5 pg/mg at 3 h to 1.2 ± 1.0 pg/mg at 6 h ($P = .146$, $F_{1,30} = 2.23$; data not shown).

Tissue IL-1β

Carrageenan injection at 6h there was a significant elevation in hind paw IL-1β concentration compared with those found after saline injection ($P < .001$, SNK; Fig 4). Muscle IL-1β concentration was elevated significantly only at 24 h after carrageenan injection into the muscle compared with saline injection ($P < .001$, SNK; Fig 4). The IL-1β concentration in the muscle, unlike that of hind paw tissue, was significantly higher 24 h after carrageenan injection compared with the concentrations found at 3 and 6 h after carrageenan injection ($P < .05$, SNK, Fig 4). The IL-1β concentration in the muscle (20.1 ± 5.2 pg/mg) was significantly higher 24 h after carrageenan injection than that found in the hind paw (10.9 ± 4.8 pg/mg, $P < .01$, $F_{2,30} = 9.02$), but not 3 or 6 h after carrageenan injection.

Tissue IL-6

Injection of carrageenan into the hind paw induced a significant elevation in hind paw IL-6 concentrations 3 h

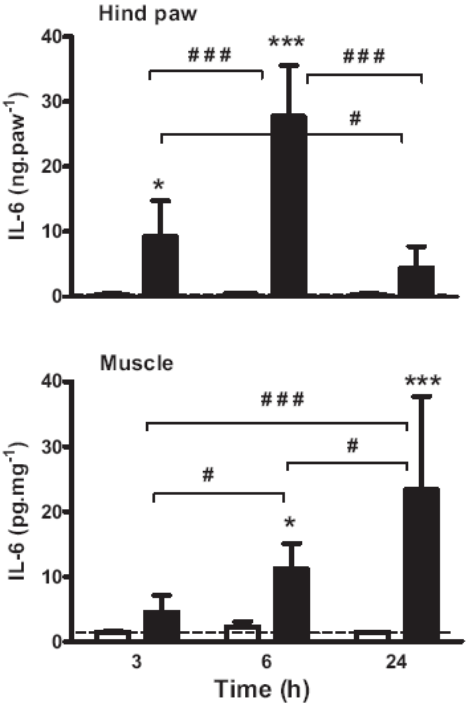

Figure 5. Concentration of IL-6 in tissue at the site of injection after injection of carrageenan (2 mg/100 μL saline, ■) or saline (□, 100 μL) into the hind paw and gastrocnemius muscle of the rat. A significant difference was found between IL-6 concentration in the hind paw tissue after carrageenan injection compared with saline injection 3 and 6 h after injection ($P < .0001$, SNK), but only 6 and 24 h after injection in the muscle ($P < .05$, SNK). There was a significant difference in both hind paw and muscle IL-6 concentration after carrageenan injection between all 3 time intervals ($P < .001$, SNK), but the maximum occurred at different times. Data expressed as mean ± SD, n = 6. *Dashed line* indicates level of detection of assay. *$P < .05$; ***$P < .001$, carrageenan vs saline groups. *$P < .05$; ###$P < .001$, between time intervals after carrageenan injection.

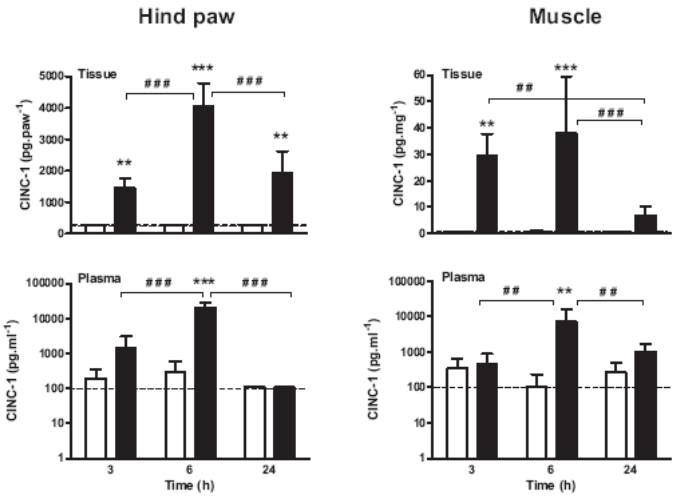

Figure 6. Concentration of CINC-1 in tissue at the site of injection and plasma after injection of carrageenan (2 mg/100 μL sterile saline, ■) or saline (□, 100 μL) into the left hind paw and gastrocnemius muscle of the rat. A significant difference was found between CINC-1 concentrations in hind paw tissue after carrageenan injection compared with saline injection 3, 6, and 24 h after injection ($P < .001$, SNK), but only 3 and 6 h after injection into the muscle ($P < .01$, SNK). Tissue CINC-1 concentration after carrageenan injection was significantly higher at 6 h than at 24 h in both hind paw and muscle and also significantly different, in hind paw only, at 3 h. The CINC-1 plasma concentrations were significantly elevated 6 h after carrageenan injection into both the hind paw and muscle compared with the saline-injected animals and, after carrageenan injection, compared with those at 3 and 24 h. Data expressed as mean ± SD, n = 6. *Dashed line* indicates level of detection of assay. $^{**}P < .01$; $^{***}P < .001$, carrageenan vs saline groups. $^{\#\#}P < .01$; $^{\#\#\#}P < .001$ between time intervals after carrageenan injection.

($P < .01$, SNK; Fig 5) and 6 h ($P < .001$, SNK) after injection compared with saline. The IL-6 concentration in the hind paw of animals injected with carrageenan peaked at 6 h and was significantly higher than at 3 and 24 h after carrageenan injection ($P < .01$, SNK). By 24 h after carrageenan injection, hind paw IL-6 concentration was no longer significantly higher than the concentrations found in animals injected with saline. Six hours, but not 3 h, after carrageenan injection into the muscle, the IL-6 concentration in the muscle was elevated significantly compared with animals injected with saline ($P < .05$, SNK), and muscle IL-6 concentrations remained elevated 24 h after carrageenan injection ($P < .01$, SNK). The IL-6 concentrations in the muscle were significantly higher 24 h after carrageenan injection compared with the concentrations found at 3 and 6 h after carrageenan injection ($P < .05$, SNK). The IL-6 tissue concentration in the hind paw was significantly higher than that found in muscle 3 h ($P < .01$) and 6 h ($P < .01$) but not at 24 h after carrageenan injection. The hind paw tissue concentration was 65.8 ± 13.4 pg/mg 6 h after carrageenan injection, which was 6 times higher than that found in muscle 6 h after carrageenan injection (11.1 ± 4.0 pg/mg).

Tissue CINC-1

Injection of carrageenan into the hind paw induced a significant elevation in tissue CINC-1 concentration 3 h ($P < .01$, SNK, Fig 6), 6 h ($P < .001$, SNK), and 24 h ($P < .01$, SNK) after injection compared with injection with saline. Only 3 h ($P < .01$, SNK) and 6 h ($P < .001$, SNK) after carrageenan injection into the muscle was tissue CINC-1 concentration significantly different from that found in the animals receiving saline. The CINC-1 concentrations in the hind paw of animals injected with carrageenan were significantly higher at 6 h compared with 3 h ($P < .001$, SNK) and 24 h ($P < .001$, SNK), but concentrations at 6 h after carrageenan injection into the muscle were significantly different only from those 24 h after injection ($P < .01$, SNK). The CINC-1 concentration in the muscle was significantly higher than that found in the hind paw tissue 3 h (4.8 ± 1.5 pg/mg, $P < .001$) and 6 h (10.7 ± 2.2 pg/mg, $P < .001$) after carrageenan injection, with hind paw concentrations 6 times less than muscle tissue 3 h after and 3.5 times less 6 h after carrageenan injection.

Plasma Cytokine Concentrations

Compared with saline injection, carrageenan injection into either the hind paw or into the muscle produced no significant increase in plasma TNF-α, IL-1β, or IL-6 3, 6, or 24 h after injection, with most values below detection limits of the assay (TNF-α of 24 pg/mL, IL-1β of 5.4 pg/mL, IL-6 of 16 pg/mL; data not shown). Plasma CINC-1 concentrations were elevated significantly 6 h after carrageenan injection into the hind paw ($P < .01$, SNK) or into the muscle ($P < .01$, SNK) compared with those after

saline injection (Fig 6), and they peaked at this time, for injection into either hind paw ($P < .001$, SNK) or muscle ($P < .01$, SNK).

Discussion

We have compared the effect on plasma and tissue cytokine concentrations, and on the associated behavioral responses to subsequent noxious challenges, of injecting carrageenan into the hind paw and gastrocnemius muscle of rats, using isovolumetric saline as the control injection. Carrageenan injection into the hind paw produced a mechanical hyperalgesia from 3 to 48 h after injection. Injection of carrageenan into the rats' gastrocnemius muscles also resulted in primary mechanical hyperalgesia to blunt pressure from 3 to 24 h after injection, and secondary hyperalgesia, to sharp pressure, was present 24-48 h after carrageenan injection.

Carrageenan-induced inflammation in the hind paw was accompanied by an early increase in TNF-α in the hind paw (3 h after injection), followed later by elevations in IL-1β and IL-6 (6 h after injection). This temporal pattern of cytokine release differed from that evident after an injection of carrageenan into the gastrocnemius muscle. In the muscle, there was no elevation in TNF-α concentration at any time, and IL-1β and IL-6 concentrations were not elevated until 24 h after carrageenan injection. The actual peak concentration, expressed per unit of tissue mass, of IL-1β in muscle tissue was double that in the hind paw, that of IL-6 one-sixth of that in the hind paw, and that of TNF-α the same, with the increase in paw TNF-α resulting from the increase in paw mass. Plasma TNF-α, IL-1β, and IL-6 were not elevated by carrageenan injection into either hind paw or muscle, compared with saline injection, at any time in our measurement period. The CINC-1 concentrations, however, rose in both plasma and tissue after carrageenan injection into both the hind paw and the muscle, with a peak concentration of plasma and tissue CINC-1 occurring 6 h after injection. This parallel increase in both plasma and tissue concentration may indicate that CINC-1 has not only a function in the local tissue but also an endocrine action.

The temporal pattern of IL-1β and IL-6 release in tissue correlated with the temporal pattern of the behavioral withdrawal threshold to a noxious sharp mechanical challenge in the hind paw and in the muscle. However, elevations of muscle tissue IL-1β and IL-6 concentration following carrageenan injection occurred only about 24 h after the primary hyperalgesia, to a blunt noxious stimulus, in the muscle. Indeed, of the 4 cytokines that we measured in muscle after carrageenan injection, only CINC-1 had elevated concentration at the time primary muscle hyperalgesia occurred. We have shown, therefore, for the first time, that the initiation of primary muscle hyperalgesia is not associated with elevations in the local concentration of TNF-α, IL-1β, or IL-6, cytokines which are known to activate the cycloxygenase enzymes[31] that regulate prostaglandin synthesis, and, conversely, that IL-1β and IL-6 concentrations are elevated at a time interval when there is no hyperalgesia.

Sharp pressure over the gastrocnemius muscle is more likely to activate cutaneous nociceptors, whereas the blunt noxious pressure activates both cutaneous and muscle nociceptors.[39] The secondary hyperalgesia, as measured by sharp pressure over the muscle, followed the same temporal pattern as that of the IL-1β and IL-6 concentration in the muscle. It is possible that elevation of IL-1β and IL-6 in muscle produced central sensitization, evident by the presence of secondary hyperalgesia of pathways leading from overlying skin but not from the muscle itself. Alternatively, there was leakage of IL-1β and IL-6 from the muscle into the overlying cutaneous tissue and direct sensitization of the cutaneous nociceptors. Yet another possibility is that the parallel development of secondary hyperalgesia in overlying skin has nothing to do with muscle IL-1β and IL-6 production.

For our investigation of cutaneous hyperalgesia, we used single injections of carrageenan into the hind paw of rats. Previous studies have reported that carrageenan injected into the hind paw induces an acute inflammation that usually peaks 2-4 h after injection,[14] that mechanical hyperalgesia develops, and that both inflammation and hyperalgesia usually resolve within 24 h.[8,11,14] In our study, however, mechanical hyperalgesia after carrageenan injection into the hind paw persisted for 48 h. Our dose of carrageenan was at least twice that of previous studies.[11,14] A dose-dependent duration of secondary hyperalgesia, but not primary hyperalgesia with injection of carrageenan has been reported previously,[29] and the intensity of both primary and secondary hyperalgesia has been shown to be positively correlated to carrageenan dose.[11,19,32] The time course of mechanical hyperalgesia present in our rats after carrageenan injection into the muscle was similar to the time course of muscle weakening reported previously in studies in which carrageenan injected into the gastrocnemius muscle of rats produced a decrease in grip force strength from 12h to 36h after injection[19]; there is a good correlation between grip strength and hyperalgesia.[19]

Carrageenan, and other inflammatory agents such as complete Freund's adjuvant, have been used frequently to explore the cytokine cascade in inflamed cutaneous tissue.[7,21,42] Consistent with previous studies, we detected increased TNF-α production in the hind paw, but not in plasma, during inflammation.[4,42] As expected, TNF-α in the hind paw was elevated 3 h after carrageenan injection. Tumor necrosis factor α is capable of activating or sensitizing nociceptors in both skin and muscle,[7,8,32] and injections of recombinant TNF-α into muscle produce mechanical hyperalgesia in rats.[32] However, we did not detect any elevation of TNF-α concentration in muscle tissue after carrageenan injection. Similarly, TNF-α concentrations do not appear to be elevated in human muscle after eccentric or concentric exercise.[24,35,38] Injecting TNF-α into a rat's muscle does not excite, but rather desensitizes, group IV afferents.[15] Also, TNF-α plays a minor role in regulating macrophage and monocyte accumulation in muscle damage.[7,36] There

have been no reports of elevated TNF-α concentration in muscle during experimental inflammation. It is possible that we missed a transient increase in muscle TNF-α in the period between 6 and 24 h after carrageenan injection, a time interval when we did not measure cytokine concentrations. However, we believe it more likely that TNF-α is not a primary mediator of hyperalgesia associated with muscle inflammation, and that injection of TNF-α into muscle does not mimic any natural process in muscle.

In the cytokine cascade accompanying inflammation, elevations in concentration of the chemoattractant cytokine CINC-1 occur later than elevations in TNF-α. Hind paw CINC-1 peaked 6 h after we injected carrageenan into the rat hind paw, following a peak of TNF-α 3 h earlier. Similarly, injection of carrageenan into a murine cutaneous air pouch resulted in peak pouch TNF-α concentrations 2 h after carrageenan injection, followed by a peak in CINC-1 around 6 h after injection.[12] We found that tissue CINC-1 concentrations were elevated, and with similar latency to onset and peak, in muscle and hind paw of our rats, in spite of there being no elevation in TNF-α concentration during muscle inflammation. The release of CINC-1 in muscle, therefore, was independent of TNF-α release in muscle, and may have been so in skin, too. Although TNF-α is capable of inducing CINC-1 release,[21] prior TNF-α release is not required for CINC-1 release following carrageenan injection.[8]

Cytokine-induced neutrophil chemoattractant 1 produces cutaneous hyperalgesia either by sensitizing nociceptors directly or by stimulating the release of sympathetic amines.[8] Cytokine-induced neutrophil chemoattractant 1 attracts neutrophils to damaged tissue, regardless of the site of injury.[12,36] However, the role of CINC-1 itself in contributing toward muscle hyperalgesia during inflammation is unclear. Interleukin-8, the human homolog of CINC-1, is released from myocytes during concentric (usually nonpainful) and eccentric (often producing delayed pain) muscle contractions. In our study, the time course of muscle and hind paw primary hyperalgesia correlated with the elevation in tissue CINC-1 concentration, so CINC-1 may play a role in sensitizing both cutaneous and muscle nociceptors.

Interleukin-6, like CINC-1, is elevated after events that lead to hyperalgesia, and also may be involved in the development of central sensitization and secondary hyperalgesia. Injection of recombinant IL-6 into the rat hind paw produces bilateral mechanical hyperalgesia.[7] The role of IL-6 in mediating muscle inflammation and hyperalgesia is less clear. Interleukin-6 is released by contracting muscle, but muscle IL-6 concentrations are elevated after exercise without concomitant muscle hyperalgesia, and IL-6 does not excite high-threshold mechanoreceptors in muscle.[15] Interleukin-6 helps to mobilize substrate for energy during exercise,[35] and that may also be its role in inflammation. In our study, the temporal pattern of hind paw IL-6 concentration correlated with the behavioral nociceptive response after carrageenan injection into the hind paw, and therefore IL-6 may contribute to cutaneous hyperalgesia. However, the delayed increase in IL-6 tissue concentration in muscle may contribute not to the initiation of hyperalgesia, but rather to maintaining hyperalgesia once the IL-6, IL-6 receptor, and gp130 complex has been established[34] and to secondary hyperalgesia, as evident in cutaneous inflammatory hyperalgesia.[7] It is equally possible that IL-6 did not contribute at all to the muscle hyperalgesia after carrageenan injection, especially considering the low IL-6 concentration during muscle inflammation compared with concentrations found during hind paw inflammation.

What is likely is that IL-1β, which followed the same temporal pattern as IL-6 in our study, contributed to the hind paw hyperalgesia and muscle hyperalgesia associated with inflammation. Interleukin-1β is a potent hyperalgesic agent,[9] and its release can be induced, together with that of IL-6, by TNF-α–dependent and TNF-α–independent pathways.[35,42] Interleukin-1β is the major cytokine stimulus for central COX-2 expression during inflammation.[31] Interleukin-1β stimulates IL-6 production during muscle injury, and the coordinated activities of both cytokines are necessary for repair and regeneration of muscle.[22,37] Interleukin-1β can be expressed constitutively by myocytes and from resident macrophages[2,36] and is elevated in human muscle for up to 5 days after eccentric exercise.[6,10] In our study, IL-1β was the only cytokine that reached a higher concentration in muscle than in hind paw after carrageenan injection. Many studies have investigated the impact of IL-1β on muscle injury, degeneration, and repair, but none previously has investigated the effect of IL-1β on muscle pain. Interleukin-1β concentration was elevated significantly 24 h after inducing muscle inflammation, which is congruent with the secondary hyperalgesia induced but not the primary hyperalgesia. It is possible that small increases in IL-1β concentrations in the muscle, undetectable in our assay, are sufficient to activate muscle nociceptors and that the large increase in IL-1β noted 24 h after induction of inflammation may contribute to sustaining the hyperalgesia and generating secondary hyperalgesia. Interleukin-1β may initiate hyperalgesia directly or via stimulation of nerve growth factor, another potent hyperalgesic agent that is present during inflammation.[30,42]

The muscle hyperalgesia we induced was experimental. Clinical muscle pains also may be attributed to the increased production and release of cytokines. Delayed-onset muscle soreness is a muscle pain, thought to be partly induced through inflammation, occurring after unaccustomed or eccentric exercise and is experienced 24-48 h after the conditioning exercise event.[23] Patients with delayed-onset muscle soreness complain of increased sensitivity to pressure applied to the affected muscles, a hyperalgesia that has been attributed to both peripheral and central sensitization,[13,20,25,26,29] possibly mediated in part by cytokines. Chronic muscle pain following injury, such as low back pain and whiplash pain, appear to occur as a result of prolonged excitation of the muscle nociceptors by neurogenic inflammation and possibly an associated cytokine production.[15,20,27,41]

Until now, no one appears to have compared experimental inflammatory muscle pain and inflammatory cutaneous pain. Our finding that both elevation in tissue IL-1β and IL-6 concentrations and secondary hyperalge-

sia are delayed after carrageenan injection into the muscle provides evidence that pharmacologic interventions targeted at preventing the release or action of IL-1β, while sparing IL-6, may attenuate secondary muscle hyperalgesia without detrimental effects on muscle regeneration. Also, our results imply that preventing the release or action of TNF-α is unlikely to be fruitful in attenuating muscle hyperalgesia.

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CHAPTER 4

Rofecoxib and tramadol do not attenuate delayed-onset muscle soreness or ischaemic pain in human volunteers.

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Rofecoxib and tramadol do not attenuate delayed-onset muscle soreness or ischaemic pain in human volunteers

L.C. Loram, D. Mitchell, and A. Fuller

Abstract: We assessed the effect of rofecoxib, a cyclo-oxygenase-2 inhibitor, and tramadol, a centrally acting analgesic, on both delayed-onset muscle soreness (DOMS) and experimentally induced ischaemic pain. We induced DOMS in 10 male and 5 female healthy volunteers by downhill running for 30 min at a 12% decline and a speed of 9 km·h^{-1}. We also induced ischaemic pain by finger movements with an arterial tourniquet around the arm. In a randomized, double-blind crossover format, we administered rofecoxib (50 mg, daily), tramadol (50 mg, 3 times per day), and a placebo (orally for 3 days), starting immediately after exercise. A 100 mm visual analogue scale (VAS) and McGill pain questionnaire were used to describe muscle soreness and ischaemic forearm pain 24 h after the exercise. The pressure pain threshold (PPT) in the thigh and ischaemic pain tolerance in the forearm were measured before exercise and 24 and 72 h after exercise. PPT decreased 24 h after exercise, compared with pre-exercise values (ANOVA, $p < 0.05$), but neither drug had any significant effect on the PPT. Neither rofecoxib nor tramadol had any effect on time of ischaemia tolerated or amount of finger activity during ischaemia. The VAS and pain-rating index, for both muscle soreness and experimental ischaemic pain, were not affected significantly by either drug. Both DOMS and ischaemic pain share peripheral and central mechanisms, yet neither are attenuated by rofecoxib or tramadol.

Key words: ischaemia, eccentric exercise, COX-2, opioid.

Résumé : Nous avons examiné l'effet du rofécoxib, un inhibiteur de la cyclooxygénase 2, et du tramadol, un analgésique d'action centrale, sur la douleur musculaire d'apparition retardée (DOMS) et sur la douleur ischémique produite en laboratoire. Nous avons induit la DOMS chez des volontaires en santé, 10 hommes et 5 femmes, par une course en descente de 30 min, sur une pente inclinée de 12 %, à une vitesse de 9 km·h^{-1}, et la douleur ischémique, par des mouvements des doigts alors qu'un garrot était placé autour du bras. Dans une étude croisée à double insu, randomizée, nous avons administré du rofécoxib (50 mg, quotidiennement), du tramadol (50 mg, 3 fois par jour) et un placebo (par voie orale pendant 3 jours), dès la fin de l'exercice. Nous avons utilisé une échelle visuelle analogue (ÉVA) de 100 mm et le questionnaire sur la douleur McGill pour décrire la douleur musculaire et la douleur ischémique de l'avant-bras 24 h après l'exercice. Nous avons mesuré le seuil de douleur à la pression (SDP) sur la cuisse et la tolérance ischémique dans l'avant-bras avant, 24 et 72 h après l'exercice. Le SDP a diminué 24 h après l'exercice, comparativement aux valeurs pré-exercice (ANOVA, $p < 0,05$), mais aucun des médicaments n'a eu d'effet significatif sur lui. Ni le rofécoxib ni le tramadol n'ont influé sur la durée d'ischémie tolérée ou sur le taux d'activité des doigts durant l'ischémie. L'ÉVA et l'index d'estimation de la douleur, pour la douleur musculaire ainsi que pour la douleur ischémique expérimentale, n'ont pas été modifiés de manière significative par l'un ou l'autre médicament. La DOMS et la douleur ischémique partagent des mécanismes périphériques et centraux, mais ne sont ni l'une ni l'autre atténuées par le rofécoxib ou le tramadol.

Mots clés : ischémie, exercice excentrique, COX-2, opioïde.

[Traduit par la Rédaction]

Introduction

Delayed-onset muscle soreness (DOMS) is a sensation of muscular pain during active contractions or passive stretch of a muscle after unaccustomed or eccentric exercise, which peaks 24–48 h after the exercise (Armstrong 1984). DOMS is thought to be caused by structural damage to muscle that leads to the release in the muscle of algogenic substances such as prostaglandins (MacIntyre et al. 1995; Tegeder et al. 2002; Cheung et al. 2003; Mense 2003). These algogenic substances activate or sensitize Aδ and C nociceptive fibres (Mense 2003). Large mechanoreceptor afferents from muscle, muscle spindles, and tendons are activated in DOMS, and may also contribute to the pain syndrome (Barlas et al. 2000*b*; Weerakkody et al. 2001, 2003). Large

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fibre contribution to nociception is characteristic of the phenomenon of central sensitization of nociceptive pathways, for which there is also evidence in DOMS (Graven-Nielsen and Arendt-Nielsen 2003; Mense 2003; Taguchi et al. 2005). Central sensitization results from changes in nociceptor transmission within the spinal cord, predominantly in the dorsal horn (Woolf 2004; Taguchi et al. 2005).

During peripheral and central sensitization, various substances activate or sensitize nociceptors. One such substance is prostaglandin. Cycloxygenase (COX) enzymes that synthesize prostaglandins appear to be activated both in the damaged tissue itself and in the central nervous system (Samad et al. 2002). Peripheral and central prostaglandin synthesis may also occur in muscle pain (Mense 2003). Therefore, it is highly anomalous that COX-inhibitors apparently do not attenuate DOMS (Hasson et al. 1993; Trappe et al. 2002), at least when administered after the exercise that caused it. Numerous studies have investigated the effect on DOMS of nonselective COX-inhibitors (see Table 1), which do not target the inducible isoenzyme cycloxygenase-2 (COX-2), the principal isoenzyme involved both centrally and peripherally in the synthesis of prostaglandin in inflammatory pain (Samad et al. 2002). We therefore investigated the effect on DOMS of the COX-2 specific inhibitor rofecoxib at a dose effective against acute pain (Fine 2002).

It is not only nonselective COX-inhibitors that fail to attenuate DOMS; the weak opioid analgesic codeine also fails to do so (Barlas et al. 2000*a*). The centrally acting analgesic tramadol hydrochloride has a mild affinity for μ-opioid receptors and also inhibits serotonin and noradrenaline reuptake, predominantly in the spinal cord (Oliva et al. 2002). Tramadol has been used successfully to manage many clinical pain syndromes, including postsurgical pain (Gibson 1996), but has not been assessed for DOMS.

Algogenic substances released in muscle, particularly prostaglandin, have the potential to contribute not only to the pain, but also to hyperalgesia, the enhanced sensitivity at the nociceptive system evident after trauma and other conditioning events (Mitchell 1999). Primary hyperalgesia manifests as an exaggerated response to a noxious challenge at the affected site, and has been identified at affected muscles in DOMS (Trappe et al. 2002). Given that central sensitization occurs in DOMS (O'Connor and Cook 1999; Graven-Nielsen and Arendt-Nielsen 2003), one would expect DOMS to be attenuated by both a COX-2 inhibitor and a centrally acting analgesic. We therefore assessed whether primary hyperalgesia to a noxious mechanical challenge occurred during DOMS and whether any hyperalgesia that did occur was attenuated by rofecoxib and tramadol.

Another type of pain that is refractory to COX-inhibitors, and indeed weak opioids (Sher et al. 1992; Gelgor and Mitchell 1995), is ischaemic pain. Ischaemic pain has a distinctive quality, so we generated this form of pain by exercising an ischaemic forearm and investigated whether the experimental ischaemic pain in the forearm was attenuated by rofecoxib and tramadol, since it is not known whether the refractoriness of ischaemic pain extends to these agents.

To address the questions we have posed, we induced DOMS in healthy young volunteers by downhill treadmill running. We subsequently administered courses of rofecoxib and tramadol at doses known to be effective in acute pain, as well as a placebo, in a double-blind crossover study. We also challenged the subjects with ischaemic stimuli, applied to the forearm, under the 3 treatment regimens.

Methods

Subjects

Fifteen subjects (10 males and 5 females; age, 24.0 ± 4.5 years; height, 1.73 ± 0.07 m; and mass, 68.8 ± 10.8 kg; mean ± SD), who were physically active but not competitive sportspersons, participated in the study. Subjects were not taking analgesics or any chronic medication. The subjects were healthy and free of pain. The subjects gave voluntary informed written consent and were free to withdraw at any time. The study was approved by the University of the Witwatersrand Committee for Research on Human Subjects (M020619) and conformed to the requirements of the International Association for the Study of Pain for studies on human subjects. Drug doses were within ranges for acute pain approved by the South African Medicine Control Council. The subjects were young and healthy, and given a single, short course of rofecoxib. Thus, they were not at risk for the adverse effects that led to the withdrawal of rofecoxib from the market (Bresalier et al. 2005).

Study procedures

Subjects reported to our exercise laboratory on 3 separate occasions. The time between the first and second trials was 13.9 ± 8.0 weeks (range, 4–30 weeks), and between the second and third trials was 11.9 ± 5.9 weeks (range, 6–27 weeks). We asked the subjects to refrain from exercise, massage, electrotherapy modalities, hot packs, caffeine ingestion, alcohol, and anti-inflammatory medication for 4 days before testing and for the 4 days of measurements. At the beginning of each trial, subjects ran downhill on a treadmill (Powerjog M30 with reversible direction motor, Sport Engineering Ltd., UK) for 30 min at a 12% decline and a speed of 9 $km \cdot h^{-1}$. Heart rate was measured at 5 min intervals during the exercise and for 30 min after exercise (Polar S610, Polar Electro Oy, Finland).

In random order and under double-blind conditions at the 3 trials, subjects were given a placebo (3 times per day), rofecoxib (50 mg in the morning, followed by 2 placebo capsules; Vioxx®, MSD, South Africa), and tramadol hydrochloride (50 mg, 3 times per day; Tramal®, Jannsen-Cilag, South Africa). The agents were administered in identical opaque gel capsules. The subjects received the first dose of each agent within 20 min of completing exercise, and then in the morning, at midday, and in the evening for 4 days. Three subjects did not complete the tramadol trial, with 1 subject displaying known adverse effects of tramadol (nausea and vomiting), the second subject developing acute cholecystitis 1 day after the exercise, and the third withdrawing from the study for undisclosed reasons. The results of these 3 subjects were used for the placebo and rofecoxib trials only.

Blood sampling

Venous blood was collected in EDTA collecting tubes immediately before, and 24 h after, the exercise. Creatine kinase concentration was measured with a colorimetric assay

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Table 1. Summary of previous studies investigating the pharmacological treatment of DOMS.

Author	Drug intervention	Exercise	Effect on muscle soreness
Barlas et al. 2000*a*	Codeine (60 mg), paracetamol (1000 mg), aspirin (900 mg)	Eccentric elbow flexion (n = 12 per group)	No change
Cannavino et al. 2003	Transdermal 10% ketoprofen	Isometric concentric and eccentric quadriceps contractions (n = 8 per group)	Change in VAS from 5.5 (placebo) to 3.0 (ketoprofen)
Donnelly et al. 1990	Ibuprofen (1200 mg before and 600 mg, every 6 h)	Downhill running (n = 16 per group)	No change
Donnelly et al. 1988	Diclofenac (50 mg, before and every 8 h)	Downhill running (n = 20)	No change
Hasson et al. 1993	Ibuprofen (400 mg)	Bench stepping (n = 5 per group)	Prophylactic ibuprofen decreased muscle soreness 24 and 48 h after excercise; therapeutic ibuprofen decreased muscle soreness 48 h after exercise
Lecomte et al. 1998	Naproxen (500 mg)	Eccentric leg extensions (n = 20)	VAS changed from 5.3 (placebo) to 3.9 (naproxen)
O'Grady et al. 2000	Diclofenac (75 mg every 12 h)	Bench stepping (n = 54)	Decrease in pain rating with prophylactic treatment
Pizza et al. 1999	Ibuprofen (800 mg 5 days before and after excercise, every 8 h)	Eccentric arm curls (n = 10)	No change
Sayers et al. 2001	Ketoprofen (25 mg, 100 mg)	Eccentric elbow flexors (n = 48)	19% change in VAS after 100 mg ketoprofen
Tokmakidis et al. 2003	Ibuprofen (400 mg) every 8 h	Eccentric leg curls (n = 19)	VAS changed from 5.5 (placebo) to 3.8 (ibuprofen)
Trappe et al. 2001, 2002	Ibuprofen (400 mg) every 8 h; paracetamol (1500 mg) every 8 h	Eccentric knee extension (n = 24)	No change
Tegeder et al. 2003	Morphine-6-β-glucuronide, morphine (infusion)	Concentric and eccentric calf exercise (n = 10)	Decrease in VAS by 37% with morphine-6-β-glucuronide and 71% decrease with morphine

Note: VAS, visual analogue scale.

(Roche Diagnostics, South Africa) before exercise and 24 h after exercise.

Pressure pain

We applied a pressure algometer with a 100 mm^2 probe (Somedic, AB, Sweden) at 3 points on the thigh, midway between the medial femoral condyle and the patella, and midway between the lateral femoral epicondyle and the patella, 70 mm superior to the superior border of the patella. These points targeted the vastus medialis and vastus lateralis muscles, near the musculotendinous junction. The third point targeted the rectus femoris muscle, midway up the thigh, in the same line as the midpoint of the patella. Pressure pain threshold was taken as the pressure at which the subject first indicated that the sensation induced by the algometer was painful. The mean of 1 measurement taken at each of the 3 sites was recorded as the pressure pain threshold. Pressure pain threshold was assessed before exercise and 24 and 72 h after the exercise, by the same experimenter, to maintain the same rate of increase of pressure and technique of applying the algometer. The subjects were unable to read the measurements on the pressure algometer and were not informed of their results, and thus were blinded to their pressure pain measurements.

Ischaemic pain

We induced ischaemia in the forearm of the dominant arm by inflating a tourniquet cuff, placed around the upper arm, to 140 mmHg (above systolic pressure; 1 mmHg = 0.133 kPa). The subject rested the arm on a shelf that left the wrist and hand free to move over a series of vertically arranged bolts, with nuts initially screwed down at the base of the bolt (Fig. 1). Once the tourniquet was inflated, the subject rested the arm for 1 min, and then turned the nuts up the bolts, with the thumb and at least 1 finger, as fast as possible for 45 s. The rest and active phases were repeated until the subject could no longer tolerate the pain associated with turning the nuts, at which point we released the tourniquet. We measured the time to the subject's volitional termination of the test and cumulative distance the subject had moved nuts up bolts, and calculated an ischaemic pain tolerance index by multiplying the duration of ischaemia (s) by the total distance over which nuts were moved (m). We measured the subject's response to ischaemic pain before exercising and 24 and 72 h after exercise.

Subjective pain assessments

Twenty-four hours after the exercise, subjects completed 3 McGill Pain Questionnaires (MPQ) and visual analogue scales to the pain they felt following application of pressure to thigh muscles, and the pain induced by exercise of an ischaemic forearm. The subject was asked to describe the pain experienced during squatting. This description was used to describe the DOMS pain and was measured 24 h after the

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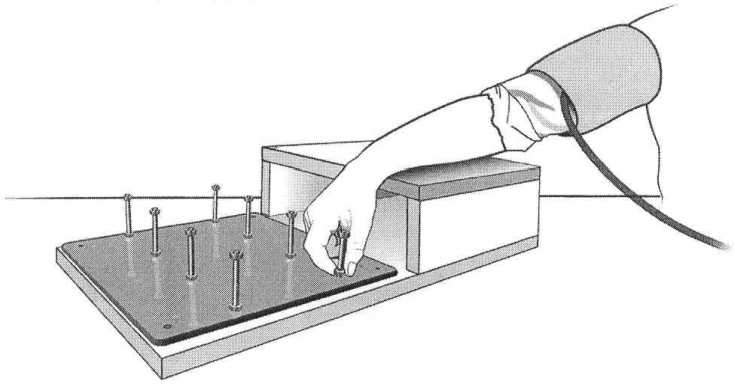
Fig. 1. The thumbscrew rack used to produce the pain in the forearm muscles during forearm ischaemia. A tourniquet was placed around the nondominant arm and inflated to 140 mmHg (1 mmHg = 0.133 kPa). The subject was asked to turn the nuts up the bolts with the thumb and at least one finger as fast as possible for 45 s followed by 1 min of rest, until that process became intolerably painful. The time to the subject's volitional termination of the test and cumulative distance the subject moved the nuts up the bolts were measured and an index of ischaemic pain tolerance was calculated by multiplying the time (s) and the total distance (m) nuts were moved.



exercise. The 100 mm visual analogue scale was anchored at "no pain" and "worst pain ever experienced".

Statistical analysis

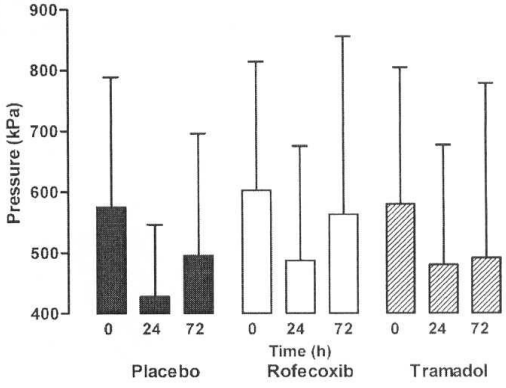
Data are shown as mean ± SD unless otherwise stated. One-way analysis of variance (ANOVA) was used to test for differences in heart rate among the 3 exercise trials and among the 3 drug trials. We used nonparametric Kruskal–Wallis analyses to test for differences in plasma creatine kinase concentrations before and after exercise. Two-way repeated measures analysis of variance, with time and drug as the main effects, was used to compare measurements of pain unless otherwise stated. Tukey post-hoc tests were carried out when significant differences were found. Visual analogue scale data were normalized using the arcsine transform and data were recorded as mean (±SD). We used a Chi-square and Fisher's exact test to compare the frequency of word choice on the McGill Pain Questionnaire. For the tramadol trial, the number of subjects choosing the selected words was converted to an integer proportion of 15, since only 12 subjects completed the trial. We considered p-values ≤0.05 significant. Post-hoc power analyses are reported in the results.

Results

Equivalence of exercise trials

The heart rate for each exercise trial was analysed according to the order in which the trials were completed, irrespective of the agent administered. The average heart rate of the subjects when they were exercising in the first trial was 142 ± 17 beats per min, 131 ± 16 beats per min in the second trial, and 133 ± 13 beats per min in the third trial. There were no significant differences among the 3 trials for either the average heart rate ($F_{[2,36]}$ = 1.83, p = 0.18), maximum heart rate ($F_{[2,36]}$ = 1.55, p = 0.23) or the heart rate during the first 30 min of the subject's recovery ($F_{[2,36]}$ = 0.19, p = 0.83).

Fig. 2. Pressure pain threshold (mean ± SD, n = 15), calculated as the average of threshold over the vastus medialis, vastus lateralis, and rectus femoris muscles following application of the pressure algometer to the thigh, measured before (represented as 0) and 24 and 72 h after downhill running followed by administration of placebo, rofecoxib, and tramadol. Two-way repeated measures ANOVA showed a significantly different variation of threshold with time ($F_{[2,22]}$ = 19.17, p < 0.0001) but not with agent administered ($F_{[2,22]}$ = 0.36, p = 0.70). Tukey post-hoc tests revealed that pressure pain threshold were significantly lower at 24 h following exercise than at other times.



Creatine kinase

No significant differences were found in the difference in creatine kinase concentration induced by the downhill running following the administration of the placebo (40 ± 38 U·L^{-1}), rofecoxib (30 ± 42 U·L^{-1}), and tramadol (34 ± 41 U·L^{-1}; Kruskal–Wallis = 0.48; p = 0.79). The plasma creatine kinase concentrations also did not change significantly

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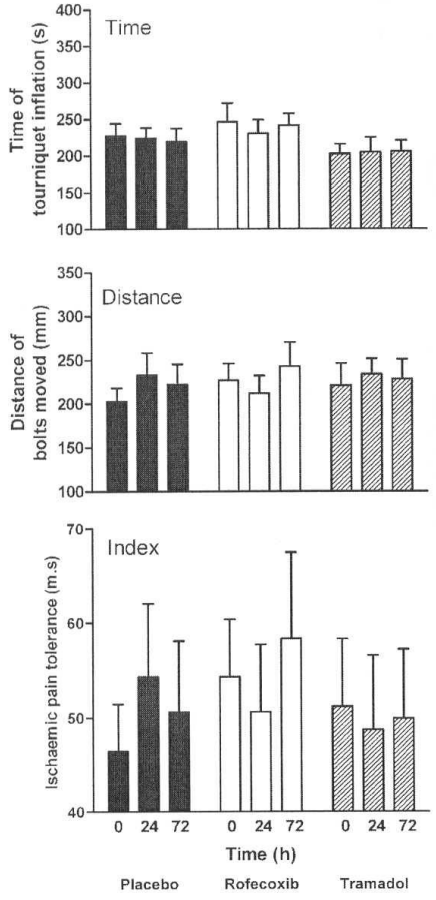
Fig. 3. Time of tourniquet inflation, distance of movement of the bolts, and ischaemic pain tolerance index (mean ± SD, n = 15), measured using the thumbscrew, before (represented as 0) and 24 and 72 h after downhill running followed by administration of placebo, rofecoxib, and tramadol. Two-way repeated measures ANOVA showed no significant variation of index with time ($F_{[2,22]}$ = 1.30, p = 0.292) or with agent administered ($F_{[2,22]}$ = 1.18, p = 0.33). There was no significant variation in the distance of the bolts moved or time of inflation over time or among agents administered (p > 0.05).

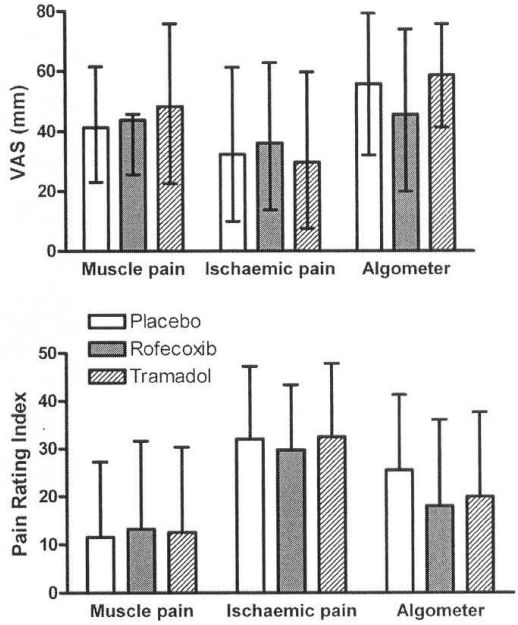


among the 3 trials, irrespective of the agent administered (Kruskal–Wallis = 0.83; p = 0.66).

Pressure pain

Figure 2 shows the pressure pain threshold (PPT) of the thigh muscles, measured before and 24 and 72 h after downhill running, for subjects receiving the 3 agents. For all 3 treatment regimens, the threshold was significantly lower at 24 h after the exercise than at all other times, but there was no significant effect of rofecoxib or tramadol on PPT 24 h after downhill running (Fig. 2), or at any other time, compared with that of the placebo. A post hoc power analysis revealed (1– β) = 0.95 (n = 15, α = 0.05) to detect a 20% change in PPT.

Fig. 4. Pain intensity (mean ± SD) of the muscle soreness from the downhill running (delayed-onset muscle soreness; DOMS), the pressure pain on the affected thigh, and the pain of the exercise in the ischaemic forearm measured on a 100 mm visual analogue scale (VAS) and pain rating intensity (PRI) scale 24 h after downhill running. VAS data were normalized by arcsine transform for statistical analysis. ANOVA showed no significant differences in the VAS among responses during administration of the 3 agents for DOMS ($F_{[2,30]}$ = 0.26, p = 0.77), pressure pain ($F_{[2,21]}$ = 0.15, p = 0.86), or ischaemic pain ($F_{[2,27]}$ = 0.69, p = 0.51). There was no significant difference in PRI after administration of any agent when used to rate the muscle pain (Kruskal–Wallis = 0.13, p = 0.94), ischaemic pain (Kruskal–Wallis = 0.10, p = 0.95), or pain from the algometer (Kruskal–Wallis = 0.05, p = 0.97).



Ischaemic pain in the forearm

Figure 3 shows the ischaemic pain tolerance index measured before and after downhill running under the 3 regimens. There were no significant differences among the index, length of time that the tourniquet was inflated, and amount of finger activity at any time interval. Rofecoxib and tramadol administration had no significant effect on index ($F_{[2,22]}$ = 1.18; p = 0.32), length of time the tourniquet was inflated ($F_{[2,22]}$ = 0.76; p = 0.48), or amount of finger activity ($F_{[2,22]}$ = 0.13; p = 0.88). A post hoc power analysis revealed (1– β) = 0.7 (n = 15, α = 0.05) to detect a 50 $m \cdot s^{-1}$ change in ischaemic index.

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Pain intensity and quality

DOMS was induced successfully, with a pain intensity at 24 h after downhill running and after placebo administration, according to the VAS, of 33 mm (61 mm (+SD), 10 mm (–SD); see Fig. 4). Administration of rofecoxib or tramadol did not significantly change the intensity ($1 - \beta = 0.85$, $\alpha = 0.05$, to detect a 20 mm change in VAS). The pain rating intensity (PRI) scale, calculated from the words selected from the McGill Pain Questionnaire (MPQ), was not different after administration of any agent when used to rate the muscle pain, ischaemic pain, or pain from the algometer. Figure 4 shows the PRI and VAS for muscle pain, pain after applying the pressure algometer, and the pain experienced during ischaemia in the forearm after placebo, rofecoxib, and tramadol administration.

The subjects complained of muscle soreness during muscle contraction and described the pain, using the MPQ, as tiring, tender, and tight. Table 2 shows the words chosen by 50% or more of the subjects from the MPQ to describe either the leg muscle pain experienced after downhill running, the pain experienced when the pressure algometer was applied to the quadriceps muscle, or pain during exercise of the ischaemic forearm 24 h after downhill running. Administration of rofecoxib or tramadol made no significant difference to the words chosen frequently to describe the 3 types of pain.

Table 2. Words chosen from the McGill Pain Questionnaire by 50% or more of the subjects to describe the muscle soreness in the thighs felt 24 h after downhill running, the pain perceived when a pressure algometer was pressed onto the affected thigh, or ischaemic pain induced in an exercising forearm.

	Word chosen	Placebo	Rofecoxib	Tramadol
Muscle soreness	Tiring	5	7	8
	Tender	7	4	5
	Tight	6	6	7
Pressure pain	Penetrating	8	12	11
	Pressing	9	6	6
	Tender	7	7	5
Ischaemic pain	Throbbing	9	6	6
	Pressing	8	6	3
	Tiring	8	5	6

Note: There was no significant association between word choice and agent administered for muscle soreness ($\chi^2 = 1.45$, $p = 0.75$), pressure pain threshold ($\chi^2 = 1.91$, $p = 0.83$), or ischaemic pain ($\chi^2 = 1.04$, $p = 0.90$).

Discussion

The presence of DOMS in the quadriceps muscles after downhill running was confirmed by the subjects' assessment of muscle soreness on the VAS (33 mm) and PRI as moderate pain 24 h after exercise and by the significant reduction in pain tolerance to a noxious mechanical challenge on the quadriceps muscles at that time; i.e., primary hyperalgesia. Neither rofecoxib nor tramadol, at the conventional doses we used, attenuated the intensity of the DOMS, nor did tramadol and rofecoxib reduce muscle sensitivity to mechanical pressure. Tramadol and rofecoxib also had no effect on experimentally induced forearm ischaemic pain. Muscle soreness and ischaemic pain also differed in quality: DOMS pain was described as tender, while ischaemic pain was described as pressing and throbbing. Therefore, both DOMS and ischaemic pain were not attenuated by a COX-2 inhibitor, rofecoxib, or the multi-action analgesic tramadol.

The failure of the 2 drugs to attenuate DOMS might have arisen because the doses of the agents were too low to alleviate muscle pain of the intensity that we generated. However, rofecoxib and tramadol have been used successfully for the treatment of pain more intense than the DOMS experienced by the subjects, as rated on the VAS (Bamigbade and Langford 1998; Fine 2002). The subjects scored the intensity of the DOMS pain as 33 mm on the VAS and 25 on the PRI, which is a score considered to reflect modest pain (O'Connor and Cook 1999). It may be, however, that musculoskeletal pain requires a larger dose of analgesic or antihyperalgesic agents to be effective (Gibson 1996; Kalso et al. 2004). However, 4 times the usual dose of ibuprofen has been shown to be ineffective in treating DOMS (Donnelly et al. 1990).

Exercise studies with crossover designs, such as ours, may be confounded by training adaptations that occur over time. However, we detected no change in either heart rate or creatine kinase concentration of subjects among the 3 trials. The period of 13 weeks, on average, between trials was, therefore, sufficient to allow subjects to begin each trial in an equivalent state. A previous study employing a 9 week interval between eccentric exercise bouts also showed no significant differences between bouts in muscle soreness or plasma creatine kinase concentrations (Byrnes et al. 1985). The creatine kinase changes in our study were similar to those recorded in previous studies, which induced DOMS by eccentric leg curls or bench stepping (Hasson et al. 1993).

The effect of COX-inhibitors on DOMS pain has been reviewed by Baldwin-Lanier (2003), in which she produced a table that included, for each study, the size effect of the drug on muscle soreness. A further possible reason for the lack of effect of rofecoxib and tramadol in treating DOMS may be type II errors in our statistical analyses. However, we performed post hoc power analyses on all our outcome measures and found a power of 70% or higher to be able to detect a 20% change in the VAS and PPT, which is considered to be a clinically relevant change in pain. Also, when comparing the size effect (based on PPT) and sample size of our study with those calculated in other studies, we found similar results, with a size effect of 0.22 for rofecoxib and 0.52 for tramadol (Baldwin-Lanier 2003).

The DOMS we produced had characteristics similar to DOMS produced in other studies, including increased sensitivity to pressure on the affected muscle (Hasson et al. 1993; Barlas et al. 2000*b*). The DOMS also had a similar intensity, as measured by the VAS (Byrnes et al. 1985; Lecomte et al. 1998; Barlas et al. 2000*b*; Tokmakidis et al. 2003). Also, the words selected to describe DOMS in our study were similar to those in other studies in which DOMS was described as tiring and tender (MacIntyre et al. 1995; Bajaj et al. 2001). We showed that DOMS present 24 h after exercise was not attenuated by the selective COX-2 inhibitor rofecoxib. Similarly, Trappe et al. (2002) found that 400 mg ibuprofen, a nonselective COX-inhibitor with low activity for COX-2, failed to decrease soreness in the affected muscle 24 and 48 h after the exercise. A 400 mg dose of ibuprofen is

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equivalent to 50 mg rofecoxib, at least in reducing dental pain after surgery (Langford and Evans 2002). DOMS also was unaltered by another nonselective COX-inhibitor, naproxen, and by a putative COX-3 inhibitor, paracetamol (acetaminophen) administered after the exercise (Lecomte et al. 1998; Barlas et al. 2000*b*). Thus, the failure of COX-inhibitors to relieve DOMS extends to the inhibition of all 3 isoenzymes, i.e., COX-1, COX-2, and COX-3. Therefore, muscle nociceptors are predominantly activated or sensitized by some mechanism other than prostaglandin, unless the central sensitization occurs in the time after the exercise is completed but before the agent is absorbed. Thus, we confirm that, though prostaglandin is synthesized in the affected muscle and probably in the CNS during DOMS, agents that inhibit its synthesis, at doses effective for other pains, do not affect the intensity of the DOMS when administered after the exercise.

Another illustration of the intractability of DOMS to the agents' effectiveness in relieving other pain is evident in the failure of tramadol to attenuate the DOMS we generated. Central sensitization, the increased excitability of neurons in the dorsal horn of the spinal cord, occurs with longer lasting effects in muscle pain than those found in cutaneous pain (Wall and Woolf 1984). Initially, central neurons are activated by nociceptive input from the periphery. If the input is sustained, molecular changes within the spinal cord occur, and central sensitization results (Woolf 2004). Tramadol is likely to attenuate pain transmission by inhibiting noradrenaline and serotonin reuptake in the brain and spinal cord, particularly by targeting the descending inhibitory pathways, thereby decreasing the impact of central sensitization (Oliva et al. 2002). Tramadol also binds to opioid receptors, and codeine (60 mg), another opioid, given to subjects within 90 min of performing eccentric elbow flexion exercise, had no effect on either pressure pain tolerance during DOMS or intrinsic pain in the muscle (Barlas et al. 2000*b*). However, DOMS was effectively attenuated when morphine-6-glucuronide, a morphine metabolite unable to penetrate the blood–brain barrier, was administered in a 2 h intravenous infusion 24 h after DOMS (Tegeder et al. 2003). Peripheral opioid receptors, therefore, may play a role in attenuating muscle pain. Also, morphine administered to rats after eccentric muscle contractions led to suppression of c-Fos activation in the dorsal horn of the spinal cord (Taguchi et al. 2005), suggesting that there is some spinal neuronal activation occurring in DOMS, but not necessarily nociceptor activation. Activation of peripheral opioid receptors, therefore, may attenuate DOMS, but the centrally acting multitarget antinociceptive tramadol, which is effective against other acute pains (Bamigbade and Langford 1998), did not attenuate DOMS.

We showed that the process that leads to DOMS leads to primary hyperalgesia (decreased tolerance to noxious pressure at the site); however, it is unusual because, unlike other hyperalgesias (Mitchell 1999), it did not respond to either COX inhibition or tramadol, implying a complex polymodal mechanism (Woolf and Mannion 1999). Ischaemic pain also is notoriously intractable to agents other than strong opioids (Sher et al. 1992). The mechanisms producing the hyperalgesia during DOMS and ischaemic pain must involve parallel and independent pathways, which rely on different neurochemical substrates. The hyperalgesia of DOMS and ischaemic pain differs from reperfusion hyperalgesia, for which there also are multiple neurochemical substrates, but they occur in series, since COX-inhibitors, opioids, antihistamines, excitatory amino acid antagonists, and benzodiazapines all individually abolish the hyperalgesia (Cartmell and Mitchell 1993).

Although administration of COX-inhibitors after the exercise does not attenuate DOMS, administration of COX-inhibitors before exercise indeed does do so (Hasson et al. 1993; Tokmakidis et al. 2003). That observation makes DOMS different from other trauma-induced hyperalgesia, for which pre-emptive COX administration is no more effective than post-trauma administration (McQuay 1995). However, it does imply that prostaglandins are synthesized during the exercise that induces and contributes to DOMS, even though the DOMS does not appear until many hours later, and when it appears, is not attenuated by blocking prostaglandin synthesis. Although we have some information about muscle pain (Mense 1991) and about the muscle events that sustain DOMS (MacIntyre et al. 1995), we still do not understand the neural mechanisms that lead to DOMS. The pain does not arise predominantly through local inflammatory or traumatic phenomena, otherwise rofecoxib and other COX-inhibitors would abolish the pain. It is a centralized pain, as evident from the secondary hyperalgesia and large fibre input, but unusual in that the maintenance of central sensitization apparently is not prostaglandin dependent. Future investigations on antinociception in DOMS, therefore, will require more than the conventional antinociceptives at conventional doses and may require concurrent administration of more than one class of analgesic.

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CHAPTER 5

CONCLUSION

The main roles of cytokines during cutaneous inflammation have been elucidated. However, the exact mechanism of how cytokines contribute to hyperalgesia, in particular muscle hyperalgesia is less clear. For example, whether cytokine receptors are present on muscle nociceptors, and if the same cytokines that sensitize cutaneous nociceptors are able to sensitize muscle nociceptors has not been determined. If the pro-inflammatory cytokines are able to induce muscle hyperalgesia, it is yet to be established whether the cytokines produce an intensity and temporal profile of hyperalgesia in muscle comparable to that found in inflamed cutaneous tissue. Finally, it is not clear whether other chemical mediators known to sensitize cutaneous nociceptors, such as prostaglandin, produce a similar effect in muscle. In this thesis I have addressed some of the issues raised above in order to further understand the mechanism of inflammatory muscle pain, in particular the role chemical mediators play in peripheral sensitization of muscle nociceptors during inflammation. The results obtained from the studies I undertook are summarized in this chapter.

5.1 Inflammatory hyperalgesia, cytokines and histology

The hind paw of rats is the conventional site used to investigate inflammatory hyperalgesia (Vinegar *et al.*, 1987; Hargreaves *et al.*, 1988). Despite extensive studies investigating the mechanisms of inflammatory hyperalgesia (Winter *et al.*, 1962; Vinegar *et al.*, 1987; Cunha *et al.*, 1991; Cunha *et al.*, 1992; Safieh-Garabedian *et al.*, 1995; Cunha *et al.*, 2000; Lorenzetti *et al.*, 2002), no studies have investigated concurrent histological changes and changes in hind paw and plasma pro-inflammatory cytokines. The

glabrous epidermis and underlying fat pad of the hind paw are different to that of other cutaneous tissue within the body in that the glabrous tissue is thicker and hair follicles are absent. I therefore also investigated the effects of injecting carrageenan, an inflammatory agent, into a tissue different to the cutaneous tissue of the hind paw, namely the keratinized tissue of the rat tail, and measured the behavioural response to a noxious pressure and noxious heat (Chapter 2). I found that an injection of carrageenan into the rat tail induces both mechanical and thermal hyperalgesia, with thermal hyperalgesia induced within three hours and mechanical hyperalgesia within six hours of injection. Both the thermal and mechanical hyperalgesia resolved 24h after injection. The behavioural responses following injection of carrageenan into the rat tail (Chapter 2) are comparable to those found in other studies, including my own study (Chapter 3), where carrageenan was injected into the hind paw (Vinegar *et al.*, 1987; Hargreaves *et al.*, 1988)

Since inflammatory hyperalgesia can be induced by carrageenan injection into the rat tail, I then measured the cytokine changes in both the plasma and the tail tissue, and the histological changes (Chapter 2), to identify whether the temporal profile of the hyperalgesia is comparable to that of the histological changes and the cytokine concentrations. I found that the peak in hyperalgesia, measured at the site of the carrageenan injection, coincided with moderate to severe inflammatory cell infiltration. My results also showed a substantial increase in pro-inflammatory cytokine concentrations at the site of injection with the concentrations of all four pro-inflammatory cytokines, TNF-α, IL-1β, IL-6 and CINC-1, peaking at the same time as

that of the hyperalgesia and the cell infiltration. Therefore, at any site of cutaneous inflammation, the hyperalgesia, histology and cytokine release are likely to be similar.

Although it has been well established that cytokines contribute to inflammatory hyperalgesia, the exact mechanism is yet to be determined. TNF-α, IL-1β and IL-6 are able to sensitize nociceptors, but is not clear how this sensitization is attained, or whether cytokine receptors are present on nociceptors. It is possible that pro-inflammatory cytokines stimulate the release of other mediators, such as neurotrophic growth factor (NGF), substance P and prostaglandins, all of which sensitize nociceptors (Baldwin Lanier, 2003). IL-1β and IL-6 both stimulate the release of cyclo-oxygenase, a rate-limiting enzyme in the synthesis of prostaglandins. Prostaglandins sensitize cutaneous nociceptors and IL-1β- and IL-6-induced hyperalgesia is attenuated by a COX-inhibitor, indomethacin (Cunha *et al.*, 1992). Therefore, IL-1β and IL-6 may not directly sensitize nociceptors, but rather sensitize nociceptors by stimulating the release of prostaglandins. Nonetheless, IL-6 and IL-1β do contribute directly or indirectly to cutaneous inflammatory hyperalgesia.

My findings do not resolve whether these cytokines directly contribute to the hyperalgesia induced. However, my findings are in agreement with previous studies (Winter *et al.*, 1962; Vinegar *et al.*, 1987; Cunha *et al.*, 1992; Safieh-Garabedian *et al.*, 1995; Lorenzetti *et al.*, 2002), the results of which infer that the inflammatory cell infiltration and the pro-inflammatory cytokines released during inflammation, regardless of the cutaneous site injected, contribute to the inflammatory hyperalgesia induced by carrageenan injection. I believe that both TNF-α and IL-1β play an

important role in inflammatory hyperalgesia in cutaneous tissue, but through stimulating the release of other mediators such as prostaglandins, NGF and substance P (Safieh-Garabedian *et al.*, 1995; Woolf *et al.*, 1997). Further work that is able to identify receptor molecules found on nociceptors and the receptor activity is required to confirm any direct role that pro-inflammatory cytokines play in inflammatory hyperalgesia.

The role that one of the pro-inflammatory mediators, the chemokine, CINC-1, plays in inflammatory hyperalgesia has received little attention. It is involved in sympathetically-mediated pain and inflammatory hyperalgesia (Lorenzetti *et al.*, 2002). CINC-1 may play a more important role in central sensitization which follows peripheral activation of nociceptors by other inflammatory mediators (Yamamoto *et al.*, 1998). However, my findings show a relationship between the hyperalgesia and the CINC-1 concentration in the inflamed tissue. It seems that CINC-1 may play a role locally and also at a site remote from the site of inflammation, such as within the central nervous system. The function of CINC-1 at a more remote site would explain the increased plasma CINC-1 concentration. I propose that one of the roles that CINC-1 may have at the site of inflammation, during inflammatory hyperalgesia, is to increase the infiltration of inflammatory cells which subsequently release cytokines to the area of inflammation and contribute to neurogenic inflammation. This theory would explain why atenolol and propanolol, adrenergic-receptor antagonists, attenuated CINC-1 induced hyperalgesia (Cunha *et al.*, 1991). It would also explain why following an injection of carrageenan, in my rats CINC-1 concentrations increased early, attracting neutrophils and increasing infiltration of further inflammatory mediators. The function of CINC-1

during inflammatory hyperalgesia needs further research, especially to identify CINC-1 receptors and their location.

5.2 Inflammatory hyperalgesia in muscle

In addition to studying inflammatory hyperalgesia in cutaneous tissue, I also investigated inflammatory hyperalgesia in muscle and compared the hyperalgesia to that of inflamed cutaneous tissue. Muscle pain is different to cutaneous pain in quality, pattern of pain and duration, but the mechanisms underlying these differences are yet to be determined. Carrageenan has been used previously to induce muscle inflammation, and in particular inflammatory muscle hyperalgesia (Berberich *et al.*, 1988; Radhakrishnan *et al.*, 2003). I induced muscle hyperalgesia in rats, similar to that found in previous studies (Kehl *et al.*, 2000), by injecting carrageenan into the muscle. Unlike Radhakrishnan *et al* (2003) who were able to produce bilateral secondary hyperalgesia in the hind paw after unilateral injection of carrageenan into the gastrocnemius muscle, I found no secondary hyperalgesia in the contralateral limb.

Although muscle inflammatory hyperalgesia has been induced previously, no studies have measured the cytokine changes that occur with the inflammation. My findings show that cytokines are elevated following injection of carrageenan into the muscle, but that the temporal patterns of the change in IL-1β and IL-6 are different to those found after cutaneous inflammation, in the hind paw, and different to the temporal pattern of muscle hyperalgesia. Also, at no time interval that I measured was muscle TNF-α concentration elevated above that of muscle injected with saline.

There are conflicting results regarding the effect that TNF-α has on muscle pain. Exogenous TNF-α, injected into muscle induces mechanical hyperalgesia (Schafers *et al.*, 2003). TNF-α, however, also desensitizes muscle nociceptors (Hoheisel *et al.*, 2005), but does so within 20min following TNF-α injection, with mechanical hyperalgesia still evident 6h to 28h after injection. Therefore, TNF-α may initially desensitize muscle nociceptors, and later sensitize muscle nociceptors, resulting in hyperalgesia.

Although TNF-α injected into muscle induces hyperalgesia (Schafers *et al.*, 2003), muscle TNF-α concentrations are not elevated following eccentric exercise (Malm *et al.*, 2000; Toft *et al.*, 2002) or, as my study shows, after carrageenan injection (Chapter 3). TNF-α is a key inflammatory mediator in cutaneous tissue but it does not appear to be important in muscle inflammation. TNF-α has catabolic effects in muscle such that increased concentrations of TNF-α in muscle results in proteolysis and muscle wasting (Goodman, 1991; Li & Reid, 2001), as evident in patients with sepsis, rheumatoid arthritis and human immunodeficiency virus (Rall *et al.*, 1996; Moldawer & Sattler, 1998; Coletti *et al.*, 2005). However, it still remains controversial as to whether TNF-α alone is able to result in cachectic effects in experimental conditions in vivo (Flores *et al.*, 1989; Michie *et al.*, 1989; Mullen *et al.*, 1990; Goodman, 1991; García-Martínez *et al.*, 1993; Peterson *et al.*, 2006).

TNF-α also plays a role in insulin resistance by impairing glucose storage and glucose uptake, as evident in patients with diabetes mellitus (Saghizadeh *et al.*, 1996; Zhang *et al.*, 2000). TNF-α may also play a role in sarcopenia associated with aging (Dirks & Leeuwenburgh, 2006). Although TNF-α is produced in resting muscle (Saghizadeh *et*

al., 1996), the release of TNF-α into normal muscle appears to be suppressed by IL-6 (Starkie *et al.*, 2003). In a study conducted in our laboratory on healthy male volunteers, where mild skeletal muscle injuries were induced by eccentric leg presses, TNF-α concentrations in muscle are not appear elevated (Rice TL, Chantler I, Loram LC, unpublished data). However, in extreme injuries, such as a freeze injury in rats (Warren *et al.*, 2002), and rheumatoid arthritis in humans (Flagg *et al.*, 2005), muscle TNF-α concentration is elevated. Therefore, under normal homeostatic conditions the release of TNF-α in muscle is suppressed, but if the skeletal muscle injury is more severe or the muscle homeostasis is disturbed, TNF-α concentration in muscle is elevated.

Unlike TNF-α, IL-1β and IL-6 concentrations are elevated following muscle injury, but their release is different in muscle inflammation compared to in cutaneous inflammation, with the release of IL-1β and IL-6 being delayed in muscle inflammation. No work has investigated the role of IL-1β in muscle pain, but it is a potent sensitizer of nociceptors in cutaneous tissue (Ferreira *et al.*, 1988). It still is not clear whether IL-1β is able to sensitize nociceptors directly by acting on IL-1 receptors, but it does stimulate the release of cyclo-oxygenase enzymes, which synthesize prostaglandins.

If IL-1β sustains inflammatory hyperalgesia in muscle, it would be worthwhile investigating the rat's behavioural response following an intramuscular injection of carrageenan and the administration of an inhibitor of IL-1β, such as a caspase-1 inhibitor, at the site of inflammation and within the spinal cord. In addition, further investigations should attempt to identify the presence of prostaglandins and NGF in

the muscle, and possibly block their effects. Nonetheless, my study investigating the cytokine profile following an injection of carrageenan into the muscle is a useful model to investigate the mechanisms of inflammatory muscle pain. Targeting possible key mediators in this model of inflammatory muscle pain may illuminate some novel therapeutic targets in treating clinical muscle pain.

5.3 Clinical muscle pain

Animals models used to mimic clinical conditions allow us to measure variables invasively and manipulate conditions in a manner that is difficult to do ethically in human studies. However, if we are to transfer conclusions drawn from animal-based experiments to human pathology, it is best to confirm the results obtained using a human experimental model. Therefore, I investigated muscle pain in humans using an experimental model where pain can be induced in the laboratory under controlled conditions, but the pain induced also is experienced during daily activities, particularly unaccustomed exercise (Chapter 4). DOMS is the most appropriate model for inflammatory pain as DOMS is produced in part from muscle inflammation and micro trauma. Much is understood of DOMS regarding the muscle injury and the associated symptoms, but a few key questions are unanswered. Firstly, what causes the pain associated with the micro trauma to the muscle after unaccustomed exercise and, secondly, why is there a delay in the pain experienced?

In the study reported in chapter 4, I attempted to identify possible mechanisms behind the pain associated with DOMS assuming that inflammation played a role. IL-1β is elevated during muscle inflammation (Chapter 2), and is elevated following eccentric

muscle contractions (Cannon *et al.*, 1989; Fielding *et al.*, 1993). IL-1β stimulates the release of prostaglandins, via cyclo-oxygenase stimulation, which sensitize nociceptors. Therefore, COX-inhibitors should attenuate DOMS. Previous studies have attempted to investigate which COX inhibitors attenuate DOMS, but with little success (Baldwin Lanier, 2003). Trappe *et al.* found that 400mg ibuprofen, a non-selective COX inhibitor, with low activity for COX-2, administered after the exercise failed to decrease soreness in the affected muscle (Trappe *et al.*, 2002). DOMS also was unaltered by another non-selective COX inhibitor, naproxen, and by a putative COX-3 inhibitor, paracetamol (acetaminophen), administered after the exercise (Lecomte *et al.*, 1998; Barlas *et al.*, 2000a). I showed that DOMS induced after exercise was not attenuated by the selective COX-2 inhibitor, rofecoxib (Chapter 4). COX-inhibitors therefore fail to relieve DOMS, irrespective of what iso-enzyme they target. Therefore, at the time that DOMS is evident, it is not being sustained by newly synthesized prostaglandin. Thus, I confirm that, though prostaglandin may be synthesized in the affected muscle during DOMS (Trappe *et al.*, 2001), agents that inhibit its synthesis, at doses effective for other pains, do not affect the intensity of the DOMS, when administered after the exercise.

It is possible that DOMS has a unique mechanism of pain distinct from other muscle pains, or, as is the case in cutaneous tissue, IL-1β stimulates the release of mediators not acting through prostaglandins, such as NGF and substance P (Safieh-Garabedian *et al.*, 1995; Woolf *et al.*, 1997), which may be important mediators in muscle pain. Although previous studies have found an elevation in prostaglandins when muscle

soreness was apparent 24h after eccentric exercise (Trappe *et al.*, 2001; Tegeder *et al.*, 2002), during carrageenan-induced muscle inflammation there were only some muscle nociceptors desensitized by acetylsalicylic acid treatment. Therefore, although prostaglandins are produced in muscle, only some muscle nociceptors are sensitized by prostaglandins (Diehl *et al.*, 1988).

Therefore, IL-1β may be important in inflammatory muscle pain by sensitizing muscle nociceptors either via prostaglandin synthesis or via other mediators such as NGF. Regardless of the mechanism of nociceptor sensitization, IL-1β does not initiate the hyperalgesia. In Chapter 3, my results show that the inflammatory hyperalgesia in the muscle developed before the IL-1β concentration in the muscle was elevated. However, it is possible that IL-1β may sustain the inflammatory muscle hyperalgesia once it has been established.

It is possible that peripheral sensitization, via prostaglandin or other mediators may not be a key player in muscle pain, but that the key player is central sensitization. Central sensitization, the increased excitability of neurones in pain pathways in the dorsal horn of the spinal cord, occurs with longer lasting effects in muscle pain than in cutaneous pain (Wall & Woolf, 1984). Initially, central neurones are activated by nociceptive input from the periphery. If the input is sustained, molecular changes within the spinal cord occur, and central sensitization results (Woolf, 2004). Injecting hypertonic saline into a muscle produces a short-lasting muscle pain, while injecting hypertonic saline into a muscle that has DOMS does not produce pain in excess of that in a muscle that does not have DOMS (Weerakkody *et al.*, 2001). In addition, in the same study, applying a

vibratory stimulus over the affected muscle increased muscle soreness following DOMS, but a vibratory stimulus did not induce muscle soreness in unexercised muscle. The implication of these findings is that the peripheral nociceptors, activated by the rapid change in sodium concentration after hypertonic saline injection (Graven-Nielsen *et al.*, 1997b), are not sensitized during DOMS, as evident by the lack of increased pain following a hypertonic saline injection. However, it is likely that central sensitization, rather than peripheral sensitization, is a key player in DOMS, and possibly all muscle pain.

Tramadol decreases the impact of central sensitization by attenuating pain transmission through activating of opioid receptors and inhibiting noradrenaline and serotonin reuptake in the brain and spinal cord, therefore targeting the descending inhibitory pathways (Oliva *et al.*, 2002). However, in my study, tramadol like codeine (60mg), another opioid, had no effect on pressure pain tolerance or intrinsic pain in the muscle during DOMS (Barlas *et al.*, 2000a). However morphine administered intravenously produced a significant attenuation of the muscle pain during DOMS (Tegeder *et al.*, 2003). Also, morphine-6-glucuronide (M6G), a morphine metabolite unable to penetrate the blood brain barrier, administered in a two hour intravenous infusion attenuated DOMS effectively (Tegeder *et al.*, 2003). Morphine administered to rats after eccentric muscle contractions led to suppression of c-Fos activation in the dorsal horn of the spinal cord leading Taguchi *et al.* (2005) to infer that there is some spinal neuronal activation occurring in DOMS, but not necessarily nociceptor activation. Opioid therapy, such as levorphanol, attenuates inflammatory muscle hyperalgesia in

rats and clinical muscle pain, such as fibromyalgia (Bengtsson *et al.*, 1989; Sorensen *et al.*, 1997; Kehl *et al.*, 2000). Therefore, clarity is required as to whether opioids are effective in treating muscle pain, in particular DOMS. It is likely that effectively treating muscle pain requires larger doses of opioids than conventionally prescribed for other pains. In addition, agents targeting central rather than peripheral mechanisms may be more effective in treating the complex polymodal mechanism of DOMS and possibly clinical muscle pain (Woolf & Mannion, 1999).

DOMS and clinical muscle pain such as whiplash and myofascial pain, are likely to differ, in mechanisms underlying the pain and in resultant muscle function. DOMS induced by unaccustomed exercise results in pain for no more than five days with a concomitant loss in muscle strength (Proske & Morgan, 2001). However, once the muscle soreness of DOMS has subsided there is muscle hypertrophy and improved muscle function (Proske & Morgan, 2001). Similarly clinical muscle pain, such as myofascial pain and fibromyalgia, results in muscle pain with a concomitant decrease in muscle function (Zedka *et al.*, 1999). In contrast though, clinical muscle pain, regardless of whether the pain subsides or becomes chronic, results in muscle atrophy (Friden & Lieber, 2001). During DOMS, and possibly clinical muscle pain too, muscle strength impairment may be caused by impaired proprioception. However, no change or damage in the muscle spindles themselves has been identified during DOMS. Therefore, it is likely that there is a change in signal from the muscle spindle, or to the muscle spindle, relating to the effort required to produce a contraction, and resulting in instability of the muscle (Proske, 2005).

If muscle spindles are not damaged during DOMS, large fibre mechanoreceptors in muscle spindles may have nociceptive properties at a high threshold, which are activated during muscle injury or after excessive load. During muscle injury, the muscle spindles may become sensitized, so that muscle spindle activation, such as during muscle contraction or passive stretch, results in pain being perceived (Weerakkody *et al.*, 2001). Applying a vibratory stimulus (6Hz) to the skin overlying a muscle, and activating large fibre mechanoreceptors decreases muscle pain induced by hypertonic saline. However, after DOMS has been induced, the muscle becomes more painful when applying the vibratory stimulus, indicating that group I large diameter fibres from muscle spindles may play a role in DOMS (Weerakkody *et al.*, 2001; Weerakkody *et al.*, 2003). The involvement of muscle spindles during muscle pain supports the concept of a predominant central mechanism and a complex polymodal mechanism underlying muscle pain.

In conclusion, I have demonstrated that the cytokine release during inflammatory pain is the same in different types of cutaneous tissue, namely hind paw tissue with the underlying fat pad and the thick keratinised tissue of the rat tail. I also have demonstrated that the cytokine release during inflammatory pain in muscle is different to that of inflammatory pain in cutaneous tissue. I have shown that, in cutaneous tissue, the cytokine release in the inflamed tissue coincides with the presence of the inflammatory cells, and the resultant hyperalgesia. However, in muscle inflammation, the cytokine release does not coincide with the hyperalgesia, with a delayed release of IL-1β and IL-6, and no increase in muscle TNF-α. Therefore, IL-1β and IL-6 may play

a role in sustaining the hyperalgesia and TNF-α may play little role in the inflammatory hyperalgesia induced. And finally, a complex polymodal mechanism likely underlies muscle pain since COX-inhibitors and a central-acting analgesic do not attenuate the muscle pain.

Future research should include investigations into the central mechanisms of muscle pain and therapeutic agents to treat muscle pain are more likely to be successful if they target changes within the spinal cord rather than within the muscle itself. A study also should be directed at investigating the rat's behavioural response following inhibition of IL-1β and CINC-1 at the site of muscle inflammation, in the circulation and within the spinal cord. Blocking the action of IL-1β and CINC-1 at all three sites would enable us to identify whether these two cytokines indeed are key mediators in inflammatory muscle hyperalgesia, and if so, where they play the greatest role. Should they play a role in carrageenan-induced inflammatory muscle hyperalgesia, then blocking the action of IL-1β and IL-8 in DOMS would be worth investigating. Not only blocking the action of IL-1β following carrageenan-induced muscle inflammation, but also blocking the action of NGF and prostaglandins should be investigated. Once the key pro-inflammatory mediators have been identified in inflammatory muscle pain, then these mediators should be measured in clinical muscle pain conditions such as myofascial pain, lower back pain and whiplash. Lastly, the mechanism by which these key pro-inflammatory mediators produce hyperalgesia should be tested, for example, by locating the appropriate receptors and determining how the mediators sensitize muscle nociceptors. There is much research still to be done to fully understand the underlying mechanisms of inflammatory muscle pain, requiring animal-based

experiments and human models of muscle pain followed by translational research into clinical muscle pain conditions.

CHAPTER 6

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