SERGIO MARQUES BORGHI

PARTICIPAÇÃO DAS CITOCINAS TNF-α, IL-1β E IL-10 EM MODELO DE DOR MUSCULAR DE INÍCIO TARDIO (DMIT) EM CAMUNDONGOS – MODULAÇÃO PELA PENTOXIFILINA E TALIDOMIDA

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Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito à obtenção do título de Doutor em Patologia Experimental.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Jr.

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RESUMO

A dor muscular de início tardio (DMIT) pode ocorrer como consequência de exercício excêntrico isolado ou de resistência, sendo descrita como dor ou sensação desconfortável após o exercício extenuante ao qual o corpo não está acostumado. Caracteriza-se por se desenvolver entre 24-48 horas após a sessão de exercício, principalmente em indivíduos não treinados. Apesar de todos os tipos de exercício físico envolverem a contração excêntrica em determinado momento, um exemplo clássico deste tipo de condição é correr em uma descida, ou quando se toca o solo após um salto em distância, ambos os exemplos levam em consideração a ativação de fibras musculares do músculo quadríceps da coxa. O processo de transmissão desta informação dolorosa envolve eventos inicialmente periféricos, com ativação de neurônios sensoriais aferentes primários no músculo esquelético, com posteriores sinapses no sistema nervoso central (medula espinhal e centros encefálicos superiores). Este tipo de dor muscular é acompanhada de inflamação local, com lesões ultra-estruturais dos miócitos e suas unidades funcionais, os sarcômeros, associada a liberação de substâncias álgicas, como por exemplo, citocinas, prostaglandinas, glutamato, bradicinina e fator de crescimento do nervo (NGF). Neste sentido, a administração intramuscular de citocinas como o fator de necrose tumoral alfa (TNF-α) induz dor muscular. Adicionalmente, o miócito do músculo esquelético expressa ambos os receptores do TNF-α, TNFR1 e TNFR2. A interleucina (IL)-1β, por sua vez, foi a primeira citocina hiperalgésica descrita na literatura. Ambos, TNF-α e IL-1β também são capazes de mediar respostas inflamatórias relacionadas à dor, como por exemplo, recrutamento de leucócitos, modulação das funções celulares e estresse oxidativo. Por outro lado, a interleucina-10 (IL-10) é conhecida por ser uma citocina com funções anti-inflamatórias e anti-hiperalgésica. De fato, estudos evidenciaram que o tratamento com IL-10 reduz o comportamento nociceptivo em modelos experimentais de dor inflamatória e neuropática. A literatura mostra que estudos experimentais e clínicos têm utilizado terapias farmacológicas a base de anti-inflamatórios e glicocorticoides como principal escolha para o tratamento da DMIT. Neste contexto, o uso de terapias farmacológicas alternativas, como as terapias anti-citocinas ainda não foram avaliadas. As drogas “imunosupressoras” pentoxiflina e talidomida, embora descritas como potentes agentes anti-inflamatórios e imunomoduladores ainda não foram especificamente testadas em estudos que tem como alvo a dor muscular, notavelmente a DMIT. Assim, neste trabalho foi investigado o papel do TNF-α e IL-10 endógenos e IL-1β (através do tratamento com o antagonista do receptor da IL-1 – IL-1ra) no desenvolvimento e mecanismos da DMIT induzida por protocolo de natação aguda em camundongos, através da avaliação da hiperalgesia mecânica muscular induzida pelo movimento. Animais não treinados (sedentários) foram expostos à água em tanque de vidro, separados individualmente em diferentes compartimentos durante 30 segundos (falso-nado), ou para uma única sessão de 30, 60 ou 120 minutos de natação aguda, e a hiperalgesia mecânica muscular, produção de TNF-α, IL-1β e IL-10 (músculo esquelético e medula espinhal), recrutamento de leucócitos [através da atividade da mieloperoxidase (MPO)], edema (através do

diâmetro distal do membro posterior e peso dos músculos sóleo e gastrocnêmio), estresse oxidativo [refletido pelos níveis de glutationa reduzida (GSH) e ânion superóxido no músculo esquelético e medula espinhal] e biomarcadores plasmáticos (cortisol, glicose, lactato e creatina quinase) foram analisados. A sessão de natação aguda com duração de 120 minutos induziu hiperalgesia mecânica muscular de maneira tempo dependente, alcançando o pico em 24 horas após o exercício, apresentando desta forma características de DMIT. Camundongos deficientes para o receptor TNFR1, ou tratados com IL-1ra, pentoxifilina e talidomida apresentaram significativa redução da hiperalgesia mecânica muscular induzida pela natação aguda. Ainda, os níveis de TNF-α, IL-1β e IL-10 aumentaram 2 e 4 horas após a natação aguda no músculo sóleo e medula espinhal, respectivamente, permanecendo sem alterações no músculo gastrocnêmio. Foi também observado aumento bifásico no edema do membro posterior distal (2 e 12-24 horas), e aumento do peso individual do músculo sóleo, mas não do músculo gastrocnêmio após a sessão de natação aguda (24 horas). Por fim, a sessão de exercício agudo induziu aumento na atividade da MPO no músculo sóleo, mas não no gastrocnêmio, e modulação dos níveis do antioxidante não enzimático GSH no músculo sóleo e medula espinhal, novamente sem alterações no músculo gastrocnêmio, com estas últimas respostas sendo dependentes do TNF-α/TNFR1 e da IL-1β, e revertidas pelo tratamento com pentoxifilina e talidomida. Ainda, os elevados níveis plasmáticos de lactato e creatina quinase induzidos pela natação aguda foram reduzidos pelo tratamento com IL-1ra. Por outro lado, nos animais deficientes para a IL-10, foram observados aumentos adicionais na hiperalgesia mecânica muscular, atividade de MPO, concentrações do ânion superóxido e redução dos níveis de GSH. Concluindo, as citocinas TNF-α (através de sinalização por TNFR1) e IL-1β, e também IL-10 têm papéis cruciais no desenvolvimento e controle, respectivamente, da DMIT induzida por sessão de natação aguda, com participação inicialmente periférica, no músculo sóleo, e em um segundo momento em regiões centrais, especificamente na medula espinhal, incluindo hiperalgesia mecânica muscular, resposta inflamatória e estresse oxidativo. O tratamento com pentoxifilina e talidomida foi capaz de reduzir todas as respostas comportamentais e bioquímicas induzidas pela sessão de natação aguda, tornando possível o seu uso como potencial terapia para o tratamento da DMIT. O conhecimento dos mecanismos moleculares envolvidos na gênese e evolução da DMIT é importante na intenção de se buscar novas alternativas terapêuticas, incluindo farmacológicas, que possam ser usadas isoladamente ou em associação com outros fármacos, visando minimizar os sintomas deletérios relacionados à DMIT, pois o seu controle, além de contribuir para aprimorar o treinamento de atletas ou mesmo de indivíduos com limitações físicas relacionadas à idade, peso ou lesões musculares e também treinamento físico intenso, como por exemplo, os militares, favorece a recuperação mais rápida de atletas e inclusão de pacientes com doenças crônicas em protocolos de reabilitação que incluem atividade física, levando-se em consideração que a dor muscular é um ponto negativo para o início e/ou continuidade da prática de exercícios físicos regulares.

Palavras-chave: DMIT, natação aguda, hiperalgesia, dor muscular, TNF-α, IL-1β, IL-10, estresse oxidativo, pentoxifilina e talidomida.

BORGHI, Sergio Marques. **Role of cytokines TNF-α, IL-1β and IL-10 in a model of delayed-onset muscle soreness in mice – modulation by pentoxifylline and thalidomide.** 2013, 280 pages. PhD thesis (Doctorate in Experimental Pathology) - State University of Londrina, Londrina, 2013.

ABSTRACT

Delayed-onset muscle soreness (DOMS) may occur as a result of isolated eccentric or resistance exercise, and is described as pain or uncomfortable sensation, after strenuous exercise, in which the body is unaccustomed. DOMS is characterized by developing between 24-48 after the exercise session, mainly in untrained people. Despite all types of physical exercises involve eccentric contractions at a given moment, a classical example of this condition is downhill running, or when it touches the ground after a long jump, both examples considering activation of thigh quadriceps muscle fibers. The process of transmission of this painful information involves initially peripheral events, with activation of primary sensory afferent neurons in skeletal muscle, with subsequent synapses in central nervous system (spinal cord and higher brain centers). This type of muscle pain is accompanied by local inflammation, with ultrastructural damage of myocyte and its functional unit, the sarcomere, associated with the release of algesic substances, such as cytokines, prostaglandins, glutamate, bradykinin and nerve growth factor (NGF). In this sense, the intramuscular administration of cytokines like tumor necrosis factor alpha (TNF-α) induces muscle. Additionally, skeletal muscle myocyte expresses both receptors of TNF-α, TNFR1 and TNFR2. The interleukin (IL)-1β, in turn, was the first hyperalgesic cytokine described in the literature. Both TNF-α and IL-1β are also able to mediate inflammatory responses related to pain, e.g. leucocyte recruitment, modulation of cellular function and oxidative stress. On the other hand, interleukin-10 (IL-10) is known to be a cytokine with anti-inflammatory and anti-hyperalgesic action. Indeed, studies have shown that the treatment with IL-10 reduces nociceptive behavior in experimental models of inflammatory and neuropathic pain. The literature shows that experimental and clinical studies have used pharmacological therapies based on anti-inflammatory and glucocorticoids as the main choice for the treatment of DOMS. In this context, the use of alternative pharmacological therapies, like anti-cytokines therapies, not yet been evaluated. “Immunosuppressive” drugs like pentoxifylline and thalidomide though described as potent anti-inflammatory and imunomodulatory agents, have not been specifically tested in studies that targets muscle pain, notably DOMS. Thus, it will be investigated the role of endogenous TNF-α and IL-10 and also IL-1β (by the treatment with IL-1 receptor antagonist – IL-1ra) in the development and mechanism of intense acute swimming-induced DOMS in mice, by evaluation of movement-induced muscle mechanical hyperalgesia. Untrained animals (sedentary) were exposed to water in a glass tank, individually separated in different compartments during 30 seconds (sham), or for a single session of 30, 60 or 120 minutes of intense acute swimming, and the muscle mechanical hyperalgesia, TNF-α, IL-1β and IL-10 production (skeletal muscle and spinal cord), leucocyte recruitment [by myeloperoxidase (MPO)], edema (through the distal hind limb diameter and weight of the soleus and gastrocnemius muscles), oxidative stress [reflected by glutathione reduced Levels (GSH) and superoxide anion production in skeletal muscle and spinal cord] and plasmatic biomarkers (cortisol, glucose, lactate and creatine kinase) was analyzed. The intense acute swimming induced muscle mechanical hyperalgesia in time dependent-manner, peaking 24 hours after the

exercise, thereby presenting characteristics of DOMS. TNFR1 deficient or IL-1ra, pentoxifylline and thalidomide treated mice showed significant reduction in muscle mechanical hiperalgesia induced by intense acute swimming. Moreover, TNF-α, IL-1β and IL-10 levels were increased 2 and 4 hours after the intense acute swimming in the soleus muscle and spinal cord, respectively, remaining unchanged in gastrocnemius muscle. Were also observed a biphasic increase in edema distal hind limb (2 e 12-24 hours), and increases in individual weight of the soleus, but not in the gastrocnemius muscle after the intense acute swimming session (24 hours). Lastly, the intense acute swimming session induced increases in MPO activity in the soleus, but not gastrocnemius muscle, and modulations of the levels of non-enzymatic antioxidant GSH in the soleus muscle and spinal cord, again without alterations in gastrocnemius muscle, with these latter responses being dependent on TNF-α/TNFR1 and IL-1β, and reversed by pentoxifylline and thalidomide treatment. Furthermore, the intense acute swimming-induced increased plasmatic levels of lactate and creatine kinase were reduced by IL-1ra treatment. By your turn, in IL-10 deficient animals, were observed additional increases in muscle mechanical hyperalgesia, MPO activity, superoxide anion levels and reducing levels of GSH. Concluding, the cytokines TNF-α (via TNFR1 signaling) and IL-1β, and also IL-10 have key roles in the development and control, respectively of delayed-onset muscle soreness (DOMS) induced by intense acute swimming, initially by a peripheral participation, in soleus muscle, and in a second moment in central sites, specifically spinal cord, including muscle inflammatory hyperalgesia and oxidative stress. Pentoxifylline and thalidomide treatment were capable to reduce all behavioral and biochemical responses induced by intense acute swimming, making possible their use as a potential therapy for the treatment of DOMS. Knowledge of the mechanism involved in DOMS genesis and evolution is important in intension to seek new alternative therapies, including pharmacological, which may be used alone or in association with other drugs, to minimize the deleterious symptoms that occurring in parallel with DOMS, because its control, besides contribute to improve the training of athletes or even individuals with physical disabilities related to age, weight or skeletal muscle injuries and also intense physical training such as military settings, favors faster recovery of athletes and inclusion of patients with chronic diseases in rehabilitation programs that include physical activities, take into account that muscle pain is a negative point for the start and/or continuity of regular physical exercise practice.

Key words: DOMS, swimming, hyperalgesia, muscle pain, TNF-α, IL-1β, IL-10, oxidative stress, pentoxifylline and thalidomide.

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LISTA DE ABREVIATURAS E SIGLAS

AGP – glicoproteína ácida
AINEs – Anti-inflamatórios não esteroidais
AR – artrite reumatoide
ATM – articulação temporomandibular
ATP – Trifosfato de adenosina
AUC – área sob a curva
C3a – proteína 3a do complemento
C5a – proteína 5a do complemento
$Ca2^{+}$ - cálcio
cAMP – adenosina monofosfato cíclica
$CD4^{+}$ – grupo de diferenciação 4^{+}
CD28 – grupo de diferenciação 28
CD64 – grupo de diferenciação 64
CD80 – grupo de diferenciação 80
CD86 – grupo de diferenciação 86
Cdc37 – proteína chaperona do ciclo de divisão celular 37
CFA – adjuvante completo de Freund
Cg – carragenina
CGRP – peptídeo relacionado ao gene da calcitonina
cIAP-1 – proteína celular inibidora de apoptose 1
cIAP-2 – proteína celular inibidora de apoptose 2
CINC-1 – citocina quimiotática para neutrófilos
CK – creatina quinase
SNC – sistema nervoso central
COX – ciclo-oxigenase
COX-1 – ciclo-oxigenase-1
COX-2 – ciclo-oxigenase-2
CRPS – síndrome dolorosa complexa regional
DIC – domínio intra-celular
DMIT – dor muscular de início tardio
DNA – ácido desoxirribonucléico

DOMS – *delayed-onset muscle soreness*
ECAT – enzima conversora de TNF-α
FADD – domínio de morte associado a Faz
FcγRI – receptor Fcγ 1
FUNED – Fundação Ezequiel Dias
G-CSF – fator estimulador de colônia de granulócitos
GDNF – fator neurotrófico derivado de células da glia
GM-CSF - fator estimulador de colônia de granulócitos e macrófagos
gp130 – glicoproteína 130
HIV – vírus da imunodeficiência humana
Hsp90 – proteína do choque térmico 90
IASP – Associação Internacional para o Estudo da Dor
ICOS – co-estimulador induzível de células T
INFγ – interferon γ
IκB – proteína inibitória de NFκB
IκBα – proteína α inibitória de NFκB
IKK – proteína quinase de IκB
IL-1 – interleucina-1
IL-1α – interleucina-1α
IL-1β – interleucina-1β
IL-1RI – receptor tipo 1 da IL-1
IL-1RII – receptor tipo 2 da IL-1
IL-1RAcP – proteína acessória do receptor IL-1
IL-1ra – antagonista do receptor da interleucina-1
IL-2 – interelucina-2
IL-6 – Interleucina-6
IL-8 – Interleucina-8
IL-10 – Interleucina-10
IL-10RI – receptor tipo 1 da IL-10
IL-10RII – receptor tipo 2 da IL-10
IL-12 – Interleucina-12
IL-18 – Interleucina-18
iNOS – óxido nítrico sintase induzível
IRAK – proteína quinase de ativação do receptor da IL-1

JNK – quinase N-terminal c-Jun

Jak1 – janus quinase 1

KC – quimiocina derivada de queratinócitos

KD(P)T – tripeptídeo Lys-D-Pro-Thr

Kd – quilodalton

Kg – quilogramas

L – litros

LPS – lipopolissacarídeo

LTB_4 – leucotrieno B_4

LTC_4 – leucotrieno C_4

LTD_4 – leucotrieno D_4

LTE_4 – leucotrieno E_4

MAP quinases (K) – proteínas quinases ativada por mitógeno

MCP-1 – proteína quimiotática de monócitos-1

MCP-5 – proteína quimiotática de monócitos-5

memTNF – receptor de membrana de TNF-α

mg – miligramas

MHC – molécula de histocompatibilidade principal

MIP-1α – proteína inflamatória de macrófagos-1α

MIP-1β – proteína inflamatória de macrófagos-1 β

MKP-1 – map quinase fosfatase-1

mRNA – ácido ribonucléico mensageiro

MyD88 – gene de resposta primária de diferenciação mielóide 88

NEMO – modulador essencial de NFκB

NFκB – fator nuclear kappa b

NGF – fator de crescimento do nervo

NADPH-oxidase – nicotinamida adenina dinucleotídeo fosfato-oxidase

PAF – fator ativador de plaquetas

PGE_2 – prostaglandina E_2

PGI_2 – prostaciclina

pH – potencial hidrogeniônico

PKA – proteína quinase A

PKC – proteína quinase C

PKCε – proteína quinase Cε

PMNL’s – leucócitos polimorfonucleares

RANTES – regulador da ativação de células T normais

RIP – proteína adaptadora de interação com o receptor TNFR1

RES – sistema reticulo endotelial

sIL-1RII – receptor tipo 2 solúvel da IL-1

SOCS-1 – supressor da sinalização de citocinas 1

SOCS-3 – supressor da sinalização de citocinas 3

SODD – silenciador os domínios de morte

STAT-1 – transdutor de sinal e ativador de transcrição-1

STAT-3 – transdutor de sinal e ativador de transcrição-3

STAT-5 – transdutor de sinal e ativador de transcrição-5

sTNFR – receptor solúvel de TNF-α

sTNFR1 – receptor tipo 1 solúvel de TNF-α

sTNFR2 – receptor tipo 2 solúvel de TNF-α

TENS – estimulação nervosa elétrica transcutânea

TGF-β – fator de crescimento e transformação β

T_H1 – T *helper* 1

T_H2 – T *helper* 2

TIMP-1 – inibidor tecidual de metaloproteinases-1

TIR – domínio do tipo Toll-receptor da IL-1

TLR2 – receptor do tipo Toll 2

TLRs – receptores do tipo Toll

TNF-α – fator de necrose tumoral-α

$TNFR1^{-/-}$ – camundongos deficientes para o receptor tipo 1 de TNF-α

TNFR1 – receptor tipo 1 de TNF-α

TNFR2 – receptor tipo 2 de TNF-α

TRADD – proteína de domínio de morte associada ao receptor tipo 1 de TNF-α

TRAF2 – fator 2 associado ao receptor de TNF

Treg – T regulatórias

TRPV1 – receptor de potencial transiente vanilóide 1

TRPV4 – receptor de potencial transiente vanilóide 4

TTx-r – resistentes à tetrodotoxina

Tyk2 – tirosina quinase 2

SUMÁRIO

1 INTRODUÇÃO

1.1 DOR MUSCULAR DE INÍCIO TARDIO

Segundo a Associação Internacional para o Estudo da Dor (IASP), dor pode ser definida como experiência sensorial e emocional desagradável, em geral associada à lesão tecidual. O termo nocicepção esta relacionado com o processo de transmissão da informação dolorosa, como por exemplo, uma terminação sensorial que detecta potenciais danos teciduais. O limiar de estimulação do nociceptor está logo abaixo da intensidade de dano tecidual, porque sua principal função não é sinalizar a existência de um dano tecidual, mas sim de alertar o sistema nervoso central de possível situação de perigo (MENSE & HOHEISEL, 2008).

Fibras aferentes de pequeno diâmetro devem ser ativadas para provocar dor muscular. O músculo esquelético é inervado tanto por fibras grandes e mielinizadas como por fibras finas e mielinizadas (grupo III ou fibras Aδ), e não-mielinizadas (grupo IV ou fibras C) aferentes, presentes principalmente no tecido conectivo do músculo, que apresentam terminações nervosas não especializadas e podem responder a estímulos nocivos mecânicos e / ou químicos (Figuras 1A e B) (STAUBER, 1989; MACINTYRE et al., 1995; GRAVEN-NIELSEN & MENSE, 2001). De maneira geral, as fibras aferentes nociceptivas de pequeno diâmetro e não as fibras de grandes diâmetros são equipadas com canais de sódio controlados por voltagem (Na_v 1.9 e 1.8) resistentes à neurotoxina tetrodotoxina (TTX-r). Neste sentido, fibras aferentes de receptores musculares de alto limiar sensíveis a estímulos mecânicos (grupos III e IV) não são bloqueados pela tetrodotoxina, o que indica que elas possuem canais de sódio TTX-r (STEFFENS et al., 2003; MENSE & HOHEISEL, 2008)

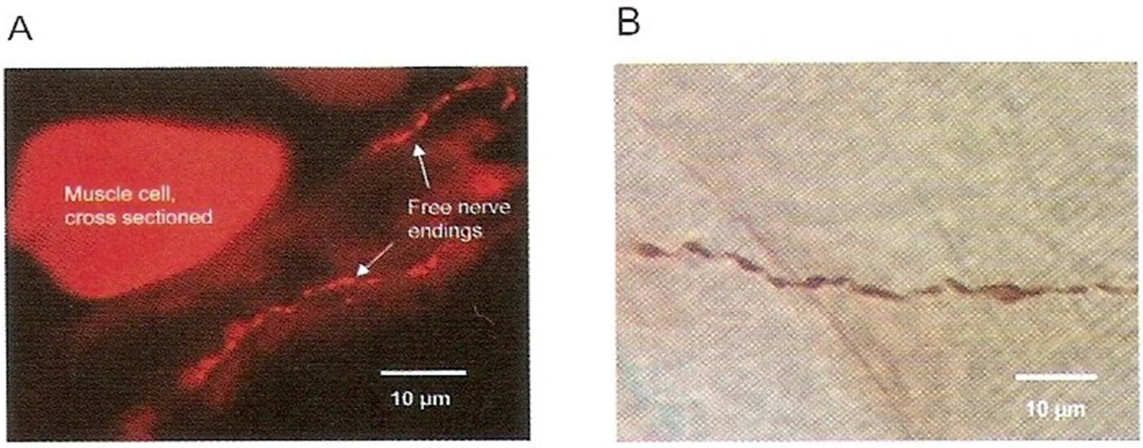

Figura 1 – (A) Terminações nervosas livres nos músculos sóleo-gastrocnêmio de rato, visualizados com anticorpos fluorescentes para substância P. (B) Axônio pré-terminal no músculo multifídeo de rato fixado com anticorpo para o peptídeo relacionado ao gene da cacitonina (CGRP). Fonte: Mense & Hoheisel, 2008.

Fisiologicamente, as fibras Aδ e C são diferenciadas pela velocidade de condução da informação dolorosa, sendo que, nos grandes mamíferos, a velocidade de condução das fibras Aδ é de 2.5-35 m/s e das fibras C menos de 2.5 m/s (GRAVEN-NIELSEN & MENSE, 2001; DJOUHRI & LAWSON, 2004). Em mamíferos menores como o rato, potenciais de ação nos nervos musculares sugerem que a velocidade de condução das fibras Aδ e C são em torno de 2-10 m/s e <2 m/s, respectivamente (BRUNETTI et al., 2003; DJOUHRI & LAWSON, 2004). Os nociceptores que inervam os músculos esqueléticos fazem sinapse predominantemente na lâmina I do corno dorsal, mas também nas lâminas mais profundas IV / V da medula espinhal (MENSE & CRAIG et al., 1988; HOHEISEL et al., 1989; MENSE et al., 1993; PANNETON et al., 2005).

A dor muscular de início tardio (DMIT) é descrita como sensação desconfortável ou dor após sessão de exercício extenuante ao qual o corpo não está acostumado. A DMIT é caracterizada por não aparecer imediatamente após o exercício, mas sim, por emergir dentro de 24 horas, alcançando picos dentro de 24-48 horas, e desaparecendo naturalmente dentro de 3-7 dias (ARMSTRONG, 1984; MACINTYRE et al., 1995; GRAVEN-NIELSEN & ARENDT-NIELSEN, 2003; MUNEHIRO et al., 2012). A DMIT pode diminuir o desempenho relacionado ao esporte e também comprometer a adesão de pacientes a programas de reabilitação

que incluam atividade física, representando um fator de risco para o desenvolvimento de doenças mais graves (MUNEHIRO et al., 2012).

Contrações excêntricas (associadas ao estiramento do músculo, p. ex. correr em uma descida) podem induzir DMIT mais facilmente do que contrações concêntricas (associadas ao encurtamento do músculo, p. ex. correr em uma subida) (ARMSTRONG, 1984). Dor muscular, dano do tecido muscular esquelético e níveis de inflamação e produção de espécies reativas de oxigênio são maiores após contrações excêntricas quando comparadas as contrações concêntricas (PEDERSEN et al., 1998; GIBALA et al., 2005; KON et al., 2007).

Está bem estabelecido que o músculo esquelético tanto em repouso quanto em contração produz espécies reativas de oxigênio e nitrogênio, e que o exercício intenso e prolongado pode resultar em dano oxidativo de proteínas e lipídios dos miócitos contráteis. Além disso, os oxidantes podem modular vias de sinalização celular regulando a expressão de múltiplos genes nas células. Esta alteração mediada pelos radicais livres na expressão de genes envolve alterações de nível transcricional, na estabilidade do mRNA e sinais de transdução. Numerosos produtos associados com os genes modulados pelos radicais livres têm sido identificados e incluem enzimas antioxidantes, proteínas de estresse e de reparo do DNA, e proteínas da cadeia de transporte de elétrons mitocondriais. Interessantemente, níveis fisiológicos de radicais livres são necessários para a produção de força normal no músculo esquelético, no entanto, altos níveis de espécies reativas de oxigênio promovem disfunção contrátil do miócito, resultando em fraqueza muscular e fadiga (POWERS & JACKSON, 2008).

Devido a esses achados, acredita-se que a alta tensão imposta ao músculo esquelético e tecido conectivo durante o exercício extenuante causam dano tecidual muscular, inflamação e estresse oxidativo, que podem sensibilizar os nociceptores (PYNE et al., 1994). Ademais, é importante relatar que a dor muscular não se desenvolve isoladamente após a sessão de exercício não usual, mas em associação a outros sintomas como danos musculares, fraqueza, alteração da mecânica articular e função muscular, refletidas por diminuição da amplitude de movimento articular e diminuição da força muscular, respectivamente, perda do senso proprioceptivo, edema e ativação muscular dessincronizada após contrações excêntricas (HORTOBAGYI et al., 1998; ROWLANDS et al., 2001; BOTTAS et al., 2005; PEAKE et al., 2005; PROSKE & ALLEN, 2005). Adicionalmente, alodinia e

hiperalgesia podem ocorrer juntamente com a somação temporal (BAJAJ et al., 2000; BARLAS et al., 2000; GIBSON et al., 2006). É importante ressaltar que, aparentemente, não existe relação entre o desenvolvimento da dor e a perda da força muscular, levando-se em consideração que o tempo dos dois eventos é diferente. A perda da força muscular é quase sempre observada imediatamente após o exercício, embora um segundo período de perda da força tenha sido relatado no camundongo, 1-3 dias pós-exercício. (MACINTYRE et al., 1995).

Diversos mecanismos têm sido propostos para DMIT, no entanto, eles são relacionados principalmente à sua geração e não explicam o componente neural da hiperalgesia mecânica (MIZUMURA & TAGUCHI, 2008). O ácido lático, encontrado em níveis elevados nos músculos esqueléticos e plasma imediatamente após o exercício foi inicialmente considerado como causa da DMIT. No entanto, esta hipótese tem encontrado forte resistência pelo fato de que o exercício concêntrico, que envolve alto metabolismo, falha em produzir DMIT (SCHWANE et al., 1983). Adicionalmente, os níveis de ácido lático retornam aos níveis basais do pré-exercício dentro de uma hora após o exercício. Neste sentido, é provável que o ácido lático contribua para a dor aguda associada com a fadiga durante e imediatamente após o exercício intenso, o que não se aplica a DMIT (MIZUMURA & TAGUCHI, 2008).

Danos ao tecido conectivo que formam as bainhas ao redor dos feixes das fibras musculares também tem sido propostos como causa para DMIT. De acordo com os dados descritos acima, estudos histológicos e investigações ultra-estruturais em humanos e animais revelaram micro lesões em músculos exercitados, incluindo ruptura das linhas Z que mecanicamente conectam os sarcômeros vizinhos por provável aumento da tensão imposta às pontes cruzadas individuais, rupturas focais no padrão estriado associadas a desorganização dos sarcômeros (FRIDEN et al., 1983; NEWHAM et al., 1983; FAULKNER et al., 1993; MACINTYRE et al., 1995; CHEUNG et al., 2003; HUME et al., 2004; LEWIS et al., 2012).

WATERMAN-STORER, 1991, propõe que a lesão pode ocorrer como resultado de dano aos filamentos intermediários do citoesqueleto (desmina, vimentina e sinemina), ou reciprocamente, enzimas proteolíticas podem ser liberadas como resultado da lesão inicial, que levaria à degradação do citoesqueleto. Esta teoria de mecanismo de lesão estrutural na DMIT é reforçada pela evidência de que a desmina (expressa em grandes quantidades no miócito após a diferenciação celular) é essencial para a manutenção da integridade da miofribila durante a tensão

que ocorre na contração associada ao estiramento (contrações excêntricas), levando-se em consideração que este filamento intermediário é responsável por conectar lateralmente as linhas Z, e também por conectar as linhas Z ao citoesqueleto subsarcolemal, desta forma desempenhando papel chave na manutenção da integridade mecânica e estrutural do aparato contrátil nos tecidos musculares (LI et al., 1997; PAULIN & LI, 1004; BÄR et al., 2004). Neste sentido, LIBER & FRIDEN, 1993, reforçam a hipótese de que não apenas a alta tensão, mas também a distensão (estiramento) ativa da fibra muscular pode exceder o limite de outros componentes estruturais do citoesqueleto extra-sarcomérico (talina, veniculina, α-actinina entre outras) que liga o citoesqueleto ao sarcolema, danificando esta arquitetura. Ainda, defeitos nos mecanismos do acoplamento excitação-contração também foram descritos (INGALLS et al., 1998; WARREN et al., 1999; LEWIS et al., 2012). Assim, é possível que os danos ultra-estruturais induzam a liberação de substâncias álgicas.

Análises bioquímicas tem mostrado liberação de enzimas estritamente intracelulares como, por exemplo, creatina quinase e lactato desidrogenase a partir dos músculos exercitados, o que evidencia perda da integridade do sarcolema (ARMSTRONG et al,. 1983; CHEUNG et al., 2003; HUME et al., 2004; LEWIS et al., 2012). Estes dados dão respaldo à hipótese de que o dano muscular ocorrido durante o exercício intenso possa ser a causa da DMIT. A análise de infiltrados celulares (macrófagos e neutrófilos) no músculo reforça a teoria de inflamação (SMITH, 1991; ARMSTRONG et al., 1983; MACINTYRE et al., 1995; CHEUNG et al., 2003; HUME et al., 2004). Por outro lado, em estudo prévio em humanos (MALM et al., 2004), não foram observadas diferenças entre marcadores de inflamação entre indivíduos que experimentaram sessões de contrações concêntricas versus excêntricas.

O processo inflamatório e consequente edema no músculo após a injúria tecidual podem contribuir significativamente para o desenvolvimento da DMIT. A elevada pressão dos tecidos edemaciados, associados ao aumento da temperatura local podem então sensibilizar e ativar terminações nervosas dentro das fibras musculares e junção musculotendínea, com estes eventos levando à sensação de DMIT. A dor pode ser mais perceptível e aumentada pelo movimento, assim que o aumento adicional da pressão intramuscular cria um estímulo mecânico para os nociceptores, já sensibilizados previamente por mediadores químicos, como

por exemplo, bradicinina, histamina, serotonina e acetilcolina, mas principalmente pela prostaglandina 2 (PGE_2), secretada por macrófagos e neutrófilos dentro da fibra muscular, levando ao aumento da sensibilidade nos nociceptores, causando dor muscular tardia (ARMSTRONG et al., 1983; SMITH, 1991; MACINTYRE et al., 1995; CHEUNG et al., 2003; HUME et al., 2004).

O envolvimento de resposta inflamatória após o exercício intenso foi também avaliada pelo tratamento com drogas anti-inflamatórias e glicocorticoides. Os estudos que utilizaram essas classes de drogas têm mostrado resultados variados baseados nas dosagens, tempo de administração e protocolo de exercício, com a maioria dos relatos evidenciando resultados efetivos quando o tratamento é realizado profilaticamente (antes do exercício), do que quando administrado terapeuticamente (após o exercício), principalmente com ibuprofeno, aspirina e dexametasona (FRANCIS et al., 1987; HASSON et al., 1992; HASSON et al., 1993; TOKMAKIDIS et al., 2003; CHEUNG et al., 2003; HUME et al., 2004; MIZUMURA & TAGUCHI, 2008). Por outro lado, dados divergentes de outros estudos não observaram nenhum efeito na percepção da dor muscular após utilização de AINEs ou bloqueadores de canais de cálcio (Cardizem) (JANSSE et al., 1983; DONNELLY et al., 1990; DVORAK, 1997; ALMEKINDERS, 1999). É possível que a resposta inflamatória seja responsável por iniciar, amplificar e / ou resolver a injúria musculoesquelética (MACINTYRE et al., 1995). No entanto, nenhum dos mecanismos propostos acima é suficiente para explicar a DMIT.

Os mecanismos de tratamentos para DMIT são abundantes, no entanto, segundo a literatura os resultados são ainda muito divergentes, e o sucesso dos mesmos é apenas limitado na intensão de se diminuir a percepção da dor. Não existem ainda protocolos de tratamento eficientes para redução do edema ou que restaurem a força dos músculos lesados, ambos os sintomas relacionados à DMIT. Compressão, exposição magnética, antioxidantes, pomadas tópicas e calor local mostraram sucesso moderado no tratamento dos sintomas da DMIT. A utilização de condutas a base de massagens tem mostrado resultados variados, que podem ser atribuídos ao tempo e tipo de aplicação das técnicas. Homeopatia, ultrassom, crioterapia, terapia de oxigenação hiperbárica, técnicas a base de estimulação elétrica (p. ex. TENS), alongamentos e acupuntura mostraram sucesso limitado em reduzir os sintomas da DMIT (CHEUNG et al., 2003; HUME et al., 2004). Por fim, levando-se em consideração a grande capacidade de rápida adaptação do músculo

esquelético em resposta a condições estressantes (PROSKE & MORGAN, 2001), sessões repetidas de exercícios excêntricos submáximos após o desenvolvimento da DMIT podem aliviar a dor, mas não restauram a função muscular (CONNOLLY et al., 2003; CHEUNG et al., 2003; HUME et al., 2004; PAGE, 2005).

Desta forma, é aceitável a hipótese de que ocorra inicialmente lesão mecânica tecidual (micro lesões) das fibras musculares e suas unidades funcionais, os sarcômeros e / ou do citoesqueleto (Figura 2), decorrente do aumento da tensão imposta ao músculo durante o exercício intenso, especialmente excêntrico. Após o colapso dos sarcômeros e consecutivo dano ao sarcolema, ocorre aumento dos níveis intracelulares de cálcio, inibindo a respiração celular e consequentemente prejudicando a produção de ATP, causando degradação adicional dos sarcômeros. Após o dano mecânico inicial, desenvolvem-se resposta inflamatória, com edema, recrutamento de leucócitos e subsequente lesão bioquímica decorrente da liberação de mediadores inflamatórios e hiperalgésicos. Entre estes mediadores, foram relatados citocinas, prostaglandinas, glutamato, bradicinina, NGF e fator neurotrófico derivado de células da glia (GDNF) aumentados nos músculos doloridos, liberados por células imunes ativadas, células residentes e teciduais, que potencializam a percepção da dor pelas terminações nervosas livres (SMITH et al., 1991; CLEAK & ESTON, 1992; MACINTYRE et al., 1995; TEGEDER et al., 2002; CHEUNG et al., 2003; CONNOLLY et al., 2003; HUME et al;. 2004; MURASE et al., 2010; LEWIS et al., 2012; MURASE, 2012).

O envolvimento do GDNF na hiperalgesia mecânica muscular pós-exercício em ratos foi recentemente descrito como sendo dependente da ativação de COX-2 no músculo (MURASE et al., 2012; MURASE et al., 2013). Neste sentido, o receptor EP2 da PGE_2, um produto final da ativação da via da COX está presente no músculo e está envolvido com a geração de hiperalgesia mecânica muscular pós-exercício presente na DMIT (OTA, 2013). Esta citação reforça o papel de mediador hiperalgésico final da PGE_2 na hiperalgesia inflamatória, sensibilizando diretamente os noiciceptores (CUNHA et al., 1992; CUNHA et al., 2005; VERRI et al., 2006). Adicionalmente, evidências da participação dos receptores de potencial transitório V1 e V4 (conhecidos como TRPV1 e TRPV4, respectivamente) foram relatadas modulando a participação do NFG (TRPV1) e GDNF (TRPV1 e V4) (OTA et al., 2013).

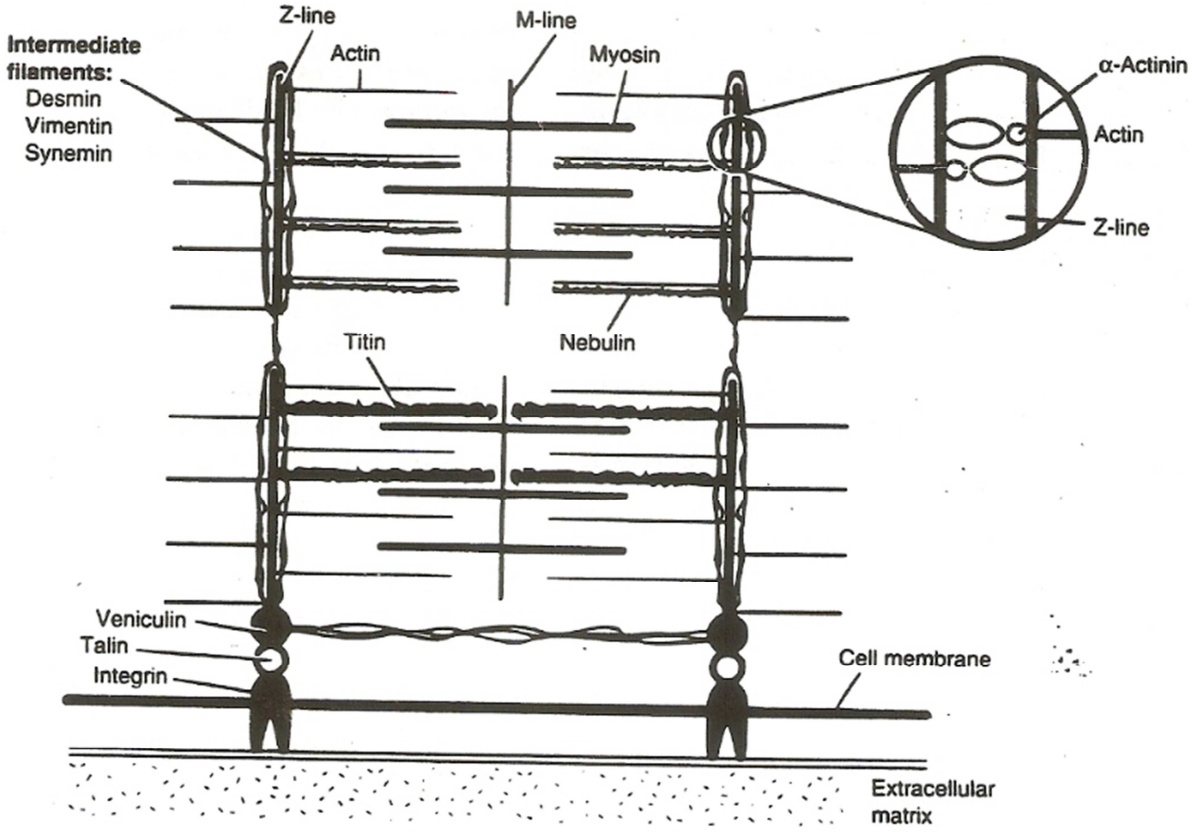

Figura 2 – Representação esquemática do sarcômero e citoesqueleto. A linha Z, linha M, actina e miosina representam estruturas do sarcômero, enquanto as estruturas do citoesqueleto são representadas pela titina, nebulina, filamentos intermedoários (desmina, vimentina e sinemina), veniculina, talina, integrina e α-actinina. Fonte: MacIntyre et al., 1995.

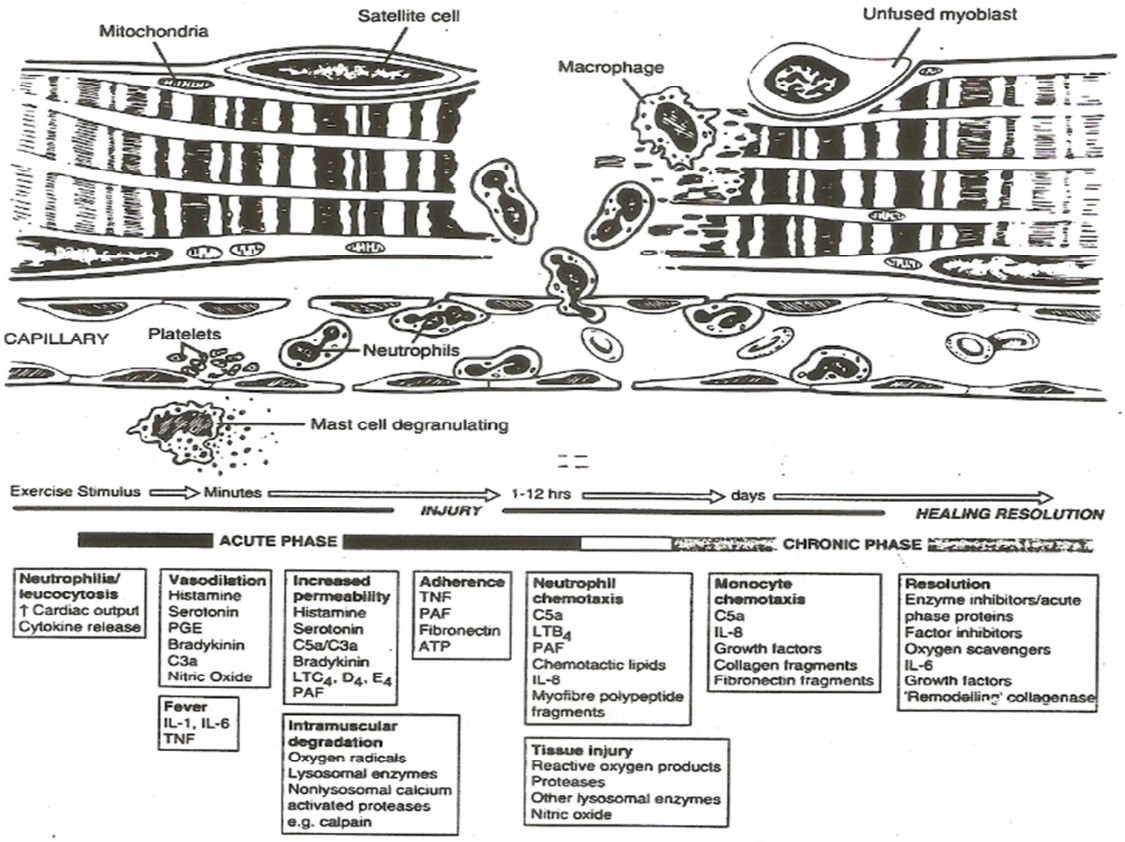

Figura 3 – Sequência de eventos envolvendo inflamação na lesão do músculo. Fonte: MacIntyre et al., 1995.

Apesar de ainda não existir consenso, pensa-se que esta teoria seja apropriada, embora experimentos adicionais sejam necessários para elucidação dos mecanismos de desenvolvimento da DMIT, inclusive através da utilização de novos modelos e / ou ferramentas experimentais de avaliação. É importante ressaltar que até o momento nenhum estudo demonstrou a existência de hiperalgesia mecânica muscular em animais (sensibilidade e dor relacionada ao movimento) (MIZUMURA & TAGUCHI, 2008).

1.2 Abordagens Farmacológicas Utilizadas na Dor Muscular de Início Tardio

Os mecanismos propostos para o desenvolvimento da DMIT permitem que os pesquisadores investiguem várias estratégias de tratamento tendo como alvo a atenuação dos sintomas da DMIT, restauração da função muscular o mais rápido possível e redução da magnitude das lesões iniciais (CHEUNG et al.,

2003). Os tratamentos podem ser utilizados de maneira profilática, na intensão de prevenir, ou terapêutica, na intensão de se tratar a DMIT. Estes tratamentos incluem crioterapia, terapia por oxigenação hiperbárica, alongamentos, ultrassom, drogas anti-inflamatórias, eletroterapia, homeopatia, compressão, massagem e exercício (CHEUNG et al., 2003; CONNELLY et al., 2003; HUME et al., 2004). Serão revisados aqui especificamente os trabalhos que utilizaram abordagens farmacológicas para a prevenção e tratamento da DMIT.

O uso de anti-inflamatórios não-esteroidais (AINEs) está entre as principais modalidades de escolha que visam recuperar a função muscular e aliviar os sintomas da DMIT. No entanto, o seu real valor no tratamento da DMIT ainda permanece equívoco com a maioria dos estudos, relatando ausência de efeitos significativos, apesar da consistente base teórica de sua eficácia. A eficácia de corticosteroides como os glicocorticoides também foi investigada. Tem sido postulado que o processo inflamatório e a presença de edema muscular após a injúria tecidual possam contribuir significativamente para o desenvolvimento da DMIT (CHEUNG et al., 2003; CONNELLY et al., 2003). Esta hipótese abre campo para a investigação dos efeitos de drogas anti-inflamatórios e analgésicas orais para a prevenção e tratamento da DMIT (JANSSEN et al., 1983; FRANCIS & HOOBLER, 1987; DONNELLY et al., 1990; HASSON et al., 1992; 1993; GULICK & KIMURA, 1996a; HERTEL, 1997).

Os AINES inibem o metabolismo do ácido araquidônico, através da inibição da via da ciclo-oxigenase, desta forma, contendo a produção de mediadores hiperalgésicos e inflamatórios como as prostaglandinas (HASSON et al., 1992; 1992; VERRI et al., 2006). Os glicocorticoides, por sua vez inibem a fosfolipase A_2, inibindo a clivagem o ácido araquidônico em fases iniciais nas membranas celulares, resultando em efeito anti-inflamatório mais amplo (CONNELLY et al., 2003). Neste sentido, a redução do processo inflamatório levaria ao controle do edema, e diminuição da pressão intramuscular, que poderiam diretamente contribuir para a redução da dor e desconforto muscular.

HASSON e colaboradores (1992; 1993) relataram significativas reduções na percepção da dor muscular 48 horas após o exercício para um grupo que recebeu administrações terapêuticas ou profiláticas de ibuprofeno, dexametasona e aspirina, comparado com um grupo placebo que recebeu iontoforese e um grupo não tratado. A contração voluntária máxima, pico de torque e

trabalho não foram diferentes entre os três grupos na 48° hora. FRANCIS & HOOBLER (1987), observaram menor restrição de movimento (50%) na extensão de cotovelo, 24 e 48 horas após o exercício (ação flexora isocinética do cotovelo) no grupo tratado com aspirina quando comparado com o grupo placebo. A dor muscular do grupo que recebeu aspirina (10 g, 4X/dia, 4 horas antes até 48 horas após o exercício) foi 25% menor do que a do grupo controle 48 horas após o exercício, com os dois grupos apresentando redução da força na 24° e 48° hora pós-exercício. No entanto, outros estudos relataram ausência de efeitos na percepção da dor muscular após a utilização de AINEs (JANSSEN et al., 1983; KUIPERS et al., 1985; DONNELLY et al., 1990; GULICK & KIMURA, 1996a).

No trabalho de HASSON e colaboradores (1992), a administração profilática de ibuprofeno (400 mg, 3X/dia) resultou em menor dor muscular do que a administração terapêutica. Corroborando, a utilização de pílulas de ibuprofeno (400 mg) a cada 8 horas dentro de um período de 48 horas diminuiu a dor muscular induzida por exercício excêntrico de flexão dos membros inferiores, mas não ajudou na restauração da força muscular em indivíduos que participaram de estudo duplo-cego (TOKMAKIDIS et al., 2003). Protocolos com administração de doses maiores de AINEs, como na utilizada por DONNELLY et al. (1990) (1200 mg de ibuprofeno, 45 minutos antes, com reforços a cada 6 horas até a 72°, totalizando 8400 mg) em que 16 participantes correram em descida com velocidade que levou a 70% da frequência cardíaca máxima, o efeito foi insatisfatório, não afetando a dor ou força muscular, com os níveis de CK e uréia sendo maiores no grupo ibuprofeno. Altas doses podem comprometer a produção de proteínas miofibrilares e atrasar o processo de cicatrização dos tecidos danificados (GULICK & KIMURA, 1996b).

O diclofenaco pode não interferir no dano, mas pode reduzir a dor muscular. Neste estudo, 20 homens não treinados foram submetidos a um protocolo de duas sessões de 45 minutos de corrida em descida com intervalo de 10 semanas, e receberam diclofenaco ou placebo antes e por 72 horas após o exercício. O diclofenaco não alterou as respostas bioquímicas séricas (CK, lactato desidrogenase, aspartato aminotransferase, creatinina e uréia), e embora a dor não tenha sido afetada pela droga de maneira geral, a dor individual foi reduzida pelo diclofenaco na primeira fase do estudo (DONNELLY et al., 1988). Foi demonstrado que o flurbiprofeno não tem nenhum efeito na dor muscular 48 horas após o exercício, em estudo com seis homens treinados que completaram três sessões de

30 minutos com 80% do consumo máximo de oxigênio em um cicloergômetro. O flurbirpofeno e o placebo foram administrados a partir do dia anterior até quatro dias após o exercício em protocolo de duplo-cego. Adicionalmente, não foram observados efeitos da droga na atividade enzimática (KUIPERS et al., 1985). O uso profilático de flurbiprofeno (40 mg, 12 horas antes) também foi avaliado na DMIT, não afetando a dor muscular tanto do grupo tratado quanto do grupo placebo (SEMARK et al., 1999).

O uso de naproxeno sódico (BOURGEOIS et al., 1999) antes e após exercício de resistência também foi avaliado em oito homens saudáveis moderadamente treinados, em estudo duplo-cego. O exercício consistia em realizar 6 sessões de 10 repetições de contrações concêntricas / excêntricas unilateralmente com o joelho a 80%-85% da potencia de uma contração máxima. O tratamento com naproxeno sódico não teve efeito na DMIT, força muscular e CK plasmática, no entanto, favoreceu a recuperação da amplitude de movimento da extensão do joelho na 48° pós-exercício. Por outro lado, indivíduos que receberam celoprofeno transdermal experimentaram redução da DMIT (CANNAVINO et al., 2003). NIEMAN e colaboradores (2005; 2006) evidenciaram que o uso de diversos AINEs (ibuprofeno, aspirina, naproxeno e inibidores específicos da COX-2) por ultramaratonistas não afeta o dano e dor muscular (escala visual análoga de dor), no entanto, podem promover elevações nos níveis plasmáticos de indicadores de endotoxemia e inflamação, incluindo citocinas. Em estudo experimental, ITOH & KAWAKITA (2002), observaram inibição da DMIT (mensurada por eletromiografia do músculo bíceps femoral) utilizando indometacina (5 mg/kg, antes, durante e após, totalizando 60 mg/kg em 12 administrações) após protocolo de exercício excêntrico em coelhos anestesiados.

Esta inconsistência nos resultados apresentados sobre o uso de anti-inflamatórios no tratamento da DMIT pode ser atribuída a diversos fatores, como os que se seguem: tempo de administração da droga, dose utilizada, e tipo de protocolo experimental / clínico utilizado para induzir DMIT. Outro ponto notável que merece ser considerado, é que na prática clínica, a medicação normalmente é administrada quando o indivíduo / paciente reclama de dores e / ou desconfortos musculares, não antes. Assim, deve-se sugerir que o consumo das diferentes drogas utilizadas seja realizado previamente ao exercício, esperando que desta maneira, os resultados possam ser mais claros (CHEUNG et al., 2003; HUME ET AL., 2004).

Para testar esta hipótese, como apresentado anteriormente, estudos têm utilizado protocolos com administração profilática (antes do exercício) e terapêutica (pós-exercício), na intensão de melhores esclarecimentos, com os dados da literatura evidenciado melhor eficácia quando o tratamento é realizado de maneira profilática (CHEUNG et al., 2003; HUME ET AL., 2004; MIZUMURA & TAGUCHI, 2008).

Apesar de sua eficiência para induzir respostas anti-inflamatórias e analgésicas, a utilização de AINEs está relacionada com a presença de efeitos colaterais indesejáveis, como por exemplo, ulcerações gastrintestinais ou efeitos renais e hipertensivos, observados após a inibição de COX-1 e -2, respectivamente. Outra potencial preocupação sobre o uso de AINEs para o tratamento da lesão muscular induzida por exercício, e consequentemente DMIT, é a possiblidade de que possam prejudicar a resposta adaptativa do músculo ao exercício, especificamente, existem evidências emergentes de que a ação das enzimas da COX, em particular a COX-2, sejam importantes e até mesmo necessárias para a atividade das células satélites durante a diferenciação do miócito, e também para a hipertrofia máxima do músculo esquelético em resposta ao trabalho funcional. A significância biológica destes achados indica que o consumo de AINEs para aliviar a DMIT e promover reparo muscular após o dano pode ser contra-indicado. Assim, a inflamação (ou pelo menos a manutenção das vias de sinalização induzidas pela COX) pode ser necessária para a adaptação e regeneração muscular em pessoas jovens e saudáveis (SCHOENFELD, 2012; PAULSEN et al., 2012). Por sua vez, o uso de corticosteroides também é acompanhado de diversos efeitos colaterais hormonais, como os observados nos tratamentos crônicos que utilizam esta classe de droga, entre eles, redução da massa óssea e muscular, edema e hipertensão (CONNELLY et al., 2003; CHEUNG et al., 2003; HUME et al., 2003).

Vale a pena salientar, que até o momento, nenhum estudo foi direcionado no sentido avaliar a participação direta das citocinas como alvo na DMIT. Embora existam fortes indícios da participação das citocinas nos mecanismos de desenvolvimento da DMIT (evidenciados por elevação nos níveis plasmáticos e musculares do mRNA/proteína) (PEDERSEN et al., 1998; NIEMAN et al., 2005; 2006; LORAM et al., 2007; SHAH et al., 2008; LIBURT et al., 2010; MURASE et al., 2010; ALVAREZ et al., 2010; CLETO et al., 2011; BORGHI et al., 2013a, submetido; 2013b, submetido), estudos direcionados com terapias anti-citocinas ainda não foram realizados. Neste sentido, a utilização de drogas que tem como alvo a inibição

da produção de citocinas seria necessária para avaliar a participação destes mediadores na DMIT. A pentoxifilina e talidomida, descritas em seções anteriores, podem ser consideradas interessantes ferramentas para serem testadas em modelos de DMIT, considerando que podem agir modulando a produção de citocinas, especificamente o TNF-α (VALE et al., 2004; VERRI et al., 2006; MAJUMDER et al., 2012). Outro ponto positivo que pode favorecer o uso da pentoxifilina e talidomida é o baixo custo destas terapias, tendo em vista que, outras terapias anti-citocinas, como etanercepte (enbrel®), infliximab (remicade®), adalimumab (humira®) ou ancinra (kineret®) além de apresentarem elevado custo, são associados com a necessidade de melhor farmacocinética (especialmente a anacinra), devido ao seu uso diário / semanal, existindo ainda, a possiblidade de ocorrer indução de anticorpo contra o imunobiológico, que pode comprometer diretamente a sua eficácia.

1.3 Fator de Necrose Tumoral-Alfa

O fator de necrose tumoral-alfa (TNF-α), uma citocina pleiotrópica, com peso molecular de 17 kDa, foi previamente chamado de caquexina, e foi identificado pela primeira vez, como fator sérico induzido por endotoxina que causa necrose de tumores *in vivo* (CARSWELL et al., 1975; TRACEY et al., 1988). Hoje é reconhecido como uma potente citocina pró-inflamatória, que é rapidamente produzida em grandes quantidades por uma variedade de células, principalmente por macrófagos (durante ativação clássica - M1), podendo também ser produzida por linfócitos T CD4+, neutrófilos, células *natural killers*, mastócitos, células endoteliais, cardiomiócitos, adipócitos, fibroblastos e neurônios em resposta a estímulos inflamatórios como, por exemplo, infecções bacterianas (MACINTYRE et al., 1995; WAJANT et al., 2003; SWARDFAGER et al., 2010; VERRI et al., 2006; MANTOVANI et al., 2011).

O TNF-α é primariamente produzido como uma proteína transmembrana do tipo 2 estruturada em homotrímeros estáveis (memTNF) (KRIEGLER et al., 1988; TANG et al., 1996). A partir desta forma integrada à membrana forma-se a citocina homotrimérica solúvel (sTNF), que é liberada via clivagem proteolítica pela metaloproteinase enzima conversora de TNF-α (ECAT) (BLACK et al., 1997). Diversas doenças humanas crônicas e progressivas estão

relacionadas com a desregulação na produção de TNF-α, entre elas, artrite reumatoide (AR), sepse, diabetes, câncer, osteoporose, esclerose múltipla, doença de Alzheimer, aterosclerose, doença inflamatória intestinal entre outras (CHEN & GOEDDEL, 2002; VERRI et al., 2006; VERRI et al., 2010; ANOGEIANAKI et al., 2011; CAMINERO et al., 2011; DAVIS et al., 2012; TANAKA, 2010, 2012; TWEEDIE et al., 2012).

A interação do TNF-α com as células-alvo ocorre através de receptores de membrana de alta afinidade, receptor do fator de necrose tumoral tipo 1 (TNFR1 ou p55) e tipo 2 (TNFR2 ou p75) (BEUTLER & CERAMI, 1988; TARTAGLIA & GOEDDEL, 1992; BAZZONI & BEUTLER, 1996, WARE, 2005). TNFR1 é constitutivamente expresso na maioria dos tecidos, enquanto a expressão de TNFR2 é altamente regulada e tipicamente encontrada em células imunológicas. Na grande maioria das células, TNFR1 aparenta ser o receptor chave para sinalização do TNF-α, enquanto no sistema linfoide, o TNFR2 parece desempenhar o papel chave. A importância do TNFR2 ainda não foi claramente determinada, porque este receptor pode apenas ser ativado pelo memTNF e não pelo sTNF (GRELL et al., 1995).

Funções biológicas distintas têm sido descritas para cada receptor. O TNFR1 é capaz de mediar migração de neutrófilos, apoptose, choque induzido por endotoxinas e está relacionado com a dor neuropática após lesão nervosa periférica, enquanto que o TNFR2 tem papel importante nos mecanismos de indução de apoptose e necrose (ROTHE et al., 1994; HEUMANN et al., 1995; SOMMER et al., 1998a, 1998b, CANETTI et al., 2001; CHEN & GOEDDEL, 2002; RAMESH & REEVES, 2003; WAJANT ET AL., 2003).

A sinalização celular induzida pela ligação do TNF-α com TNFR1 leva a uma série de respostas, incluindo ativação dos fatores de transcrição fator nuclear kappa b (NFκB) e c-Jun, induzindo sinalização pela via das proteínas quinases ativadas por mitógeno (MAP Quinases), ou induzindo sinalização de morte celular (apoptose). A ativação destas vias de sinalização é responsável pela indução da expressão de importantes genes para diversos processos biológicos, incluindo crescimento e morte celular, oncogênese, respostas imunes, inflamatórias e de estresse (CHEN & GOEDDEL, 2002; GAUR & AGGARWAL, 2003; LEE et al., 2004; TAKADA & AGGARWAL, 2004; WAJANT ET AL., 2003; GAO et al., 2006; ZHOU et al., 2006; CHEN et al., 2007; ZHOU et al., 2007; OGURA et al., 2008; KANT et al.,

2011; OECKINGHAUS et al., 2011). Assim, durante a liberação de TNF-α uma grande variedade de células com funções e condições variadas pode responder induzindo resposta inflamatória apropriada.

Um aspecto interessante da sinalização TNF-α/TNFR1 é a existência de sinalização cruzada entre apoptose, NFκB e JNK. Na ausência da atividade de NFκB, ocorre aumento da susceptibilidade de sinalização para a apoptose induzida por TNF, assim como a ativação de JNK induzida por TNF é mais intensa e duradoura na ausência de NFκB, e a produção de diversos genes relacionados à ativação de NFκB inibem a ativação e JNK pelo TNF. Além do mais, a ativação de NFκB promove a resíntese de moléculas inibitórias durante a cascata de ativação, promovendo uma fase de regulação, e maior duração da amplitude de modulação pela sinalização iniciada pelo TNF (CHEN & GOEDDEL, 2002).

O passo inicial da sinalização de TNF-α envolve a ligação da molécula trimérica de TNF ao domínio extracelular de TNFR1, liberando a proteína inibitória silenciosa dos domínios de morte (SODD) do domínio intracelular (DIC) de TNFR1. O complexo resultante DIC TNFR1 é reconhecido por uma proteína adaptadora do receptor de TNF, associada ao domínio de morte (TRADD), que por sua vez recruta outras proteínas adaptadoras adicionais, como a proteína de interação com o receptor (RIP), fator 2 associado ao TNFR (TRAF2) e o domínio de morte associado a Fas (FADD). Essas proteínas recrutam enzimas chaves ao complexo TNFR1, que são responsáveis por iniciar os eventos de sinalização. Por exemplo, a caspase 8 é recrutada por FADD ao complexo TNFR1, torna-se ativada, e inicia uma cascata de eventos que leva a apoptose. TRAF2, por sua vez, recruta as proteínas celulares inibidoras de apoptose 1 e 2 (cIAP-1 e -2), duas proteínas antiapoptóticas. TRAF2 também é responsável por iniciar a sinalização da via das MAP quinases que resultam na ativação de JNK, responsável por fosforilar c-Jun (CHEN & GOEDDEL, 2002).

Finalmente, as proteínas RIP e TRAF2 são críticas para o funcionamento de outra importante via de sinalização do TNFR1, que é a ativação do fator de transcrição NFκB (via canonical). Estas últimas ativam o complexo proteico IκB quinase (IKK), que medeia a fosforilação de IκB de maneira dependente de TNFR1. O complexo IKK possui duas subunidades catalíticas, IKKα e IKKβ e uma subunidade regulatória denominada modulador essencial de NFκB (NEMO ou IKKα). Adicionalmente o complexo IKK é composto por uma chaperona quinase-específica

que consiste de Cdc37 e Hsp90 que tem participação no deslocamento do complexo do citoplasma para a membrana (DIC do TNFR1). Na sinalização por TNF-α/TNFR1, o complexo IKKβ/NEMO parece ser mais crítico, com a subunidade IKKα desempenhando apenas participação mínima. A fosforilação de IκB acontece por ubiquitinação e posterior degradação pelo proteassoma, resultando na liberação dos dímeros de NFκB (na resposta inflamatória p50/p65), permitindo a translocação dos mesmos para o núcleo da célula, induzindo a ativação transcricional de grande variedade de genes relacionados à inflamação, como por exemplo, citocinas pró-inflamatórias (TNF-α, IL-1β, IL-8 e IL-6), complexo enzimático de membrana nicotinamida adenina dinucleotídeo fosfato-oxidase (NADPH-oxidase) resultando na produção de ânion superóxido e outras espécies reativas de oxigênio, e as enzimas ciclo-oxigenase-2 (COX-2) e oxido nítrico sintase induzível (iNOS). A atividade transcricional de NFκB é adicionalmente regulada por modificações pós-translacionais. As vias de sinalização induzidas pela ligação TNF-α/TNFR1 são resumidas na figura 4. (MING ET AL., 1987; ATKINSON et al., 1988; CHEN & GOEDDEL, 2002; WAJANT et al., 2003; JUNG et al., 2003; HAYDEN & GHOSH, 2004; AL-HANBALI et al., 2009; VERRI et al., 2010; KILPATRICK et al., 2010; OECKINGHAUS et al., 2011; ALBERTI et al., 2012; HSU et al., 2013).

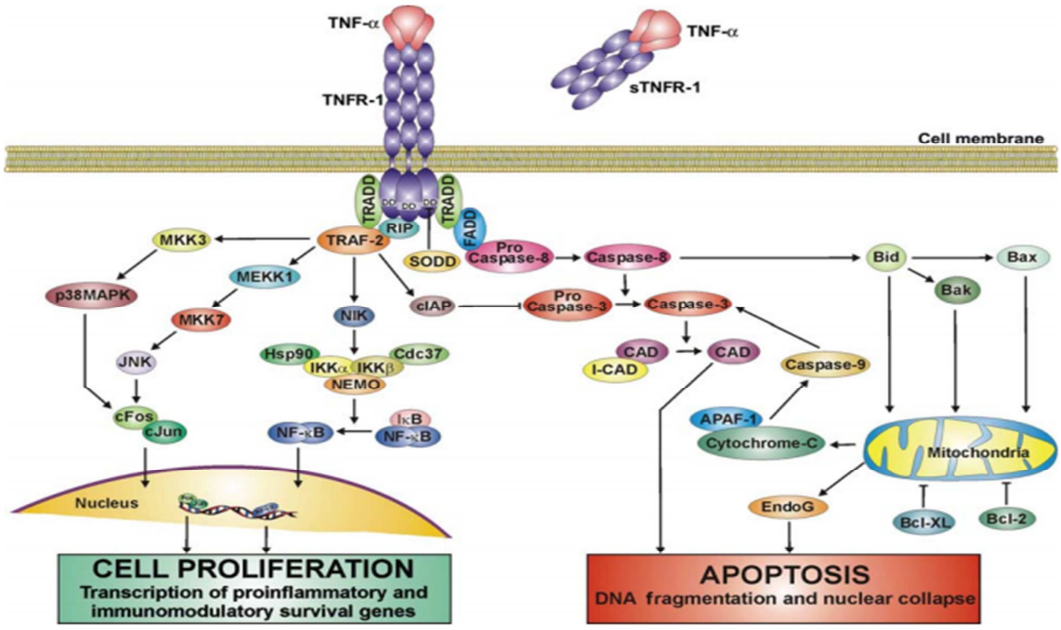

Figura 4 – Vias de sinalizações mediadas por TNF-α/TNFR1. Fonte: van Horssen et al., 2006.

A literatura tem mostrado que, além de ser um importante modulador da resposta inflamatória, o TNF-α tem papel chave nos mecanismos de indução da dor neuropática e inflamatória (CUNHA et al., 1992; WATKINS et al., 1995; SOMMER et al., 1998a; SOMMER et al., 1998b; SHUBAYEV & MYERS, 2001; CUNHA et al., 2005; ZELENKA et al., 2005; VERRI et al., 2006). Neste sentido, o TNF-α é capaz de induzir aumento do limiar mecânico nociceptivo em ratos e camundongos, após administração de estímulos como carragenina (Cg) e lipopolissacarídeo (LPS), iniciando cascatas de sinalização que levam à liberação de mediadores inflamatórios, como citocinas (IL-1β, IL-6 e quimiocinas), que induzem a produção final de prostanóides e aminas simpáticas, estas últimas sendo responsáveis pela direta estimulação do nociceptor primário, iniciando o processo de transmissão ascendente da informação dolorosa (CUNHA et al., 2005; VERRI et al., 2006). Corroborando, o pré-tratamento com anticorpo anti-TNF-α impede o aumento do limiar nociceptivo em resposta a Cg e LPS, e o tratamento com indometacina (inibidor da COX) e atenolol (antagonista de receptores β adrenérgicos) parcialmente inibem a hiperalgesia mecânica. O co-tratamento com essas drogas é capaz de abolir os comportamentos relacionados à dor (CUNHA et al., 1992), sugerindo desta forma, um papel crucial do TNF-α em modelos experimentais de hiperalgesia mecânica induzida por Cg e LPS.

Reforçando o conceito do importante papel nociceptivo do TNF-α na dor inflamatória, tem sido sugerido que o TNF-α pode estar associado com síndromes dolorosas musculoesqueléticas, levando-se em consideração que os receptores 1 e 2 do TNF são expressos no músculo esquelético e também que sua administração por via intramuscular induz hiperalgesia em ratos através da síntese de mediadores álgicos finais, como as prostaglandinas (SCHAFERS et al., 2003; FIGUERAS et al., 2005). Adicionalmente, evidências *in vivo* mostraram elevados níveis de TNF-α no músculo trapézio de indivíduos acometidos por pontos dolorosos miofasciais, relacionando esta citocina com a dor muscular e condições inflamatórias (SHAH et al., 2008). Finalmente, o TNF-α pode também ter participação efetiva no desenvolvimento de comportamentos relacionados à dor durante herniação do núcleo pulposo intervertebral, pois a aplicação de anticorpo anti-TNF-α a raízes nervosas previne parcialmente as respostas nociceptivas relacionadas à herniação em ratos (ONDA et al., 2003).

Juntos, estes dados evidenciam de forma consistente o papel chave do TNF-α nos mecanismos de gênese da dor. Neste contexto, diferentes drogas têm sido desenvolvidas para o tratamento de doenças inflamatórias como, por exemplo, a artrite reumatoide. Elas incluem infliximab (anticorpo quimérico anti-TNF-α), etanercept (proteína de fusão da imunoglobulina G/receptor de TNF p75), e mais recentemente, adalimumab (anticorpo monoclonal humano completo anti-TNF-α). Recentes evidências tem mostrado eficiência no tratamento com as drogas classificadas como “imunossupressoras” talidomida e pentoxifilina, já que ambas degradam o mRNA do TNF-α (STRIETER et al., 1988; DOHERTY et al., 1991; MOREIRA et al., 1993; SAMPAIO et al., 1998; VERRI et al., 2006). Essas terapias anti-TNF se mostraram efetivas em diferentes doenças como psoríase e principalmente artrite reumatoide (MORELAND, 1999; TOBIN & KIRBY, 2005; HARAOUI, 2005).

1.4 Interleucina-1

A interleucina 1 (IL-1) foi primeiramente descrita como uma proteína que induz febre e foi chamada de pirógeno leucocítico humano. É constituída de 2 proteínas principais, a IL-1α e IL-1β (DINARELLO et al., 1977; DINARELLO, 2009). Até o momento, existem 11 membros da família da IL-1 (IL-1F1 a IL-1F11). A IL-1α e IL-1β humanas são compostas de estruturas homotriméricas, com peso molecular de 17 kDa e 16 kDa, respectivamente, e tem mínima sequência de homologia, no entanto, apresentam propriedades biológicas similares. Também existem diferenças fundamentais na sua localização, maturação e secreção.

A IL-1α é traduzida diretamente para sua forma biologicamente ativa, enquanto a IL-1β, assim como outro membro da família da IL-1, a IL-18, é traduzida primeiramente como pró-IL-1β, não apresentando atividade biológica nesta forma, até que seja clivada pela protease intracelular enzima conversora da IL-1β, também conhecida como caspase-1, que faz parte de um complexo de proteínas intracelulares chamado de *inflammasome*. A clivagem dos precursores tanto da IL-1β quanto IL-18 também podem ser realizadas por proteases neutrofílicas extracelulares (MOSLEY et al., 1987; MARTINON & TSCHOPP, 2007; DINARELLO et al., 2011). Após sua produção, a IL-1α permanece dentro da célula ou na superfície da membrana celular, onde desempenha função de mensageiro autócrino

mais intensamente do que um mediador extracelular, enquanto que a maioria da IL-1β produzida é transportada para o exterior da célula onde age localmente ou entra na corrente sanguínea, podendo agir de forma endócrina (DINARELLO et al., 1994). A IL-1 é uma potente citocina pró-inflamatória que age como pirógeno endógeno, e apresenta diversos efeitos que potencializam a proliferação celular, diferenciação e função de muitas células inatas e imunocompetentes específicas. A IL-1 ainda é capaz de mediar doenças inflamatórias pela inicialização e potencialização das respostas imune e inflamatória (AKDIS et al., 2011).

Um terceiro componente da família, o antagonista do receptor da IL-1 (IL-1ra) é sintetizado e liberado em resposta aos mesmos estímulos que levam a produção de IL-1 (EISENBER et al., 1990). A IL-1ra carece de um domínio de interação com a proteína acessória do receptor da IL-1 (IL-1RAcP), de modo que a ligação da IL-1ra ao receptor da IL-1 inibe a sinalização da IL-1 (DINARELLO, 2009). Os genes da IL-1α, IL-1β e IL-1ra são estreitamente associados na região de 2q12-q21 do cromossomo 2 humano (AKDIS et al., 2011).

Diferentes tipos celulares incluindo macrófagos, monócitos, linfócitos, neutrófilos, células dendríticas, fibroblastos, queratinócitos, micróglia e células de linhagens sinoviais produzem IL-1, que por sua vez induz a produção de outros mediadores inflamatórios (DINARELLO, 1998; AKDIS et al., 2011; CONTASSOT et al., 2012). A síntese dos precursores da IL-1α e IL-1β começam após ativação da célula, por estimulação dos receptores do tipo Toll (TLRs) ligados à membrana (p. ex. Toll 4) através de seus agonistas, como por exemplo, endotoxinas ou LPS. A IL-1 tem sido relacionada com a patogênese de diversas doenças inflamatórias, como artrite reumatoide, osteoartrite, doença inflamatória intestinal, aterosclerose, doença pulmonar obstrutiva crônica, esclerose múltipla e doença de Alzheimer e doenças alérgicas como dermatites atópicas (COMINELLI et al., 1990; ISAACS et al., 1992; TERUI et al., 1998; KIRII et al., 2003; AKDIS et al., 2011).

São muitas as funções biológicas da IL-1β agindo como mediador inflamatório, que incluem importante papel regulador da homeostase nas fases iniciais do desenvolvimento hematopoiético de células-tronco e também modulação da atividade do centro termorregulador do hipotálamo, levando ao aumento da temperatura corpórea, induzindo febre. Adicionalmente induz a produção de quimiocinas como a IL-8, que agem como potentes quimioatraentes para neutrófilos, desta forma, promovendo recrutamento de leucócitos. A IL-1β ativa hepatócitos,

induzindo a liberação de proteínas de fase aguda, que agem como importantes opsoninas favorecendo a fagocitose por macrófagos e neutrófilos e também é uma indutora chave de proteínas antimicrobianas, como a β-defensina, presente na mucosa pulmonar, com importante função na defesa durante contatos iniciais com patógenos (DINARELLO, 1984; GOLDBLUM et al., 1988; FIBBE et al., 1988; MOSER et al., 1989; MYTSUYAMA et al., 1994; NIKOLAUS et al., 1998; SINGH et al., 1998; VERRI et al., 2006; ORELIO et al., 2008; AKDIS et al., 2011).

A IL-1α e IL-1β exercem efeitos similares pela ligação ao receptor tipo 1 da IL-1 (IL-1RI). Elas também podem se ligar ao receptor tipo 2 da IL-1 (IL-1RII), que age como um receptor chamariz para a IL-1 e não está envolvido com transdução de sinais (AKDIS et al., 2011). IL-1RI é expresso em todas as células responsivas a IL-1α e IL-1β, predominantemente células T, fibroblastos, células epiteliais e endoteliais e é frequentemente co-expresso com o IL-1RII. Ambos os receptores da IL-1 (IL-1R) contêm um domínio de ligação, composto de 3 domínios tipo imunoglobulina, e pertencem à superfamília dos receptores Toll-IL-1 (TIR), que é definido por um domínio TIR intracelular, responsável por iniciar a cascata de sinalização (GREENFEDER et al., 1995). A principal diferença entre IL-1RI e IL-1RII é o domínio intracelular que é extremamente pequeno no IL-1RII (29 aminoácidos) quando comparado com IL-1RI (213 aminoácidos), e por isso, o IL-1RII é incapaz de formar um complexo com a IL-1RAcP, que é necessária para o sinal de transdução (AKDIS et al., 2011). O papel funcional do IL-1RII é evitar a interação entre a IL-1 e o IL-1RI, agindo desta forma como inibidor natural da atividade da IL-1, função complementada pela IL-1ra, que é um inibidor endógeno da IL-1α a da IL-1β e se liga competitivamente ao receptor da IL-1 sem ativá-lo (AKDIS et al., 2011).

O domínio extracelular da IL-1RI, composto de 319 aminoácidos é responsável pela ligação e interação com afinidade similar de ambas as proteínas agonistas IL-1α a da IL-1β ou ligação da proteína antagonista IL-1ra (AKDIS et al., 2011). Após a ligação dos ligantes IL-1α ou IL-1β ao receptor de membrana IL-1RI, a aproximação do IL-1RI e da IL-1RAcP, que age como co-receptor é necessária para iniciação da sinalização, que envolve o recrutamento de moléculas adaptadoras, como MyD88 e ativação de quinases associadas ao IL-1RI (IRAKs), resultando na ativação de vias de sinalização do NFκB (via canonical) e MAP quinases como ERK 1/2, JNK e p38, que cooperativamente induzem a expressão dos genes-alvo pró-inflamatórios, como IL-6, IL-8, MCP-1, COX-2, iNOS, IκBα, IL-1α, IL-1β, MKP-1,

TLR2 entre outros, por mecanismos transcripcionais e pós-transcripcionais. Além disso, a IL-1β ativa o endotélio local, que resulta em aumento da expressão de fatores de adesão nas células endoteliais e induz vasodilatação, levando ao aumento da permeabilidade dos vasos sanguíneos, permitindo assim a transmigração de leucócitos para o local do dano tecidual. Estes dados evidenciam importante papel da IL-1 na coordenação da inflamação local e sistêmica. A figura 5 resume as via de transdução de sinais induzidas pela IL-1 através da interação com IL-1R (SINGH et al., 1998; SAKAI et al., 2004; AKIRA et al., 2006; MASSARO et al., 2006; WEBER et al., 2010; AKDIS et al., 2011; OECKINGHAUS et al., 2011; CONTASSOT et al., 2012).

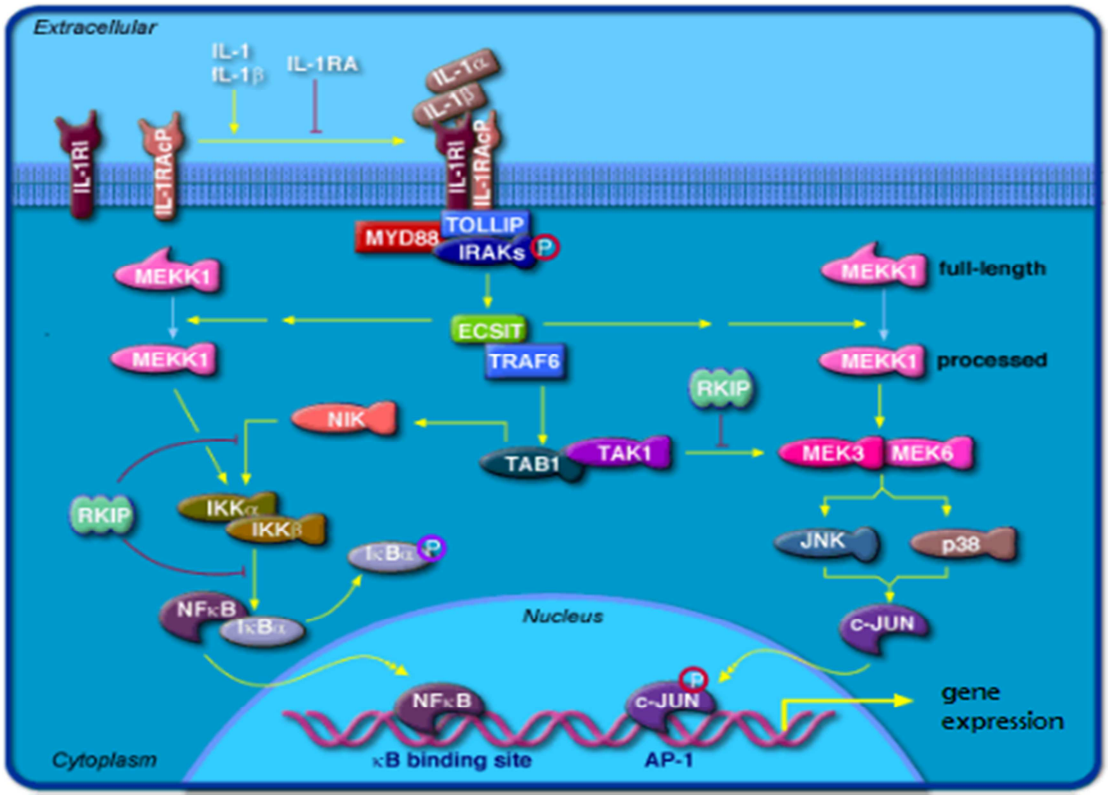

Figura 5 - Vias de sinalizações mediadas por IL-1/IL-1R. Fonte: BioCarta.

Como descrito anteriormente, a IL-1β, que tem sua expressão induzida pelo NFκB, estimula a produção de COX-2 e a subsequente liberação de seus produtos, as prostaglandinas (BERNHEIM et al., 1980; ZUCALI et al., 1986; CROFFORD et al., 1994). As prostaglandinas são os mediadores hiperalgésicos finais de uma cascata de eventos que sensibilizam os nociceptores

(HANDWERKER, 1976; FERREIRA & NAKAMURA, 1979a, 1979b), desta forma sugerindo que a IL-1β age como importante mediador hiperalgésico inflamatório (VERRI et al., 2006). De fato, a IL-1β, mas não a IL-1α, foi à primeira citocina relatada por mediar nocicepção inflamatória em modelos com animais experimentais. Foi mostrado que a injeção intra-plantar de IL-1β, mesmo que em pequenas doses produz hiperalgesia mecânica, que é dependente da liberação de prostanóides, fato confirmado pelo achado de que o tratamento com indometacina (inibidor da COX) bloqueia os seus efeitos (FERREIRA et al., 1988).

A participação da IL-1β na indução da hiperalgesia inflamatória mediada por prostanóides foi também demonstrada em modelos experimentais de inflamação com administração local de Cg ou LPS em ratos (FERREIRA et al., 1988). A hiperalgesia mecânica induzida por Cg ou LPS foi parcialmente inibida (50%) pelo tratamento local prévio com anticorpo anti-IL-1β (CUNHA et al., 1992). Reforçando este dado, resultados similares foram obtidos na hiperalgesia mecânica induzida por Cg em camundongos (CUNHA et al., 2005). Essa inibição parcial sugere que outros caminhos nociceptivos além deste mediado pela IL-1β/prostanóides estão envolvidos na hiperalgesia mecânica inflamatória induzida por Cg em ratos. De fato, quimiocinas (CINC-1 no rato e KC no camundongo) e aminas simpáticas estão também envolvidas na hiperalgesia mecânica induzida por Cg e LPS (CUNHA et al., 1991).

Esta grande compilação de resultados da literatura sugere um papel de mediador hiperalgésico intermediário da IL-1β, principalmente em tecidos periféricos. Assim, a partir de perspectiva terapêutica, esses dados experimentais suportam o fato de que a citocina IL-1β é um potencial alvo para o controle da dor inflamatória (VERRI et al., 2006). Neste sentido, drogas que tem como alvo a IL-1β tem sido utilizadas. Entre elas, o tratamento com IL-1ra exógena (inibidor solúvel da IL-1α e IL-1β) e o tripeptídeo KD(P)T consistentemente inibem a hiperalgesia mecânica induzida por Cg, LPS, bradicinina, TNF-α, IL-1β (FERREIRA et al., 1988; CUNHA et al., 2000; VERRI et al., 2006). Outra droga, IL-1ra seletiva, a Anacinra, que difere da IL-1ra natural pela presença de um grupamento metionina, foi testada em modelos de doenças inflamatórias e está sobre avaliação clínica (VILA et al., 2005; DEN BROEDER et al., 2006). Finalmente, recente perspectiva no controle da hiperalgesia induzida por IL-1β é a inibição de MAP quinases como a p38, que é

responsável por mediar a hiperalgesia inflamatória induzida por IL-1β (SUNG et al., 2005).

1.5 INTERLEUCINA-10

A interleucina-10 (IL-10) foi descrita pela primeira vez em 1989 como um fator inibidor da síntese de citocinas, mais especificamente um fator derivado de T_H2 inibidor da produção de INFγ e outras citocinas como a IL-2 em células T_H1 murinas (FIORENTINO et al., 1989; DONELLY et al., 1999). No entanto, no homem, a produção de IL-10 não é um aspecto típico de células T_H2, porque tanto células T_H1 quanto T_H2 são capazes de produzir IL-10, considerando que a principal fonte de IL-10 derivada de células T são as células T regulatórias (Treg), no entanto, outros tipos celulares como células B, monócitos, macrófagos e células dendríticas também podem produzir IL-10 (AKDIS et al., 2011). O gene da IL-10 está localizado no cromossomo 1 tanto no genoma humano (1q31-32) quanto no murino (KIM et al., 1992). Sua estrutura é altamente conservada e consiste de 5 exons e 4 introns, uma característica que é compartilhada pela maioria dos homólogos da IL-10. A IL-10 humana tem um peso molecular de 18 Kd e é secretada na forma de homodímero, consistindo de 2 subunidades de 178 aminoácidos longos (VIEIRA et al., 1991; ZDANOV et al., 1996).

A IL-10 se liga a um receptor tetramérico que pertence à família de receptores do INF e é composto de duas cadeias IL-10RI e duas cadeias IL-10RII (LIU et al., 1994). A cadeia IL-10RI é expressa em células T, células B, células *natural killers*, monócitos, mastócitos e células dendríticas, enquanto que a cadeia IL-10RII é ubiquitinamente expressa (NAGALAKSHMI et al., 2004). A IL-10 pode se ligar a cadeia IL-10RI com alta afinidade, no entanto, não tem interação direta com o IL-10RII (TAN et al., 1993). A IL-10 murina se liga tanto a IL-10RI humana quanto murina, enquanto que a humana se liga apenas a IL-10RI humana (AKDIS et al., 2011). Apesar da cadeia IL-10RII não interagir diretamente com a IL-10 e não proporcionar regiões de acoplamento para STAT3 (transdutor de sinal e ativador de transcrição-3) é essencial para a transdução de sinais mediada pela IL-10, levando-se em consideração que camundongos deficientes para a cadeia IL-10RII desenvolveram um fenótipo similar a animais deficientes para IL-10 ou STAT3, que é principalmente caracterizado por colite crônica (SPENCER et al., 1998).

A cadeia IL-10RI é associada com Janus Quinase 1 (Jak1), enquanto que a cadeia IL-10RII é associada com Tirosina Quinase 2 (Tyk2) (DONELLY et al., 1999; AKDIS et al., 2011). O complexo IL-10R sinaliza via Jak1 e Tyk2, seguido da fosforilação de STAT1, STAT3 e STAT5 (FINBLOOM & WINESTOCK, 1995; HO et al., 1995). Neste sentido, a ligação da IL-10 com o domínio extracelular de IL-10RI inicia a ativação de Jak1 e Tyk2, os quais por sua vez induzem a fosforilação dos resíduos de tirosina Y427 e Y477 ou Y446 e Y496 nos domínios intracelulares do IL-10RI humano e murinho. A fosforilação destes resíduos de tirosina e suas resultantes sequências peptídicas fornecem locais de ligação para STAT3, mas não para STAT1 e STAT5 (estas últimas podem ser ativadas de outras maneiras pela IL-10). STAT3 então se liga a cadeia IL-10RI através do domínio SH2 e é subsequentemente fosforilada. Uma vez ativada ela se dissocia de seu receptor, e passa por homodimerização ou forma heterodímeros com STAT1 ou STAT5 (WEBER-NORDT et al., 1996; DONELLY et al., 1995). Estes dímeros de STAT se translocam para o núcleo e se ligam a regiões promotoras dos genes responsivos a IL-10 e desta forma, se dá início a transcrição gênica (DONELLY et al., 1995; AKDIS et al., 2011) (Figura 6).

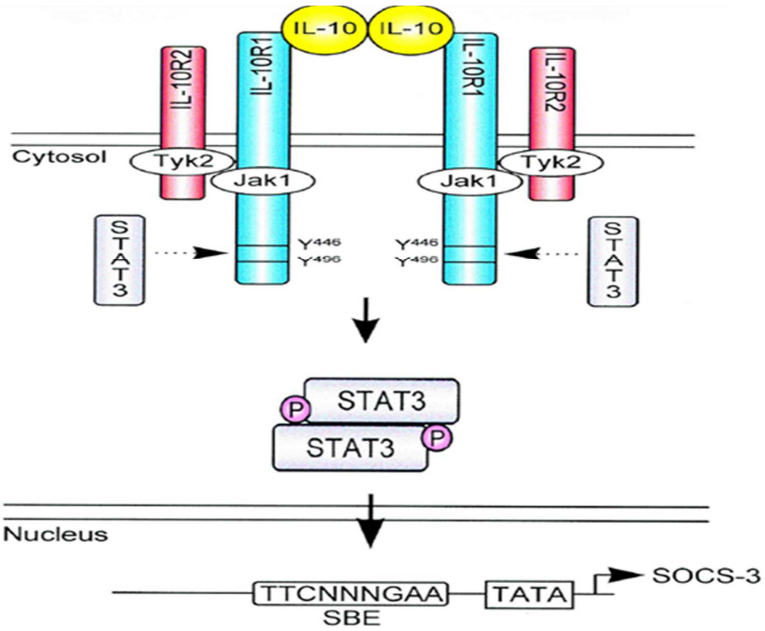

Figura 6 – Mecanismo esquemático das vias de sinalização induzidas pela IL-10 em fagócitos mononucleares humanos. Fonte: Donnelly et al., 1999.

STAT3 mostrou-se ser essencial para todos os aspectos dos efeitos anti-inflamatórios da IL-10, como demonstrado por um modelo murino com deleção gênica de STAT3 em neutrófilos e macrófagos. Nestes animais, o efeito supressivo da IL-10 na produção de citocinas inflamatórias foi completamente abolido, com esses animais ficando altamente susceptíveis a choque endotóxico e enterocotite crônica (TAKEDA et al., 1999). De fato, evidências recentes sugerem que a produção de IL-10 depende da ativação do fator de transcrição mTOR (alvo da rapamicina em mamíferos) induzida por STAT-3 (FOLDENAUER et al., 2013).

A IL-10 é um regulador chave da resposta inflamatória e através de seus efeitos imunosupressivos protege o hospedeiro de resposta inflamatória exagerada tanto durante infecções microbianas quanto durante doenças auto-imunes. Sua função primária é limitar a produção de citocinas e quimiocinas em macrófagos e células dendríticas induzidas por agonistas dos receptores do tipo toll (TLR), principalmente lipopolissacarídeos (LPS) (AKDIS et al., 2011). A IL-10 afeta diretamente a função dos monócitos e macrófagos reduzindo a expressão de moléculas MHC classe II e das moléculas co-estimulatórias CD80/CD86 nessas células (de WAAL MALEFYT et al., 1991a). Ademais, a IL-10 inibe a expressão de muitas citocinas, incluindo IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-18, GM-CSF, G-CSF e TNF-α; quimiocinas, incuindo MCP-1 (proteína quimioatraente para monócitos), MCP-5, MIP-1α (proteína inflamatória do macrófago), MIP-1β, RANTES, IL-8, assim como receptores de quimiocinas (de WAAL MALEFYT et al., 1991a; de WAAL MALEFYT et al., 1991b). Assim, a IL-10 inibe a produção de citocinas e a proliferação de células $TCD4^+$ principalmente indiretamente, através de seus efeitos nas células apresentadoras de antígenos (de WAAL MALEFYT et al., 1991a; de WAAL MALEFYT et al., 1991b). No entanto, também foi mostrado que a IL-10 afeta diretamente a produção de citocinas nas células T através da supressão de CD28 e do co-estimulador induzível de células T (ICOS) (TAYLOR et al., 2007). De forma contrária aos seus efeitos inibitórios em muitas células, a IL-10 aumenta a expressão de moléculas MHC classe II e estimula a diferenciação de células B murinas em células secretoras de anticorpos (AKDIS et al., 2011).

Apesar de a IL-10 ser reconhecida principalmente por sua habilidade de inibir a expressão de genes relacionados às citocinas, ela também ativa a expressão de vários genes nos monócitos, entre eles, o receptor FcγRI (CD64), que apresenta alta afinidade para imunoglobulina G (IgG), inibidor tecidual

de metaloproteinases-1 (TIMP-1) e o receptor de quimiocina CCR5. Adicionalmente, a IL-10 pode potencializar a expressão de IL-1ra e TNFR2 (p75) em monócitos estimulados por LPS (LARNER et al., 1993; JENKINS et al., 1994; LACRAZ et al., 1995; DICKENSHEETS et al., 1997; SOZZANI et al., 1998; ITO et al., 1999). Por fim, a indução da expressão dos genes SOCS-3 e -1 (supressor da sinalização de citocinas 3 e 1, respectivamente) leva a inibição de resposta induzidas por citocinas, o que pode explicar os amplos efeitos anti-inflamatórios da IL-10, que são dependente de STAT3 (DONELLY et al., 1999; AKDIS et al., 2011).

Foi sugerido que a IL-10 exerce suas ações imunossupressoras por interferir na ativação das vias de sinalização induzidas pelo NFκB, no entanto estes supostos efeitos inibitórios da IL-10 sobre o NFκB foi demonstrado posteriormente ser negligenciável (SCHOTTELIUS et al., 1999; MURRAY, 2005). Por outro lado, efeito inibitório indireto induzido pela sinalização da IL-10 sobre o NFκB foi recentemente demonstrado (EL KASMI et al., 2007). Outros estudos avaliaram o papel da sinalização induzida pela IL-10 na ativação de proteínas quinases ativadas por mitógenos (MAPK), apresentando resultados conflitantes, sendo observado tanto ausência de efeitos quanto inibição destas vias em diferentes modelos (DONELLY et al., 1999; CLARKE et al., 1998; FOEY et al., 1998; NIIRO et al., 1998; WARD et al., 2005; RAJASINGH et al., 2006). Neste contexto, a IL-10 parece ter papel protetor em diversas doenças autoimunes como o lúpus eritematoso sistêmico, artrite reumatóide e diabetes melito, além de estar presente em elevadas concentrações em diversos canceres incluindo melanomas e linfomas (AKDIS et al., 2011). Esta ação imunossupressora foi demonstrada em camundongos deficientes para IL-10 que desenvolvem atrite induzida por colágeno de maneira mais severa. Resposta similar foi observada em ratos tratados com anticorpo anti-IL-10 (FIORENTINO et al., 1991; KASAMA et al., 1995; JOHANSSON et al., 2001). Por outro lado, o tratamento com IL-10 reduz significativamente a severidade da artrite (TANAKA et al., 1996).

A função anti-hiperalgésica da IL-10 também foi evidenciada em diversos modelos experimentais. Em modelo inflamatório induzido por administração de Cg, uma única injeção local de IL-10 antes do estímulo aboliu a hiperalgesia mecânica na pata de ratos (POOLE et al., 1995; VERRI et al., 2006). Adicionalmente, a IL-10 é capaz de inibir a hiperalgesia induzida por injeção local de TNF-α, IL-1β e IL-6, mas não por IL-8 ou PGE_2. Estes resultados podem ser explicados pelo fato de que o efeito nociceptivo induzido pela IL-8 é mediado por

aminas simpáticas e não por citocinas, e no caso da PGE_2 pela sua direta ação de sensibilização dos nociceptors (VERRI et al., 2006). No entanto, a IL-10 pode inibir a produção de prostanóides. Neste sentido, foi observado que a hiperalgesia induzida por IL-1β (que é dependente da produção de prostanóides) foi inibida pelo tratamento com IL-10 (CUNHA et al., 1992; POOLE et al., 1995; VERRI et al., 2006). O efeito inibitório da IL-10 também foi demonstrado em modelo de hiperalgesia térmica e mecânica induzida por LPS, através da inibição de citocinas pro-inflamatórias (KANAAN et al., 1998). Adicionalmente, a administração de IL-10 atenuou a hiperalgesia térmica provocada após injúria por constrição crônica do nervo (WAGNER et al., 1998). Por fim, o efeito anti-nociceptivo da IL-10 foi demonstrado em modelos de dor manifesta, inibindo de maneira dose-dependente contrações abdominais induzidas por ácido acético e zimosan, através da inibição da produção de TNF-α e IL-1β, e também inibindo a hiperalgesia induzida pelo movimento em modelo de inflamação articular em ratos, (VALE et al., 2003).

A liberação endógena de IL-10 também pode agir como modulador negativo do processo inflamatório, porque a administração de anticorpo contra esta citocina, além da aumentar a resposta hiperalgésica, também aumenta outros eventos inflamatórios (POOLE et al., 1995; OBERHOLZER et al., 2002; VALE et al., 2003; VERRI et al., 2006). Desta forma, a administração de IL-10 pode representar uma ferramenta benéfica eficaz na intensão de se inibir a dor neuropática e inflamatória.

1.6 PENTOXIFILINA

A pentoxifilina, um derivado da metilxantina, é um agente hemorreológico ativo, que modifica as propriedades funcionais do fluxo sanguíneo, agindo sobre a sua viscosidade. É administrado por via oral, e usado no tratamento de doenças vasculares periféricas, doenças cerebrovasculares e diversas outras condições que envolvem defeitos na microcirculação regional, com mínimos efeitos colaterais (WARD & CLISSOLD, 1987; MAGNUSSON, 2009). A pentoxifilina age primeiramente por aumentar a deformidade eritrocitária prejudicada, por reduzir a viscosidade sanguínea e por diminuir o potencial de agregação plaquetária e consequente formação de trombos, e ainda por reduzir a adesão e ativação dos leucócitos no endotélio, reduzindo assim o dano endotelial resultante. A resistência

periférica pode ser levemente reduzida por administração de altas doses ou infusão rápida de pentoxifilina. Por fim, a pentoxifilina pode exercer leve efeito inotrópico no coração. A pentoxifilina é usualmente bem tolerada quando administrada como a convencional formulação de liberação controlada, e sua farmacocinética e farmacodinâmica garantem que ela é segura (WARD & CLISSOLD, 1987; VERRI et al., 2006). O principal modo de ação da pentoxifilina é a redução da produção de TNF-α em mais de 50%, por diminuir o tempo de meia vida de seu mRNA (STRIETER et al., 1988; DOHERTY et al., 1991; SAMPAIO et al., 1998).

Após administração oral, a absorção da pentoxifilina é rápida e praticamente completa. Após absorção quase completa, a pentoxifilina passa por metabolismo de primeira passagem. Quando a biotransformação da pentoxifilina foi estudada no humano, sete metabólitos de fase 1 (descritos M1-M7) foram identificados na urina humana (HINZE, 1972b). As estruturas dos metabólitos foram determinadas: a biotransformação produz três metabolitos hidroxilados (M1, M2, M3a e M3b), dois metabólitos carboxilados (M4, M5), e dois metabólitos desmetilados (M6 e M7) (MAGNUSSON, 2009) (Figura 8).

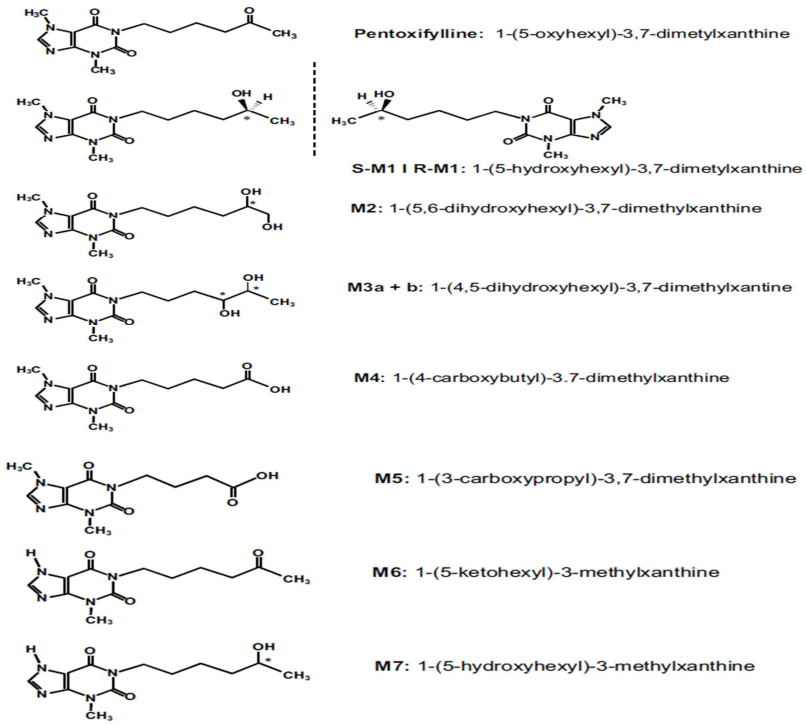

Figura 8 – Fórmula estrutural da pentoxifilina e seus metabólitos. Fonte: Magnusson et al., 2009.

O metabólito mais excretado na urina é o M5, seguido pelo M4. A excreção da pentoxifilina inalterada e do metabólito M1 é responsável por menos de 1% da dose (HINZE, 1972a). Quando a pentoxifilina é administrada a humanos saudáveis, as áreas sob a curva (AUC) de concentração plasmática para M5 e M1, mas não para M4 são maiores do que as da pentoxifilina (BEERMANN et al., 1985; SMITH et al., 1986; BRYCE et al., 1989). Estudos têm mostrado que a depuração da pentoxifilina foi muito maior do que o fluxo sanguíneo hepático, e superiores, ou no mesmo nível do débito cardíaco (INGS et al., 1982; RAMES et al., 1990). Uma vez que o metabolismo da pentoxifilina a M1 é reversível, a depuração é ainda mais subestimada, assim que a curva de concentração plasmática é aumentada devido à contínua adição da pentoxifilina formada a partir de M1. A alta taxa de depuração indica que a pentoxifilina é metabolizada também em outros locais além do fígado, como por exemplo, o sangue, uma vez que a depuração não é limitada ao fluxo sanguíneo hepático. Neste sentido, estudos mostraram que a pentoxifilina é metabolizada a M1 quando incubada em sangue (BRYCE et al., 1980; INGS et al., 1982), sugerindo que o sangue é a principal local para a interconversão da pentoxifilina em M1 (NICKLASSON et al., 2002). Os metabólitos M1 e M5 apresentam efeitos hemorreológicos significantes, mas M2, M3, M4, M6 e M7 têm poucos efeitos hemorreológicos (AMBRUS et al., 1995).

A pentoxifilina é classificada como droga hemorreológica, inibidor de fosfodiesterase e inibidor inespecífico de citocinas (VERRI et al., 2006; MIKA et al., 2008; MELO et al., 2008). Estudos têm mostrado que a pentoxifilina melhora o fluxo sanguíneo periférico, utilizando diversos mecanismos (MAGNUSSON et al., 2009). O fluxo sanguíneo dos capilares e a viscosidade sanguínea são influenciados pela deformidade e agregação dos eritrócitos, hematócrito e viscosidade do plasma. A pentoxifilina reduz de maneira significativa a viscosidade do sangue em pacientes com desordens arteriais periféricas aumentando significativamente a deformidade dos eritrócitos em indivíduos saudáveis e pacientes com doença vascular periférica (SAMLASKA et al., 1994; WARD & CLISSOLD, 1987). Adicionalmente, a pentoxifilina diminui a adesão e agregação plaquetária na parede dos vasos de pacientes com desordens vasculares periféricas, e também reduz os níveis de fibrinogênio. Em pacientes saudáveis, a pentoxifilina aumenta a filtrabilidade de monócitos e leucócitos polimorfonucleares (WARD & CLISSOLD, 1987).

Consequentemente, a pentoxifilina promove a perfusão da microcirculação pela melhora da fluidez sanguínea e pelo desenvolvimento dos efeitos anti-trombóticos.

Após incubação com a pentoxifilina, observa-se que tanto células mono quanto polimorfonucleadas tem aumento dos níveis intracelulares de adenosina monofosfato cíclica (cAMP) através da inibição da fosfodiesterase (BESSLER et al., 1986). A fosfodiesterase catalisa a hidrólise do cAMP em adenosina monofosfato (5' AMP); a inibição desta enzima leva a elevação dos níveis de cAMP (MAGNUSSEN, 2009). O conteúdo elevado de cAMP nas plaquetas interfere com a agregação plaquetária e leva a inibição da ciclo-oxigenase (SHA et al., 2003). Além disso, níveis elevados de cAMP inibem a produção de citocinas em monócitos e linfócitos ativados (MAGNUSSEN, 2009).

O mecanismo exato pelo qual a pentoxifilina modifica a fisiologia do eritrócito ainda não é completamente entendido, mas pode ser decorrente da redução intracelular de cálcio (Ca^{2+}), devido à inibição do influxo de cálcio. É sabido que os eritrócitos mais velhos acumulam mais Ca^{2+} e são menos deformáveis. A transglutaminase é uma enzima dependente de Ca^{2+} que atravessa de maneira irreversível a membrana, desta forma, agindo sobre os eritrócitos. A pentoxifilina pode reduzir o cálcio intracelular e inibir a ativação de transglutaminases dependentes de Ca^{2+} (SWISLOCKI et al., 1989; 1991). Isto permite que os eritrócitos permaneçam deformados e torna mais acessível para eles a passagem através dos capilares. No entanto, o mecanismo exato pelo qual a pentoxifilina melhora a fluidez do sangue ainda não é completamente sabida. Outro estudo mostrou que a pentoxifilina melhora a fluidez do sangue em voluntários saudáveis, no entanto, sem aumento da deformidade dos eritrócitos (CUMMINGS et al., 1992).

Estudos mais recentes mostraram que a pentoxifilina pode agir em combinação com a vitamina E, reduzindo a fibrose induzida por radiação (DELANIAN et al., 1998; 1999; 2003; OKUNIEFF et al., 2004; CHIAO et al., 2005). Previamente, a fibrose induzida por radiação foi considerada irreversível.

A pentoxifilina foi descrita como benéfica em desordens imunológicas onde o TNF-α, entre outras citocinas parecem desempenhar um papel, como por exemplo, dermatites de contato alérgico, eritema nodoso leproso, artrite reumatóide, câncer e malária cerebral (GRANINGER et al., 1991; DEZUBE et al., 1993; SCHWARZ et al., 1993; HUIZINGA et al., 1996; SAMPAIO et al., 1998). Além do mais, a pentoxifilina inibe de maneira dose dependente a produção de IL-2 e INF-

γ, concomitantemente com inibição de TNF-α em células mononucleares sanguíneas periféricas (RIENECK et al., 1993; FUNK et al., 1995). Contudo, a inibição de outras citocinas como IL-1β e IL-6 permanece controversa (WEINBERG et al., 1992; MOREIRA et al., 1993; TILG et al., 1993; SAMPAIO et al., 1998; KOO et al., 2000). Aparentemente, a pentoxifilina não afeta os níveis de outras citocinas como a IL-10, embora alguns trabalhos tenham evidenciado aumentos na produção de IL-10 (D'HELLENCOURT et al., 1996; VAN FURTH et al., 1997; VERRI et al. 2006). Em sistemas in vitro, a pentoxifilina pode tanto aumentar quanto diminuir a produção de citocinas (MARCINKIEWICZ et al., 2000). Assim, é provável que a modulação da produção de IL-10 pela pentoxifilina dependa da dosagem, tempo de tratamento e modelo experimental.

Devido ao fato de que o TNF-α e outras citocinas têm papel crucial no desenvolvimento de doenças inflamatórias (VERRI et al., 2006), a pentoxifilina pode ser considerada como uma ferramenta farmacológica promissora para o tratamento destas doenças. Quanto à dor inflamatória, foi demonstrado em humanos que o pré-tratamento com pentoxifilina, além de diminuir os níveis séricos de TNF-α e IL-1β, reduz a necessidade do uso de opióides no pós-operatório imediato de colecistectomia. Em outro estudo usando o mesmo protocolo, o pós-tratamento com pentoxifilina não modificou os mesmos parâmetros avaliados, evidenciando a importância da farmacocinética da pentoxifilina para sua ação (VERRI et al., 2006). Em pacientes com claudicação intermitente, a pentoxifilina foi eficiente em reduzir os níveis de proteína C reativa, além de melhorar a dor (DE ALBUQUERQUE et al., 2008). Corroborando este fato, a pentoxifilina reduziu os níveis sistêmicos de proteína C reativa e TNF-α em pacientes com doenças arterial coronariana, após 6 meses de tratamento (FERNANDES et al., 2008).

Além das demonstrações de efetividade da pentoxifilina em humanos, existem evidências experimentais consistentes de sua ação anti-nociceptiva. Em ratos a pentoxifilina inibe a hiperalgesia mecânica administrada pré- ou pós-injúria (WORDLICZEK et al., 2000). A pentoxifilina também inibe a dor inflamatória em diferentes modelos, como por exemplo, de contorções abdominais induzidas por ácido acético e zimosan, hiperalgia articular induzida por zimosan e hiperalgesia mecânica induzida por TNF-α, Cg ou bradicinina (VALE et al., 2004). Adicionalmente, não foram detectados efeitos centrais, indicando o seu uso para o tratamento da dor inflamatória periférica (VALE et al., 2004). Os efeitos anti-

inflamatórios e anti-nociceptivos da pentoxifilina são provavelmente indiretos, especificamente no nível da produção de citocinas, considerando que ela não inibe a hiperalgesia mecânica induzida por PGE_2 ou por compostos análogos da prostaciclina (PGI_2), já que estes últimos são os mediadores hiperalgésicos finais, estimulando diretamente os nociceptores (via seus receptores expressos nos nociceptores) (CUNHA et al. 2005; VERRI et al., 2006). Por fim, em modelo de síndrome dolorosa regional complexa em camundongos, a pentoxifilina reduz a sensibilização dos nociceptores, eventos vasculares e expressão do mRNA / níveis proteicos de citocinas (TNFα, IL-1β e IL-6) na pata homolateral após fratura de tíbia (WEI et al., 2009).

Além da inibição do TNF-α e outras citocinas, foi relatado que a pentoxifilina inibe a ativação de NFκB induzida por endotoxina in vivo no intestino de ratos durante sepse. A pentoxifilina pode reduzir a expressão de NFκB via inibição da proteína quinase C (PKC) (BISWAS et al., 1994). A pentoxifilina pode inibir inespecificamente a PKC e também a proteína quinase A (PKA) (BISWAS et al., 1994), contribuindo para o controle da dor, considerando que a participação tanto da PKCε quanto da PKA foi relacionada com indução de hiperalgesia. Neste sentido, a PKA e a PKCε têm sido implicadas na modulação da atividade de canais iônicos, diminuindo o limiar de disparo de potenciais de ação dos neurônios, facilitando a ativação neuronal (LYNN & O'SHEA, 1998; ALEY & LEVINE, 1999; PARADA et al., 2003; 2005). Outros estudos posteriores também relataram inibição da ativação de NFκB pela pentoxifilina (LIU et al., 2007; HERNANDEZ-FLOREZ et al., 2011; MAHAMOUD et al., 2012), o que pode contribuir para a ação anti-hiperalgésica da pentoxifilina, levando-se em consideração que a sua inibição leva a produção de citocinas inflamatórias (VERRI et al., 2006).

Vale a pena salientar que inibidores específicos de citocinas (imunobiológicos) estão disponíveis no mercado, como por exemplo, infliximab (anticorpo anti-TNF), etanercept (receptor solúvel para TNF), anakinra (antagonista de receptor de IL-1), no entanto, o alto custo destas terapias deve ser considerado. Neste sentido, a relação custo-benefício da pentoxifilina reforça esta droga como terapia promissora para o tratamento de doenças acompanhadas de dor induzida por citocinas. Além da questão do custo, a pentoxifilina poderia ser utilizada como alternativa terapêutica para pacientes com efeitos adversos mais intensos com o uso

de imunobiológicos como reação inflamatória no local de administração e diminuição da eficácia.

1.7 TALIDOMIDA

A talidomida, um derivado do ácido glutâmico, foi inicialmente desenvolvida como droga anti-emética, para ser usada principalmente durante a gravidez. No entanto, devido aos seus efeitos teratogênicos, o uso da talidomida ficou restrito (VERRI et al., 2006). Atualmente, é usada em alguns países, como por exemplo, o Brasil, para o tratamento do eritema nodoso leproso, uma complicação inflamatória da lepra que ocorre em aproximadamente 30% dos pacientes, usualmente com iniciação em associação durante terapia multidrogas (SARNO et al., 1991; MAJUMDER et al., 2012). Mais recentemente, em 2006, a talidomida foi aprovada para o tratamento do mieloma múltiplo, e sua eficácia tem sido relatada em grande variedade de doenças malignas e não malignas (MAJUMDER et al., 2012). Adicionalmente, o seu uso em várias condições inflamatórias, dermatológicas e oncológicas vêm sendo investigada, com resultados promissores (SARNO et al., 1991; AARESTRUP et al., 1995; FERNÁNDEZ-CAMACHO & LEON-DORANTES, 2000; RAJKUMAR & WITZIG, 2000; WINES ET AL., 2002; MOOS ET AL., 2003; YEH et al., 2004; KUMAR et al., 2004; TEO et al., 2004; TEO et al., 2005). A talidomida é composta de dois enantiômeros, talidomida–R e –S, com ligação proteica de 55% e 65%, respectivamente. Os dois enantiômeros tem propriedades distintas; a talidomida–S é um supressor mais potente da liberação de TNF-α por células mononucleares periféricas ativadas, enquanto que a talidomida–R é mais sedativa (MAJUMDER et al., 2012) (Figura 9). Mais de 90% da droga absorvida é excretada na urina e fezes dentro de 48 horas (TEO et al., 2004).

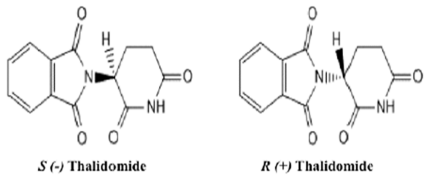

Figura 9 – Estrutura química dos dois enantiômeros da talidomida S(-) e R(+). Fonte: Majumder et al., 2012.

A talidomida e seus análogos são minimamente metabolizados pelo fígado. No entanto, em soluções aquosas com pH 7 são espontaneamente hidrolisadas em mais de 20 produtos que são responsáveis por sua atividade, e excretados pelo rim. Após a administração de uma única dose oral de 200 mg de talidomida (dose aprovada pelos Estados Unidos para formulação da cápsula) em voluntários saudáveis, a absorção é lenta e extensiva, resultando num pico de concentração de 1-2 mg/L, 3-4 horas após a administração, com tempo de retardo de absorção de 30 minutos, aparente tempo de meia-vida de eliminação de 6 horas e remoção sistêmica de 10 horas (TEO et al., 2004; MAJUMDER et al., 2012). Pela reduzida hidrossolubilidade, a absorção da talidomida pelo trato gastrintestinal é lenta e a biodisponibilidade varia entre 67% e 93%. Estudos experimentais mostraram elevadas concentrações no trato gastrintestinal, fígado e rins, e baixa nos músculo, cérebro e tecido adiposo. Nos animais, a principal via de degradação parece ser uma clivagem hidrolítica não enzimática, produzindo sete produtos principais a partir da hidrólise e no mínimo cinco produtos menos significativos. A talidomida pode sofrer biotransformação hepática, via enzimas do citocromo P450 (FUNED).

O medicamento parece não inibir seu próprio metabolismo, entretanto pode interferir na indução enzimática promovida por outros compostos. O produto final do metabolismo é o ácido ftálico. O tempo de meia-vida é de aproximadamente 8,7 horas, e a ligação às proteínas plasmáticas é elevada (FUNED). O tempo gasto para início da ação em diferentes condições clínicas é apresentado na tabela 1. A concentração plasmática máxima, o tempo necessário para alcançar o pico da concentração plasmática e o tempo de meia-vida de eliminação estão especificados na tabela 2.

Condição Clínica	Início da ação
Reação hansênica do tipo eritema nodoso ou tipo II	48 horas
Enxerto-versus-hospedeiro	1 a 2 meses
Lúpus eritematoso discóide	2 semanas
Artrite reumatóide	2 a 3 meses

Tabela 1. Tempo para início da ação da talidomida nas diferentes condições clínicas. Fonte: Fundação Ezequiel Dias (FUNED).

Condição Clínica	Dose	Pico de concentração plasmática (mg/ml)	Tempo para atingir concentração plasmática máxima (h)	Tempo de meia vida de eliminação (h)
Indivíduos saudáveis	50 mg	0,62	2,9	5,52
	200 mg	1,15 a 1,76	3,5 a 4,4	5,53
	400 mg	2,82	4,3	7,29
Portadores do vírus HIV	100 mg	1,17 +/- 0,21	3,4 +/- 1,8	6,5 +/- 3,4
	300 mg	3,47 +/- 1,14	3,4 +/- 1,5	5,7 +/- 0,6
Pacientes com hanseníase	400 mg	3,44	5,7	6,86

Tabela 2. Tempo necessário para atingir o pico de concentração sérica e concentração plasmática máxima e o tempo de meia-vida de eliminação, após administração de dose única de talidomida. Fonte: Fundação Ezequiel Dias (FUNED).

A excreção da talidomida é renal, com a taxa de depuração de 1,15 ml/minuto. Menos de 0,7% do fármaco é excretado sob a forma inalterada. O produto final do metabolismo, o ácido ftálico, é excretado conjugado com a glicina (FUNED). Idade, sexo e tabagismo não têm efeito na farmacocinética da talidomida. Embora a presença de alimentos ricos em lipídeos no estômago possa aumentar em 6 horas o tempo necessário para atingir o melhor efeito da talidomida (FUNED), o efeito do alimento não é considerado significativo (TEO et al., 2004). A talidomida não modifica a farmacocinética de contraceptivos orais, além de ser bem tolerada em pacientes com doenças hepáticas e renais graves, considerando que é principalmente hidrolisada e excretada passivamente (TEO et al., 2004; FUNED).

A talidomida é indicada para manifestações cutâneas moderadas a grave, no tratamento da reação hansênica do tipo eritema nodoso (tipo II), inclusive como terapia de manutenção para prevenção e supressão das manifestações da reação hansênica do tipo eritema nodoso recidivante. Não é recomendada como monoterapia no tratamento da reação hansênica tipo II na presença de neurite moderada a grave. É indicada ainda para o tratamento de úlceras aftosas associada à imunodeficiência em pacientes infectados ou não pelo HIV, que não respondem ao tratamento com outras drogas, lúpus eritematoso e doença do enxerto-versus-hospedeiro. No Brasil, as indicações da talidomida são restritas aos programas oficiais das doenças supra-citadas (FUNED), no entanto, em outros países ocidentais, o seu uso ainda permanece restrito.

A talidomida está relacionada quimicamente com glitetimida e clortalidona, apresentando ação sedativa e/ou hipnótica, anti-inflamatória, moduladora da resposta imune e teratogênica. Foi bem estabelecido que a talidomida não apresenta ação antibacteriana e antimicótica, desta forma, sua utilidade clínica está relacionada às suas propriedades anti-inflamatória e imunomoduladora (FUNED). Os estudos sobre os mecanismos de ação da talidomida demonstraram que ela inibe seletivamente a produção de TNF-α em macrófagos alveolares de humanos e também monócitos humanos estimulados com produtos de *E. Coli* ou *Mycobacterium Leprae* (SAMPAIO et al., 1991; MOREIRA et al., 1993; TAVARES et al., 1997). De fato, a talidomida inibe a produção de TNF-α por aumentar a degradação de seu mRNA (MOREIRA et al., 1993). Além do mais, aparentemente, a talidomida parece não afetar a produção de outras citocinas em monócitos estimulados por LPS (SAMPAIO et al., 1991), embora sua ação sobre outras citocinas, como por exemplo, IL-1β, IL-8, IL-12, IL-18 e KC tenham sido descritas em trabalhos com diferentes modelos de doenças (YE et al., 2006; ESKI et al., 2008; MELO et al., 2008). Neste sentido, a talidomida parece reduzir o TNF-α em pacientes com reação sistêmica do tipo II, provavelmente pela capacidade de reduzir os sintomas locais e sistêmicos da reação e diminuir o número de neutrófilos e linfócitos TCD4+ nas lesões (FUNED).

A seletiva e eficaz ação inibitória da talidomida sobre o TNF-α foi demonstrada pela eficiência no tratamento de doenças citadas anteriormente, caracterizadas por infiltração de leucócitos polimorfonucleares (PMNLs), como por exemplo, doença do enxerto-versus-hospedeiro (VOGELSANG et al., 1992), úlceras aftosas orais em pacientes imunodeprimidos em decorrência do HIV (JACOBSSON et al., 1997), artrite reumatoide refratária (GUTIERREZ-RODRIGUEZ et al., 1989), migração de neutrófilos para cavidade sinovial durante inflamação imune (BOMBINI et al., 2004) e reação hansênica do tipo II ou do tipo eritema nodoso (FUNED). Observa-se ainda, redução da quimiotaxia dos PMNLs, e consequentemente de sua fagocitose. A eficiência da talidomida também foi constatada em outros processos inflamatórios em que predominam células mononucleares, como por exemplo, no lúpus eritematoso discóide, diminuindo a capacidade fagocitária dos monócitos, o que sugere redução do processo inflamatório e da injúria tecidual, através do controle do estresse oxidativo e também de outros mediadores inflamatórios (FUNED).

A talidomida bloqueia a expressão de TNF-α por diferentes possíveis mecanismos. Pode inibir o NFκB em células B ativadas, um fator de transcrição considerado essencial para produção de TNF-α e outras citocinas que sob ação da talidomida leva a redução da expressão de TNF-α. Adicionalmente, a proteína adaptadora MyD88 pode regular a expressão de TNF-α sob ação da talidomida. Como descrito anteriormente, o tratamento com talidomida também leva a degradação do mRNA do TNF-α, reduzindo a expressão total da proteína TNF-α (MAJUMDER et al., 2012). A talidomida pode ainda regular a produção de TNF-α por inibir a α1-glicoproteína ácida (AGP), uma proteína de fase aguda, descrita por apresentar ações pro-inflamatórias (TURK et al., 1996; PUKHAL'SKII et al., 2001). Por fim, evidências sugerem que a talidomida pode inibir a expressão de TNF-α por interagir com regiões promotoras específicas do DNA, especificamente com o gene GC (citosina/guanina)-rich (STEPHENS et al., 2000; DRUCKER et al., 2003). A Figura 10 mostra os possíveis diferentes alvos da talidomida no bloqueio da sinalização induzida por TNF-α.

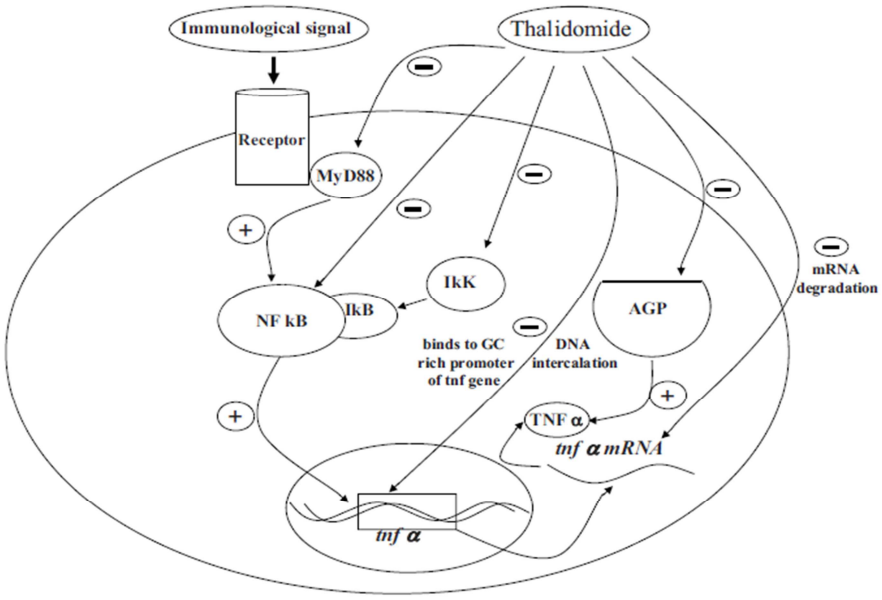

Figura 10 – Possíveis diferentes alvos da talidomida no bloqueio da sinalização do TNF-α. Fonte: Majumder et al., 2012.

Com base nas evidências sobre o potencial terapêutico do uso da talidomida em doenças relacionadas com o TNF-α, os efeitos analgésicos desta

droga foram investigados. Segundo a revisão de VERRI e colaboradores (2006), a talidomida inibe a hiperalgesia mecânica induzida por Cg em ratos e também contorções abdominais induzidas por acido acético em camundongos. Interessantemente, a hiperalgesia mecânica induzida por TNF-α ou PGE_2 não foi inibida pelo tratamento com talidomida, reforçando o conceito de que a inibição pode estar associada seletivamente com a inibição da expressão do mRNA do TNF-α nas fases iniciais dos mecanismos da gênese da dor (MOREIRA et al., 1993; RIBEIRO et al., 2000a; RIBEIRO et al., 2000b). Aparentemente, o efeito analgésico da talidomida é periférico e indireto, não afetando o edema ou estimulando a produção de citocinas anti-inflamatórias, no entanto, é importante mencionar que os efeitos modulatórios da talidomida dependem do modelo, dose e tempo de administração do tratamento.

O potencial uso terapêutico da talidomida como analgésico também foi evidenciado em modelos de hiperalgesia induzida por injúria por constrição crônica do nervo ciático relacionada à TNF-α/TNFR1 em ratos, reduzindo os comportamentos relacionados a dor e também as alterações vasculares patológicas relacionadas ao modelo (SOMMER et al., 1998a; 1998b). Ainda, outros estudos também avaliaram os efeitos da talidomida na síndrome mielodisplásica, mieloma múltiplo, dor relacionada ao câncer ósseo, modelo de dor orofacial induzida por formalina e no tratamento de dores crônicas como na síndrome dolorosa complexa regional (CRPS), com resultados promissores no controle da dor (MACKEY & FEINBERG, 2006; SEADI PEREIRA et al., 2009; GU et al., 2010; ASHER & FURNISH, 2013). Por fim, a talidomida e seus análogos são prontamente capazes de atravessar a barreira hemato-encefálica, sendo desta forma, fortes candidatos para serem utilizados na determinação do potencial valor das terapias anti-TNF-α em grande variedade de doenças. (MAJUMDER et al., 2012).

Desta forma, evidências consistentes da ação da talidomida dão respaldo ao seu uso no controle da dor inflamatória ou neuropática associada com papel nociceptivo do TNF-α. Assim como a pentoxifilina, a talidomida, devido ao seu custo-benefício favorável em relação a outras abordagens farmacológicas, pode ser considerada uma droga interessante para o tratamento da dor e doenças inflamatórias crônicas. Citações a respeito da talidomida, em especial a que se segue: “o pesadelo do passado passa a se tornar uma promessa do futuro”, ganham

a cada dia mais crédito considerando os efeitos devastadores da sua primeira aplicação clínica e a sua perspectiva terapêutica atual.

1.8 Papel das Citocinas no Desenvolvimento da Dor Muscular

A dor de origem musculoesquelética – incluindo a fibromialgia, mialgia relacionada ao trabalho excessivo, lombalgias, artrites e outras condições inflamatórias dos músculos, tendões e articulações – é um importante problema econômico e de saúde (IASP). Sendo assim, estudos que avaliem os mecanismos envolvidos na dor muscular são necessários visando novas descobertas que possam auxiliar no tratamento destas condições clínicas.

Citocinas são peptídeos extracelulares que apresentam potentes efeitos biológicos estimulatórios ou inibitórios em diversas populações celulares. Originalmente identificadas como mediadores críticos para os processos inflamatórios e respostas imunes, as citocinas hoje são reconhecidas por estar envolvidas na maioria dos processos fisiológicos. Seu papel essencial na homeostase envolve ativação de mecanismos inflamatórios e nociceptivos, modulação do reparo tecidual e remodelação do tecido danificado. Em condições inflamatórias, ocorre intenso aumento na produção de citocinas, e ao mesmo tempo, o balanço entre o controle e produção de citocinas é perdido (JOUVENNE et al., 1998). As informações disponíveis sobre o envolvimento das citocinas com a dor musculoesquelética são no geral muito limitadas, exceto para as citocinas IL-1β e TNF-α (KOPP & ALSTERGREN, 2008).

A dor articular e muscular é frequentemente associada com a ativação de nociceptores articulares e musculares por uma variedade de mediadores inflamatórios, incluindo neuropeptídios, prostanóides e citocinas (KOPP & SOMMER, 1997). Evidências mostraram que a inflamação nos tecidos sinoviais, como por exemplo, aqueles presentes na articulação temporomandibular (ATM), e provavelmente na musculatura adjacente, desempenham importante papel na determinação do desenvolvimento da dor (KOPP & ALSTERGREN, 2008).

Quando a produção de citocinas é balanceada nos tecidos saudáveis ou durante remissão de doença inflamatória, os efeitos deletérios das citocinas pró-inflamatórias são endogenamente controlados pelos efeitos de mediadores anti-inflamatórios, como por exemplo, a interleucina-10 (IL-10), IL-1ra,

receptores solúveis (sTNFR2, sIL-1RII) ou receptores chamariz (IL-1RII) (KOPP & ALSTERGREN, 2008). O desequilíbrio na liberação de citocinas, levando a maior produção de citocinas pró-inflamatórias, excede a habilidade de contenção dos efeitos pró-inflamatórios por parte dos fatores de controle endógenos, resultando em aumento da atividade inflamatória.

As citocinas TNF-α, IL-1β e IL-6, além de apresentarem efeitos pró-inflamatórios diretos, também exercem efeitos indiretos por estimularem a liberação de outros mediadores inflamatórios, entre eles, outras citocinas, prostanóides, bradicinina e serotonina. Neste sentido, os neurônios nociceptivos periféricos são sensibilizados e ativados diretamente pelo TNF-α, IL-1β e IL-6, através de suas ligações aos seus respectivos receptores. Corroborando, receptores de superfície celular nas fibras aferentes nociceptivas foram identificados para o TNF-α e IL-1β, mas não para a IL-6. No entanto, eles expressam a proteína gp130, que se liga a parte solúvel do receptor da IL-6 na superfície celular, permitindo a sua ligação e consequente resposta celular (KOPP & ALSTERGREN, 2008). Um modelo de modulação da dor articular por citocinas é apresentado na Figura 7.

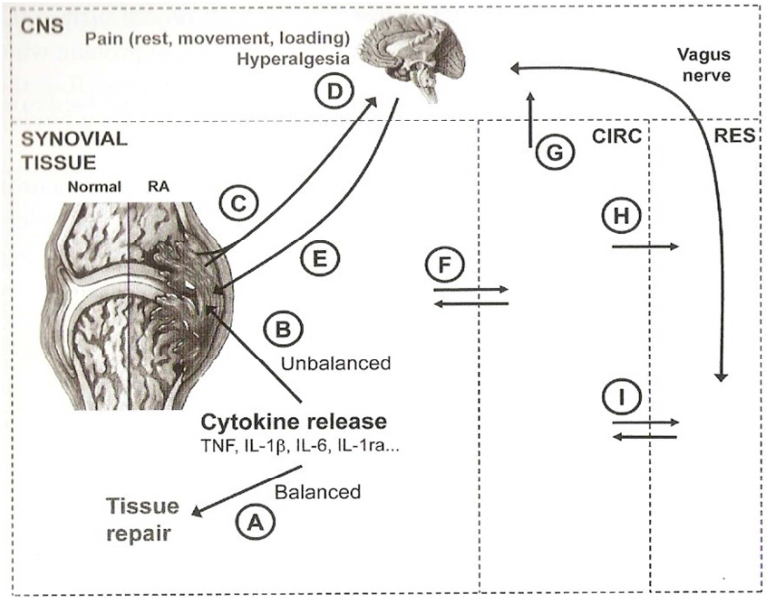

Figura 7 – Modelo de modulação da dor articular mediada por citocinas. (A) Equilíbrio na produção de citocinas, com os efeitos deletérios das citocinas pró-inflamatórias sendo controlados por fatores endógenos anti-inflamatórios. (B) Desequilíbrio na produção de citocinas, a favor das pró-inflamatórias. (C) Sensibilização e / ou ativação de neurônios nociceptivos periféricos. (D) O aumento

da sinalização nociceptiva para o sistema nervoso central (CNS) devido aos efeitos das citocinas pró-inflamatórias amplifica a sensação de dor articular e leva a sensação de hiperalgesia para as regiões adjacentes a articulação. (E) Impulsos nociceptivos contínuos ao CNS aumentam a atividade simpática, levando a liberação de aminas simpáticas no tecido sinovial, amplificando a sinalização nociceptiva das terminações nervosas. (F) Durante o processo inflamatório, as citocinas entram na circulação sanguínea (CIRC) por drenagem linfática, ou aumento da permeabilidade vascular influenciando outros tecidos distantes da sinóvia. (G) TNF-α, IL-1β e IL-6 exercem efeitos diretos no CNS, através de ligação a seus respectivos receptores em regiões paraventriculares, podendo induzir febre e hiperalgesia generalizada. (H) Na circulação as citocinas podem influenciar o CNS pela ativação de receptores no nervo vago no sistema reticuloendotelial (RES), incluindo fígado e baço, com a sinalização aferente do vago provocando febre e hiperalgesia generalizada. (I) Atividade na via anti-inflamatória colinérgica (nervo vago e RES) pode diminuir a liberação de citocinas em locais de grande produção como fígado e baço, e redirecionar o recrutamento de leucócitos para fora da periferia, desta forma reduzindo o processo inflamatório periférico. Fonte: Koop & Alstergren, 2008.

A IL-1β é capaz de diminuir o limiar nociceptivo nos tecidos periféricos por efeito excitatório direto, sensibilizando as fibras nociceptivas (FUKUOKA et al., 1994). A IL-1β é um potente agente hiperalgésico, quando administrada sistemicamente a ratos, parcialmente por ação periférica (FERREIRA et al., 1988). A presença de IL-1β não é detectável no fluído sinovial da ATM de indivíduos saudáveis, mas níveis elevados em pacientes com poli-artrite são encontrados (ASLTERGREN et al., 1998, 1999). Os níveis de IL-1β no fluído sinovial de ATMs com atrite mostrou correlação positiva significante com a dor no repouso e sensibilidade e correlação negativa com os níveis de tolerância a pressão da dor (ASLTERGREN et al., 1998), evidenciando que, a IL-1β é um dos determinantes da dor e sensibilização da ATM. Reforçando este conceito, pacientes com altas concentrações de IL-1ra e baixas concentrações de IL-1β no fluído sinovial mostraram uma resolução mais rápida da artrite, com o equilíbrio entre as concentrações de IL-1ra e IL-1β sendo determinantes para a progressão do processo inflamatório. De fato, altos níveis de IL-1ra no fluído sinovial da ATM são

associados com pouca ou nenhuma dor relacionada ao movimento mandibular (ASLTERGREN et al., 2008).

Estudos em humanos demonstraram altos níveis de IL-1β no músculo trapézio de indivíduos com pontos de nódulos miofasciais (SHAH et al., 2008). Além do mais, a administração intramuscular de estímulos inflamatórios como a Cg, induz hiperalgesia, com aumento dos níveis de IL-1β no músculo (LORAM et al., 2007). Em modelos experimentais que avaliaram a dor muscular pós-exercício físico, foi evidenciado aumento da produção de citocinas inflamatórias em resposta ao exercício extenuante, inclusive a IL-1β no músculo esquelético, sendo que o miócito mecanicamente lesado durante o exercício foi considerado uma possível fonte desta citocina (MACINTYRE et al., 1995; PEDERSEN et al., 1998; PRZYBYLA et al., 2006). Ademais, altos níveis de imunoreatividade para a IL-1β no tecido muscular foram observados em até 5 dias após sessão de exercício excêntrico e elevados níveis de repouso da atividade biológica da IL-1 *in vitro* foram mostradas em indivíduos treinados (EVANS et al., 1986; CANNON et al, 1989). Reforçando este conceito, CHEN et al., 2003, mostraram que a resposta molecular ao exercício excêntrico envolve a expressão do receptor da IL-1 no músculo humano.

Modelos animais indicam que o TNF-α induz hiperalgesia mecânica com início rápido (dentro de 30 min) quando administrado por via subcutânea. A hiperalgesia aparenta ser resultado da sensibilização de fibras C cutâneas, que podem persistir por até 30 dias ou mais, e é associada com sinais de inflamação e aumento dos níveis de mediadores inflamatórios como prostaglandinas (SOMMER & KRESS, 2004; CUNHA et al., 2005). Ainda, a administração local de TNF-α induz atividade espontânea nas fibras nervosas aferentes C e Aδ que resulta em diminuição do limiar nociceptivo, contribuindo para sensibilização central (SORKIN et al., 1997; JUNGER & SORKIN, 2000; ÖZAKATAY et al., 2006). No entanto, resultados apresentados por HOHEISEL et al., 2005, evidenciaram que a injeção de TNF-α no tecido muscular de ratos não excitou as fibras C aferentes. Adicionalmente, níveis intramusculares de TNF-α não aumentaram em modelo de inflamação muscular em ratos (LORAM et al., 2007).

O TNF-α aparenta ser um dos mediadores envolvidos em diversos modelos animais de artrite (CUNHA et al., 2005). Assim, a administração subcutânea do inibidor de TNF-α etanercept diminui a hiperalgesia mecânica quando administrado previamente a indução da artrite (pela injeção de adjuvante completo

de Freund – CFA) na articulação do joelho de ratos (INGLIS et al., 2005). Em contraste com os seus efeitos nos tecidos cutâneos e articulares, quando o TNF-α é injetado no músculo gastrocnêmio de ratos, induz sensibilização mecânica prolongada, que foi associada com aumento dos níveis teciduais de PGE_2, CGRP e fator de crescimento do nervo (NGF), embora não tenha havido inflamação muscular ou recrutamento de células inflamatórias de maneira significativa (SCHÄFERS et al., 2003). O TNF-α também foi detectado na sinóvia e fluídos sinoviais de paciente com AR (CHU et al., 1991) e em pacientes com outras doenças inflamatórias como artrite psoriática, espondilite pélvica, osteoartrite e artrite reativa (PARTSCH et al., 1997). No fluído sinovial de pacientes com lesões da ATM, foi encontrado aumento nos níveis de TNF-α, associado a dor durante movimento mandibular (SANDLER et al., 1998; NORDAHL et al., 2000).

Estudos que avaliam a relação do TNF-α com a nocicepção têm mostrado que a hiperalgesia experimental pode ser causada por administração sistêmica desta citocina (WATKINS et al., 1995). O TNF-α pode ter também efeito indireto na sensibilização do nociceptor, pelo aumento da produção e liberação de outros mediadores álgicos, como a IL-1β, serotonina e prostaglandinas, que levam a dor e hiperalgesia (WATKINS et al., 1995). Pode ainda induzir atividade ectópica em nociceptores aferentes primários em ratos quando aplicados diretamente no nervo ciático (SORKIN et al., 1997).

O papel das citocinas na dor muscular ainda é muito divergente e inconclusivo. Em modelo de DMIT em homens saudáveis, a administração de etanercept (inibidor de TNF-α), não afetou a dor muscular, mas recuperou a força do músculo mais rapidamente (RICE et al., 2007), sugerindo que a dor muscular não é modulada pelo TNF-α. No entanto, citocinas pró-inflamatórias como o TNF-α tem sido associadas com a fisiopatologia das miopatias inflamatórias (LUNDBERG, 2000) e fibromialgia (WALLACE et al., 2001).

Os efeitos pró-inflamatórios do TNF-α e IL-1β podem aumentar a adesão de leucócitos ao endotélio durante o recrutamento de células imunes, estimular a função de leucócitos e causar a ativação de macrófagos. O aumento intenso do metabolismo que leva a degradação do músculo esquelético é uma função de ambos TNF-α e IL-1β (EVANS & CANNON, 1991). Ainda, elevados níveis de TNF-α e IL-1β foram detectados em microdiálises do músculo trapézio de pacientes com dor cervical idiopática associada a nódulos miofasciais, quando

comparados com indivíduos controle (SHAH et al., 2005). Da mesma forma, CARP et al., 2007, evidenciaram que pacientes com distúrbios musculoesqueléticos causados por sobrecarga muscular apresentavam elevados níveis de TNF-α, IL-1β e IL-6, com sintomas de dor mais pronunciados. Desta forma, é possível pensar que resposta sistêmica adicional pode ser provocada pela liberação de citocinas no sangue a partir de músculos lesados, assim como citocinas (p. ex. TNF-α, IL-1β e IL-6) circulantes podem estimular resposta global mais difundida de sensibilização tecidual (BARBE & BARR, 2006).

Funções tanto pró- quanto anti-inflamatórias são atribuídas a IL-6 (VERRI et al., 2006). A IL-6 é capaz de causar dor e degeneração da cartilagem e ossos (WATKINS et al., 1994), com sua produção sendo fortemente induzida por TNF-α e IL-1β. Por outro lado, a IL-6 induz aumento dos níveis circulantes de IL-1ra e dos receptores sTNFR1. Também é a citocina chave para o início da resposta de fase aguda e consequente produção de proteína-C-reativa no fígado. A IL-6 é significativamente elevada na artrite reumatóide (KOPP et al., 2005; NORTHOFF & BERG, 1991), e foi detectada mais frequentemente no fluído sinovial de pacientes com dor na ATM, do que em indivíduos saudáveis, com os níveis de IL-6 sendo correlacionados com a intensidade da dor (SHINODA & TAKAKU, 2000). Por fim, foi observada deficiência da produção das citocinas anti-inflamatórios IL-10 e fator de crescimento e transformação β (TGF-β) em pacientes com dor na ATM (FANG et al., 1999; TOMINAGA et al., 2004).

Fica claro que novas modalidades de modelos experimentais que incluam tratamentos farmacológicos (agonistas e antagonistas de citocinas) para dor muscular crônica e severa de natureza inflamatória são necessários, visando maiores esclarecimentos sobre os mecanismos de ação das citocinas no desenvolvimento e progressão da dor muscular.

2 OBJETIVO

Avaliar a participação das citocinas pró-inflamatórias TNF-α e IL-1β e anti-inflamatória IL-10, assim como a modulação das mesmas pela pentoxifilina e talidomida em modelo de dor muscular de início tardio, induzida por natação aguda em camundongos.

2.1 OBJETIVOS ESPECÍFICOS

A) Padronizar o modelo experimental de natação aguda em camundongos para induzir dor muscular de início tardio;

B) Avaliar através de experimento tempo-resposta em modelo de dor muscular de início tardio, o tempo ideal para indução de dor muscular significativa induzida por sessão de natação aguda;

C) Avaliar a intensidade de hiperalgesia mecânica muscular induzida pelo movimento em animais selvagens, TNFR1$^{-/-}$, IL-10$^{-/-}$ e tratados com IL-1ra, pentoxifilina ou talidomida submetidos a protocolo experimental de natação aguda ou falso-nado (sham);

D) Avaliar a produção das citocinas pró-inflamatórias TNF-α e IL-1β e anti-inflamatória IL-10, assim como sua modulação pela pentoxifilina ou talidomida nos músculos sóleo e gastrocnêmio e medula espinhal de animais selvagens submetidos a protocolo experimental de natação aguda ou falso-nado (sham);

E) Avaliar o recrutamento de leucócitos (músculos sóleo e gastrocnêmico) e estresse oxidativo (músculos sóleo e gastrocnêmico e medula espinhal) em animais selvagens, TNFR1$^{-/-}$, IL-10$^{-/-}$ e tratados com IL-1ra, pentoxifilina ou talidomida submetidos a protocolo experimental de natação aguda ou falso-nado (sham), através da determinação da atividade da mieloperoxidase (MPO) e n-acetilglicosaminidase (NAG) e dos níveis de glutationa reduzida (GSH), respectivamente;

F) Avaliação da produção de biomarcadores fisiológicos de estresse e de metabolismo do músculo esquelético de animais

selvagens, TNFR1$^{-/-}$, e tratados com IL-1ra submetidos a protocolo experimental de natação aguda ou falso-nado (sham), através dos níveis plasmáticos de cortisol e glicose, lactato e creatina quinase total.

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ANEXOS

ANEXO 1

ARTIGO SUBMETIDO 1

Este trabalho foi realizado no laboratório de fisiopatologia e farmacologia da dor, neuropatia, câncer e inflamação, resultando na formação do artigo científico: Role of TNF-α/TNFR1 in intense acute swimming-induced delayed onset muscle soreness in mice, de autoria de Sergio M. Borghi, Ana C. Zarpelon, Felipe A. Pinho-Ribeiro, Renato D. R. Cardoso, Rubia Casagrande, Marli C. Martins Pinge, Roberto I. Tatakihara, Thiago M. Cunha, Sergio H. Ferreira, Fernando Q. Cunha e Waldiceu A. Verri Jr.

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Role of TNF-α/TNFR1 in Intense Acute Swimming-induced Delayed Onset Muscle Soreness in Mice

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Abstract

The injection of cytokines such as TNF-α induces muscle pain. Herein, it was addressed the role of endogenous TNF-α in intense acute swimming-induced muscle mechanical hyperalgesia in mice. Mice were exposed to water during 30s (sham) or to a single session of 30-120 min of swimming. Intense acute swimming induced a dose-dependent (time of exercise-dependent) muscle mechanical hyperalgesia, which peaked after 24 h presenting characteristics of delayed onset muscle soreness (DOMS). The intense acute swimming (120 min)-induced muscle mechanical hyperalgesia was reduced in etanercept (soluble TNF receptor) treated and TNFR1 deficient ($^{-/-}$) mice. TNF-α levels increased 2 and 4 hours after intense acute swimming in soleus muscle (but not in gastrocnemius), and spinal cord, respectively. Exercise induced an increase of myeloperoxidase activity and decrease in reduced glutathione levels in an etanercept-sensitive and TNFR1-dependent manners in the soleus muscle, but not in the gastrocnemius muscle. Concluding, TNF-α/TNFR1 signaling mediates intense acute swimming-induced delayed onset muscle soreness (DOMS) by an initial role in the soleus muscle followed by spinal cord, inducing muscle inflammatory hyperalgesia and oxidative stress. The knowledge of these mechanisms might contribute to improve the training of athletes, individuals with physical impairment and intense training such as military settings.

Key Words: DOMS; swimming; hyperalgesia; muscle pain; TNF-α; oxidative stress.

1. Introduction

Long term aerobic physical training with mild to moderate intensity (20-30 min) is able to reduce inflammatory and neuropathic pain in animal models [1-4]. However, intense acute physical exercise of moderate intensity and long duration can induce allodynia and hyperalgesia post-exercise. Furthermore, eccentric physical exercise and forced swimming, respectively, are capable of inducing mechanical and thermal hyperalgesia, and chronic muscle pain [5-7].

Previous studies have linked chemical mediators such as cytokines with pro-inflammatory characteristics to the genesis of pain [8-10]. Additionally, the acute physical exercise has also been linked to signaling pathways related to cytokines in post-exercise [5,11]. TNF-α has an early pivotal role in the development of hyperalgesia, activating two hyperalgesic pathways IL-1β-induced prostaglandin production and IL-8-induced sympathetic amines release in the carrageenan model of inflammatory hyperalgesia [8]. In line with a role of TNF-α in muscle pain, the intramuscular injection of TNF-α evokes a time- and dose-dependent muscle hyperalgesia within several hours after injection, indicating a possible critical role of TNF-α in the development of muscle hyperalgesia [10]. In agreement, TNF receptor I and II are expressed in skeletal muscle [12]. Moreover, horses submitted to high intensity exercise present elevated levels of TNF-α in blood and skeletal muscle samples [13].

The time course of cytokine production in skeletal muscle after intense exercise is related with the muscle damage, and it is probable that myocytes are mechanically damaged during the strenuous exercise, and this lesion stimulates the local production of inflammatory cytokines including TNF-α [14].

TNF-α also coordinates the recruitment of neutrophils to inflammatory foci as well as activates nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) resulting in superoxide anion production and consequent oxidative stress [15-18]. In fact, intense and prolonged physical exercise can result in oxidative damage of proteins and lipids present in contractile myocytes. High levels of reactive oxygen species can promote contractile dysfunction resulting in muscle weakness and fatigue [19]. Moreover, reactive oxygen species may modulate signaling pathways and regulate the expression of multiple genes, including cytokines. In this sense, cytokines and oxidative stress are interdependent pathways [20] involved in muscle inflammation and pain.

Taking into account the above mentioned and that TNF-α is the most clinically consolidated cytokine target, in the present study it was addressed the role of endogenous TNF-α in the development of intense acute swimming-induced muscle mechanical hyperalgesia in mice, which presents characteristics of delayed onset muscle soreness. TNFR1 deficient (-/-) mice and intraperitoneal (i.p.) treatment with etanercept (soluble TNF receptor) were used to evaluate the participation of TNF-α in the mechanisms of intense acute swimming-induced muscle pain. Additionally, TNF-α production was investigated in peripheral and spinal sites as well as its influence in muscle inflammation (myeloperoxidase activity) and oxidative stress (reduced glutathione levels).

2. Materials and Methods

2.1. Animals

The experiments were performed on male C57 BL/6 (wild type – WT) and TNFR1 deficient ($^{-/-}$) mice, 20-25g from University of São Paulo, Ribeirão Preto, SP, Brazil. Mice were housed in standard clear plastic cages with free access to water and food, light / dark cycle of 12 / 12h and controlled temperature. Mice were maintained in the vivarium of the Department of Pathology of Universidade Estadual de Londrina for at least two days before experiments. Mice were used only once and were acclimatized to the testing room at least 1 hour before the experiments, which was conducted during the light cycle. Animals' care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Institutional Ethics Committee for Animal Research of the Universidade Estadual de Londrina, process number 2066.2011. All efforts were made to minimize the number of animals used and their suffering.

2.2. Intense Acute Swimming

Mice were placed in a glass box (45×28×25 cm, divided in six compartments) with approximately 20 liters of water at 31° ± 1°C [21]. Each mouse was placed in one compartment and swam all the same time. A drop of liquid soap was added to reduce the surface tension of water diminishing the “floating” behavior [2]. After the intense acute swimming session or sham conditions, animals were dried and placed in cages together with their respective group. Mice were randomized in sham and exercised

groups. Sham animals were allowed to swim for just 30 seconds, and were immediately removed from the water after this period and dried. Mice in the swimming group were exposed to water for 1 session of 30, 60 or 120 min, or 1 session of 30 minutes per day during 5 days. The mechanical hyperalgesia was evaluated between 6-48 h after the swimming session. Intense acute swimming session of 120 min was recorded using a digital camera and time spent swimming was determined individually. The results were expressed as the mean ± S.E.M. of swimming time.

2.3. Evaluation of Mechanical Hyperalgesia

Mechanical hyperalgesia was tested in mice as previously reported [22,23]. Briefly, in a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15–30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic von Frey anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 or 4.15 mm^2 (referred to as regular and large probes, respectively) contact area polypropylene tips [22,23]. The investigator was trained to apply the probes perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the withdrawal response, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were evaluated at baseline and between 6-48h after exercise. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements (indicated time points) after stimulus from the baseline measurements. The basal

mechanical withdrawal threshold was 8.8 ± 0.1 g (mean ± S.E.M. of 29 groups, 6 mice per group) before intense acute swimming session. There was no difference of basal mechanical withdrawal thresholds between groups in the same experiment.

2.4. Cortisol and Glucose Plasmatic Concentrations

The animals were anesthetized with ethyl ether and blood samples of all groups were collected from ocular orbit immediately after (2 hours) and 24 hours after the intense acute swimming session, always in the afternoon (between 15:00h and 17:00h). Samples were centrifuged at 3.300 *g* in 4 °C for 5 min and the plasma resultant was assayed for cortisol and glucose levels. Cortisol was evaluated by Architect System Kit, which is a chemiluminescent assay of microparticles for quantitative determination of serum cortisol and glucose concentrations (Dimension® Clinical Chemistry System – SIEMENS) was quantified with spectrophotometer both according to the manufacture's guidelines. Naïve and sham groups were used as control.

2.5. Leukocyte Migration to the Skeletal Muscle Tissue

The intense acute swimming-induced leukocyte recruitment to the soleus and gastrocnemius muscles of mice was evaluated using the myeloperoxidase (MPO) kinetic–colorimetric assay [24]. Samples of skeletal muscles were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at −86 °C until use. Samples were homogenized using a Polytron (PT3100), centrifuged at 16.100 *g* in 4 °C for 2 min and the resulting supernatant

assayed spectrophotometrically for MPO activity determination at 450 nm (Spectra max), with 3 readings in 1 min. The MPO activity of samples was compared to a standard curve of neutrophils. Briefly, 10 µl of sample were mixed with 200 µl of 50 mM phosphate buffer pH 6.0, containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results were presented as MPO activity (number of total neutrophils x 10^{10}/ mg of muscle).

2.6. TNF-α Production

Mice were killed 2 and 4 h after swimming session and samples of the spinal cord (L4-L6), and gastrocnemius and soleus muscles were collected. The samples were homogenized in 300 µl (spinal cord pool of three mice) or 500 µl (skeletal muscles) of the appropriate buffer containing protease inhibitors. TNF-α level was determined as described previously [25] by enzyme-linked immunosorbent assay (ELISA). The results were expressed as picograms (pg) of TNF-α per 100 mg of tissue. Naïve and sham groups were used as control.

2.7. Reduced Glutathione (GSH) Assay

The levels of skeletal muscle GSH were determined using a spectrophotometric method [26]. Samples of gastrocnemius (100 mg) or soleus (40 mg) (1:10 dilution) were homogenized (IKA T10) in 4 and 1.6 ml of EDTA 0,02M, respectively. Homogenates (2.5 ml) were treated with 2 ml H_20 Milli Q plus 0.5 ml of trichloroacetic acid 50%. After 15 minutes, the homogenates were centrifuged at 1500 *g* for 15 min, and 1 ml from supernatant was added to 2 ml of a solution containing Tris 0.4M (pH

8.9) plus 50 ml of DTNB. After 5 min, the measurements were performed in 412 nm against white control (UV - Visible spectrophotometer [UV-1650 PC] – SHIMADZU). The GSH levels were corrected according to the total protein concentration. The results were presented as mmols of GSH per gram of protein in skeletal muscles.

2.8. Drugs

Drugs were obtained from the following sources: Carrageenan (100 µg diluted in 25 µl of NaCl 0.9 %/mice) from FMC (Philadelphia), 2% lidocaine chloride without vasoconstrictor (5µl) from Cristalia (São Paulo, Brazil), etanercept (1-10 mg/kg diluted in NaCl 0.9 %/mice) from Wyeth (São Paulo, Brazil) and saline solution 0,9% from Gaspar Viana S/A (Fortaleza, CE, Brazil).

2.9. Statistical Analysis

Results are presented as means ± S.E.M. of measurements made on 6 mice in each group. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The analyzed factors were treatments, time and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's t-test was performed for each time. On the other hand, when the hyperalgesic response were measured once after the administration or enforcement of the stimuli, the difference between responses were evaluated by one-way ANOVA followed by

Tukey's t-test. Additionally, comparative statistical analysis between two groups were done using t test. Statistical differences were considered to be significant at $P<0.05$.

3. Results

3.1. Evaluation of swimming intensity to induce mechanical hyperalgesia

In the first series of experiments, it was investigated the appropriate swimming time to induce mechanical hyperalgesia and its temporal profile. Mice were submitted to swimming sections of 30 min once a day during 5 days (Fig 1A). There was no evidence of mechanical hyperalgesia in these mice compared to sham group. The mechanical hyperalgesia was measured every day, but only the data of the 5th day is presented for clarity (Fig 1A). Using a different protocol, mice were submitted to a single session of 30, 60 or 120 min of swimming (Fig 1B). Again, 30 min did not induce mechanical hyperalgesia. On the other hand, the 60 and 120 min of swimming induced significant mechanical hyperalgesia at 6, 12 and 24 h after exercise. The mechanical hyperalgesia induced by 120 min of exercise remained significant up to 48 h with significant differences compared to 30 and 60 min at 24 and 48 h after exercise (Fig 1B). The peak of hyperalgesia was observed at 24 h after swimming session. Therefore, 120 min swimming session and evaluations at 24 h were chosen for next experiments.

In the next set of experiments, lidocaine 2% was injected intraplantarly (i.pl., 5 µl) [23] or intramuscularly (i.m., 20 µl, in the calf). The i.pl. injection of lidocaine increased the mechanical threshold of sham group resulting in a negative delta of reaction (Fig 1C). On the other hand, the control swim group presented significant mechanical hyperalgesia, which was unaffected by i.pl. treatment with saline or lidocaine or i.m. treatment with saline (Fig 1C). Intense acute swimming-induced mechanical hyperalgesia was reduced by i.m. (in the calf) treatment with

lidocaine. To confirm that the lack of effect of i.pl. lidocaine in intense acute swimming-induced muscle mechanical hyperalgesia is shared by other types of muscle pain, mice were treated with saline or lidocaine 2% (i.pl., 5 µl) 4.5 h after carrageenan (100 µg) [27] or saline (20 µl) injection in the calf (i.m.) (Fig 1D). I.pl. lidocaine increased the mechanical threshold of mice that received i.m. saline, but did not affect the mechanical threshold of carrageenan group (Fig 1D) while i.m. lidocaine reduced carrageenan-induced muscle mechanical hyperalgesia (Fig 1D). Thus, confirming that intense acute swimming- and i.m. carrageenan-induced muscle mechanical hyperalgesia depends on movement-elicited hyperalgesia rather than cutaneous paw hyperalgesia. **(Include Figure 1 here).**

3.2. Involvement of TNF-α/TNFR1 in the muscle mechanical hyperalgesia induced by intense acute swimming

In this set of the experiments, two area size probes were used 0.5 mm^2 (regular probe, Fig 2, A and C) and 4.15 mm^2 (large probe, Fig 2, B and D). Intense acute swimming (120 min) induced significant muscle mechanical hyperalgesia in WT (wild type - C57BL/6) mice compared to sham group as determined using both probes (Fig 2, A-D). The treatment with etanercept (soluble TNF receptor) inhibited in a dose-dependent manner (1-10 mg/kg, i.p., 48 and 1 h before the session, Fig 2, A and B) the intense acute swimming-induced muscle hyperalgesia. The 10 mg/kg dose was effective in inhibiting intense acute swimming-induced muscle mechanical hyperalgesia from 6^{th} to 36^{th} h with the regular probe (Fig 2A) and from 6^{th} to 48^{th} with the large probe (Fig 2B) with significant differences compared to the lower doses of etanercept between 6-24 h with the regular probe (Fig 2A) and 6-36 h with the large

probe (Fig 2B). Additionally, intense acute swimming-induced muscle mechanical hyperalgesia was almost abolished between 6th to 48th h in TNFR1 deficient ($^{-/-}$) mice compared to WT (C57BL/6) (Fig 2, C and D) as determined using both probes. There was no statistical difference between sham groups of WT and TNFR1$^{-/-}$ mice (data not shown). Thus, these results indicate that TNF-α acting on TNFR1 receptor is an important cytokine in intense acute swimming-induced muscle mechanical hyperalgesia. There was no difference in the total swimming time over 120 min of WT, TNFR1$^{-/-}$ and WT mice treated with etanercept (10 mg/kg) (103,81 ± 3,46; 105.15 ± 7,73; and 102.41 ± 3.29 min, respectively; *n=6*, P>0.05). **(Include Figure 2 here).**

3.3. Intense acute swimming induces the production of TNF-α in the spinal cord and soleus, but not in the gastrocnemius muscle

The levels of TNF-α produced upon intense acute swimming were measured in the soleus (Fig 3A) and gastrocnemius (Fig 3B) skeletal muscles and spinal cord (L4-L6, Fig 3C) 2 and 4 h after exercise session. There was significant increase of TNF-α levels in the soleus muscle 2 h after intense acute swimming, but basal levels were detected 4 h after exercise session (Fig 3A). On the other hand, there was no difference between naïve, sham and intense acute swimming groups regarding TNF-α production in the gastrocnemius muscle (Fig 3B). There was an increase of TNF-α (Fig 3C) levels in the spinal cord 4 h, but not at 2 h after exercise session. Thus, TNF-α is produced peripherally at an early phase in the soleus muscle (Fig 3A) and in a second moment in the spinal cord (Fig 3C). **(Include Figure 3 here).**

3.4. Intense acute swimming induces TNF-α/TNFR1-dependent increase of myeloperoxidase (MPO) activity in the soleus, but not in the gastrocnemius muscle

Intense acute swimming induced significant increase of MPO activity in the soleus muscle at 24 h (peak of hyperalgesia) after the session compared to naïve and sham groups (Fig 4A). On the other hand, the MPO activity achieved similar levels in naïve, sham and intense acute swimming groups in the gastrocnemius muscle (Fig 4B). Furthermore, the intense acute swimming-induced increase of MPO activity was reduced by etanercept treatment (10 mg/kg, i.p., 48 and 1 h before the session) and in TNFR1$^{-/-}$ mice (Fig 4C) in the soleus muscle, without differences among groups in the gastrocnemius muscle (Fig 4, D). There was no statistical difference between naïve and sham groups of WT and TNFR1$^{-/-}$ mice (data not shown). **(Include Figure 4 here).**

3.5. Intense acute swimming induces TNF-α/TNFR1-dependent depletion of endogenous reduced glutathione (GSH)

Intense acute swimming induced the reduction of GSH levels at 2 and 4 h after exercise session, which was detected in the soleus (Fig 5A), but not in the gastrocnemius muscle (Fig 5B). Furthermore, the GSH depletion induced by intense acute swimming was prevented in the soleus muscle by etanercept treatment (dose as in Fig 4) and in TNFR1$^{-/-}$ mice (Fig 5C) while the GSH levels were similar in all groups in the gastrocnemius muscle (Fig 5D). There was no statistical difference between naïve and sham groups of WT and TNFR1$^{-/-}$ mice (data not shown). Thus,

indicating that TNF-α acting on TNFR1 receptor contributes to the oxidative stress in the soleus muscle induced by intense acute swimming. **(Include Figure 5 here).**

3.6. Evaluation of Serum Cortisol and Glucose Levels

To disprove that the present model is a stress-induced hyperalgesia model, plasma cortisol and glucose levels were assessed. Two time points were chosen, 2 h representing the immediately time the swimming session finished and 24 h representing the peak of hyperalgesia. One session of intense acute swimming did not increase cortisol (Fig 6A) and glucose (Fig 6B) levels in WT mice at 2 or 24 h after the swimming session, presenting concentrations similar to the baseline levels (naïve and sham groups). $TNFR1^{-/-}$ mice did not present changes in the concentrations profile of plasmatic cortisol (Fig 6A) and glucose (Fig 6B) from WT mice, evidencing that TNFR1 deficiency does not affect the release of cortisol or glucose in the present experimental condition. **(Include Figure 6 here).**

4. Discussion

Moderate physical exercise of short duration carried out daily with professional guidance is beneficial and helps to control pain states in patients with chronic diseases such as lupus, fibromyalgia, rheumatoid arthritis, and type 2 diabetes [28-31]. On the other hand, the present study demonstrates that intense acute swimming induces prolonged muscle mechanical hyperalgesia, muscle inflammation and oxidative stress dependent on endogenous TNF-α activation of TNFR1 receptors.

It has been shown that intramuscular injection of TNF-α induces a reduction in the grip force test, increases the local inflammation and mechanical hyperalgesia to muscle pressure while there was no alteration in the response to von Frey hairs [9]. Schäfers et al. [9] injected TNF-α in the gastrocnemius muscle, and in the present study it was detected that intense acute swimming induces an increase of TNF-α levels only in the soleus muscle. Here, we have detected muscle mechanical hyperalgesia using an electronic version of von Frey method, which presents differences with the analog method [9] since with the electronic version there is a basal response indicating that the experiments are measuring hyperalgesia, and in the analog version the majority of research groups do not detect a basal response indicating that it is being evaluated allodynia [22,32]. Therefore, these studies are evaluating different experimental conditions. Furthermore, an important question that was raised in the development of this study was if this electronic version of von Frey test could in addition to the known detection of cutaneous hyperalgesia, also detect movement-elicited hyperalgesia. To further elucidate this issue, lidocaine was injected i.pl. in mice submitted to sham conditions, intense acute swimming or that received i.m. injection of carrageenan. The i.pl. treatment with lidocaine increased

the mechanical cutaneous threshold of negative control groups (saline i.m. and sham swim), which is consistent with the primary use of this test as previously described [22]. On the other hand, animals subjected to exercise or carrageenan-induced muscle inflammation showed a reduction of mechanical threshold that was not affected by i.pl. lidocaine. It is reasonable that mice with muscle hyperalgesia and non-sensitized cutaneous paw tissue presents lower mechanical threshold upon movement compared to cutaneous threshold, thus, explaining why i.pl. lidocaine treatment did not affect muscle hyperalgesia. Lidocaine was also injected i.m. (in the calf), and this treatment was successful to inhibit intense acute swimming- and carrageenan-induced mechanical hyperalgesia, indicating that movement-elicited muscle hyperalgesia and muscle inflammatory hyperalgesia were under investigation, respectively. Further addressing the differentiation of cutaneous and muscle hyperalgesia, the modulation of hyperalgesia was evaluated in WT mice treated with vehicle, etanercept or in TNFR1$^{-/-}$ mice using regular probe (0.5 mm^2 contact area) that elicits nociceptive responses per se and large probe (4.15 mm^2 contact area) that does not elicit nociceptive responses per se [23]. It was observed that similar results were obtained using both probes. Taking into account the experiments with lidocaine and two contact area probes, it is conceivable that the pressure exerted by the probe on the plantar surface induces the dorsal flexion of the ankle joint, promoting the stretch of the Achilles tendon, generating muscle distention (movement-elicited hyperalgesia), which is sufficient to trigger muscle nociceptive responses. It is possible that the paw withdrawal reflex of mice during the nociceptive test may be a stretch reflex response resulting from activation of muscle receptors related to muscle length (muscle spindles) and / or flexion reflex (removal of the member upon a painful stimulus) in an attempt to avoid further damage to the already

inflamed muscle that worked excessively during the intense acute swimming session [33-35]. Therefore, the decreased mechanical threshold observed after swimming sessions was related to muscle and not cutaneous hyperalgesia. Moreover, these data demonstrate a novel applicability for the electronic von Frey test.

Targeting TNF-α in WT mice by treatment with etanercept dose-dependently inhibited the intense acute swimming-induced muscle mechanical hyperalgesia. In agreement with this pharmacological approach, TNFR1$^{-/-}$ mice also presented reduced hyperalgesia compared to WT mice. Therefore, TNF-α acting on TNFR1 is an important cytokine in intense acute swimming-induced muscle mechanical hyperalgesia. There was no difference in the effectively swimming time of WT, WT treated with etanercept, and TNFR1$^{-/-}$ mice indicating that the difference in the muscle hyperalgesia, and immune and biochemical parameters can not be attributed to difference in the swimming time. The importance of TNF-α is emphasized by the therapeutic approaches targeting this cytokine in diseases. Pre-clinical studies have been demonstrating the role of TNF-α in inflammatory hyperalgesia [8] and that the TNFR1 receptor is of importance in carrageenan-, antigen- and zymosan-induced mechanical hyperalgesia [10,36-38]. Targeting TNF-α or TNFR1, but not TNFR2 also reduces experimental neuropathic pain [39,40], which corroborates evidence that nerve injury in the chronic constriction injury model induces the increased production of TNF-α [41,42]. The production of TNF-α seems to be an early event in both inflammation and nerve injury models [8,11,42]. In the intense acute swimming-induced muscle inflammation and hyperalgesia, it is likely that the production of TNF-α is an early event in the soleus muscle. Moreover, in carrageenan-induced paw inflammation, TNF-α is also an early cytokine [8,10]. In agreement with the present results, TNF-α mRNA expression increased in response to formalin-induced muscle

inflammation [43]. *In vivo* evidence showed elevated levels of TNF-α in the trapezius muscle of subjects with myofascial trigger points, linking this cytokine with muscle painful and inflammatory conditions [44]. It is noteworthy to mention that in a model of 4 sets of 15 repetitions at 80% of individual one-repetition maximum using leg press, two treatments with etanercept (25 mg/kg) with at least 6 weeks of interval apart did not alter the muscle pain (e.g. visual analog scale and pressure pain threshold) [45]. Additionally, non-painful exercise induces an increase in TNF-α mRNA expression [46]. However, these apparent contradictions with our study might have some explanations. The etanercept package insert recommends a dose of 25 mg twice a week and advises that the effectiveness may be compromised by reduction of dose or increase of treatment interval in humans [47], and in fact, increase of effectiveness was observed with higher doses of etanercept such as 50 mg twice a week [48,49]. In this line, in the present study a dose-response curve was performed using 48 and 1h of pretreatment with etanercept as well as TNFR1$^{-/-}$ mice, which guaranteed proper conditions to evaluate the role of TNF-α in intense acute swimming-induced muscle mechanical hyperalgesia. Murase et al. [46] detected an increase of TNF-α mRNA expression in non-painful exercise while did not perform functional studies targeting TNF-α to reduce muscle hyperalgesia in painful exercise. Herein, TNF-α protein levels were determined and TNF-α and TNFR1 were target using pharmacological and genetic tools, respectively. Importantly, pain evaluation method, doses of treatments, exercise and muscle characteristics might have influenced these different results.

Intense acute swimming induced an increase of TNF-α production in the spinal cord at 4 h, which occurs after the increase of TNF-α production in the soleus muscle, at 2 h. Furthermore, the time profile of TNF-α production at 2h in the soleus

muscle and at 4 h in the spinal cord suggests that the peripheral TNF-α may be responsible for the increase of TNF-α production in the spinal cord. In this sense, it has been described that the depolarization of primary afferent nociceptors induces the release of the chemokine CX3XL1 by neurons in the spinal cord, which in turn, activates CX3CR1 receptors in microglia resulting in MAP (mitogen-activated protein) kinase p38 phosphorylation that induces TNF-α production. TNF-α activates second order neurons in the spinal cord to induce hyperalgesia [50]. Another possibility is the retrograde axonal transport of TNF-α and TNFR1 from the peripheral site to the dorsal root ganglia and spinal cord [51]. An anterograde transport has also been described from the dorsal root ganglia to the nerve injury site and innervated muscle, but not skin [52]. These results corroborate a peripheral and central (spinal cord) integrated nociceptive mechanism in the pathophysiology of intense acute swimming-induced muscle mechanical hyperalgesia.

It is noteworthy to mention that TNF-α does not excite group IV neurons, which are sensitive to mechanical stimuli [53]. On the other hand, i.m. injection of TNF-α induces muscle hyperalgesia together with the production of calcitonin-gene related peptide, nerve growth factor and prostaglandin E_2 (PGE_2) [9]. These data may be seen as contradictory, but rather indicate that TNF-α is a hyperalgesic cytokine in muscle pain by sensitizing the nociceptor that depolarizes upon mechanical stimulus, but does not inducing itself nociceptor depolarization in the muscle. In agreement, in peripheral inflammation TNF-α induces mechanical hyperalgesia dependent on the production of other molecules such as PGE_2 that is ultimately responsible for nociceptor sensitization [8]. Moreover, TNF-α detection in the spinal cord indicates that it might have a role as a nociceptor sensitizing cytokine in this foci and the hyperalgesic role of TNF-α is not restricted to the muscle tissue. In agreement,

peripheral stimuli induce hyperalgesia dependent on activation of spinal cord nociceptive mechanisms [54-56].

TNF-α is known for its chemoattractant effect [16-18,57] and to activate NADPH oxidase in recruited neutrophils resulting in the production of superoxide anion [19,58]. Therefore, we reason that TNF-α could be responsible for inflammatory cell recruitment and oxidative stress induced by intense acute swimming in the soleus muscle. In fact, the treatment with etanercept or deficiency of TNFR1 reduced the increase of MPO activity and GSH depletion induced by intense acute swimming. MPO activity has been used as a marker of neutrophil recruitment, and was previously used to demonstrate that neutrophils contribute to inflammatory pain by further producing nociceptive mediators [59,60]. Oxidative stress involves many oxygen and nitrogen reactive species with an intracellular signaling role that have also been implicated in the genesis of inflammatory and neuropathic pain since its inhibition by varied strategies reduced nociceptive responses [20,26,61,62].

The relative amount of antioxidant enzymes present in skeletal muscle fibers differ according to the types of fibers. Slow type I fibers are more actively recruited during submaximal endurance exercise compared to the fast type IIX or IIb fibers [63] and increases in muscle glutathione peroxidase activity are limited to highly oxidative muscles, which preferably have type I and IIa fibers [64]. Soleus muscle contains predominantly slow type I (37%) and IIa (38%) myofibers. In turn, gastrocnemius has predominantly fast type IIb (54%) or IIX (19%) myofibers, with a small percentage of type I (5%) myofibers [65]. Thus, soleus muscle is considered a highly oxidative muscle compared to gastrocnemius muscle [19,64,65]. Therefore, these myofiber characteristics of soleus (type I and IIa) and gastrocnemius (types IIb and IIX)

corroborates the present data in which the oxidative stress (reduction of GSH levels) occurred in the soleus, but not in the gastrocnemius muscle.

The long duration and high intensity of swimming recruited especially slow oxidative fibers which are resistant to fatigue. It is probable that during the exercise session, the gastrocnemius muscle was used in the initial phase of exercise and rapidly entered into fatigue due to the predominance of fast fibers activity in the initial phase, producing smaller amounts of chemical mediators and hence causing less oxidative stress. The soleus muscle, in turn, due to the fatigue-resistant fibers was responsible for producing chemical mediators such as TNF-α resulting in the recruitment of leukocytes and generation of oxidative stress, responding more intensely to the inflammatory stimulus caused by intense acute swimming. Nevertheless, it is noteworthy to mention that the participation of gastrocnemius muscle was not completely ruled out since it might depend on the nature and intensity of exercise and time point evaluated.

Importantly, the present study used a model of exercise avoiding stress and hypoalgesia, and focusing in exercise induced hyperalgesia. To reach this fine line some studies were taken into account. Stress is a confounding factor in models of swimming capable of generating stress-induced hyperalgesia, which is inversely related to water temperature [2,3,6,7,66]. Temperatures between 24-26^{o}C are used in models to evaluate the mechanisms of stress-induced hyperalgesia and higher temperatures are not effective to induce stress-induced hyperalgesia [6,7,66]. In fact, protocols using water at 37^{o}C are known to induce hypoalgesia in inflammatory and neuropathic pain models [2,3]. Therefore, an intermediary temperature between those necessary to induce stress (25°C) and hypoalgesia (37°C) was selected for the present study (31°C) to focus in exercise-induced hyperalgesia. Additional concepts

corroborate that the present model is not evaluating stress-induced hyperalgesia or hypoalgesia. Although mice could present some stress in the initial phase of the swimming exercise, the habituation of the animal to the condition dissipates the stress [2,3]. In this sense, a decrease in feces in the water can be observed after the initial phase (20 min) of our intense acute swimming session, which is considered a valid parameter to indicate habituation [2,3]. Other animal model of swimming, like forced swim test, is used as an efficient toll to evaluate abandonment or for screening antidepressants [67]. These models, besides being used to evaluate depressive behaviors, often use 5-15 min of swimming session as standard time, with cold water temperatures (20-24°C) [68,69], a different protocol from our model that present long duration (120 min) and intermediate water temperature to evaluate movement-induced muscle hyperalgesia. Further reinforcing this concept, stress-induced hyperalgesia has a limited duration of few hours, but not prolonged and increasing hyperalgesia within 1-2 days [70]. Finally, the maintenance of basal serum cortisol and glucose levels evidenced at the end of the swimming section and at the peak of hyperalgesia in animals that received one session of intense acute swimming disproves that stress is responsible for the hyperalgesia observed in our study.

In fact, we speculate that the present model shows characteristics closely related to delayed onset muscle soreness (DOMS). DOMS is an uncomfortable feeling or pain after strenuous exercise session, especially induced by eccentric contractions, in which the body is unaccustomed, mainly in untrained individuals, reaching peaks close to 24-48h after the exercise session, accompanied by peripheral inflammatory character, with enhanced cytokine levels [71]. The uncomfortable pain sensation of DOMS is triggered by movement in a similar fashion to the present model in which the electronic von Frey was used to induce the

movement of mice hind leg, and since the sensitized muscle presented a lower mechanical threshold than the intact cutaneous paw skin, a nociceptive behavior could be detected. The present model adds to the literature by allowing the evaluation of movement-elicited muscle hyperalgesia, complementing the data using Randall & Selitto test or pressure tests directly applied to the muscle.

4.1. Conclusions

The present study provides evidences on the role of endogenous TNF-α/TNFR1 in intense acute swimming-induced muscle mechanical hyperalgesia in mice. There is a muscle specific role in that the production of this cytokine occurs in the soleus, but not gastrocnemius muscle. The soleus muscle is also the foci of inflammatory neutrophil recruitment and oxidative stress upon intense exercise. The mechanical hyperalgesia induced by intense acute swimming depends on peripheral (soleus muscle) and central (spinal cord) production of TNF-α. This advance in the understanding of exercise-induced muscle pain might contribute to the improvement of the recovery and training of professional athletes or even individuals with physical limitations related to age, weight, or skeletal muscle injuries, because the pain limits training time and increases recovery time. Furthermore, TNF-α/TNFR1 could be considered a potential target to the prevention of DOMS symptoms. The proposed participation of TNF-α in intense acute swimming-induced muscle mechanical hyperalgesia was summarized in Fig 7. **(Include Figure 7 here).**

Disclosures

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The authors declare no conflicts of interest.

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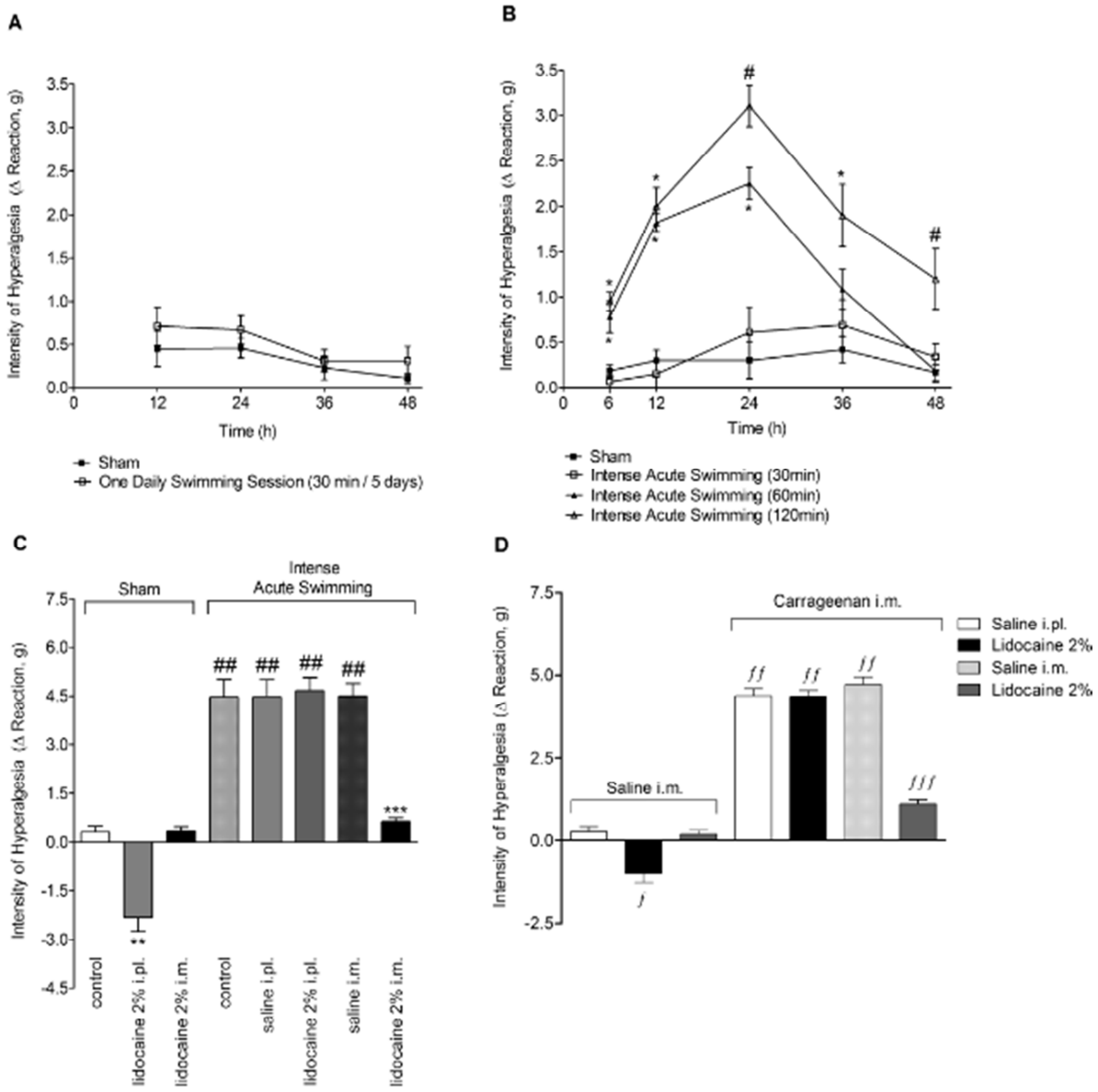

Fig. 1 – Time of swimming-dependent mechanical hyperalgesia. WT mice were submitted to one daily swimming session of 30 min over 5 days (Panel A, only the fifth day results were presented) or to one session of intense acute swimming of 30, 60 or 120 min (Panel B). The mechanical hyperalgesia was evaluated 6-48 hours after each session. Lidocaine or vehicle (saline) was injected i.pl. (5 µl) or i.m. (20 µl) 30 min before the measurements of intense acute swimming groups (24^{th} h after the session) (Panel C), or intramuscular injection of saline (20 µl) or carrageenan (100 µg) groups (5^{th} h after the stimuli) (panel D). Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. Panel B: *P<0.05 compared with the sham and intense acute swimming 30 min; #P<0.05 compared to sham and intense acute swimming 30 and 60 min; Panel C: **P<0.05 compared with control and lidocaine i.m. sham groups; ##P<0.05 compared

with sham groups; ***P<0.05 compared with control, saline/lidocaine i.pl. and saline i.m.; Panel D: ^{f}P<0.05 compared with saline i.m./i.pl. and saline/lidocaine i.m.; ^{ff}P<0.05 compared with saline i.m. groups; ^{fff}P<0.05 compared with carrageenan i.m. groups (One-way ANOVA followed by Tukey's t test).

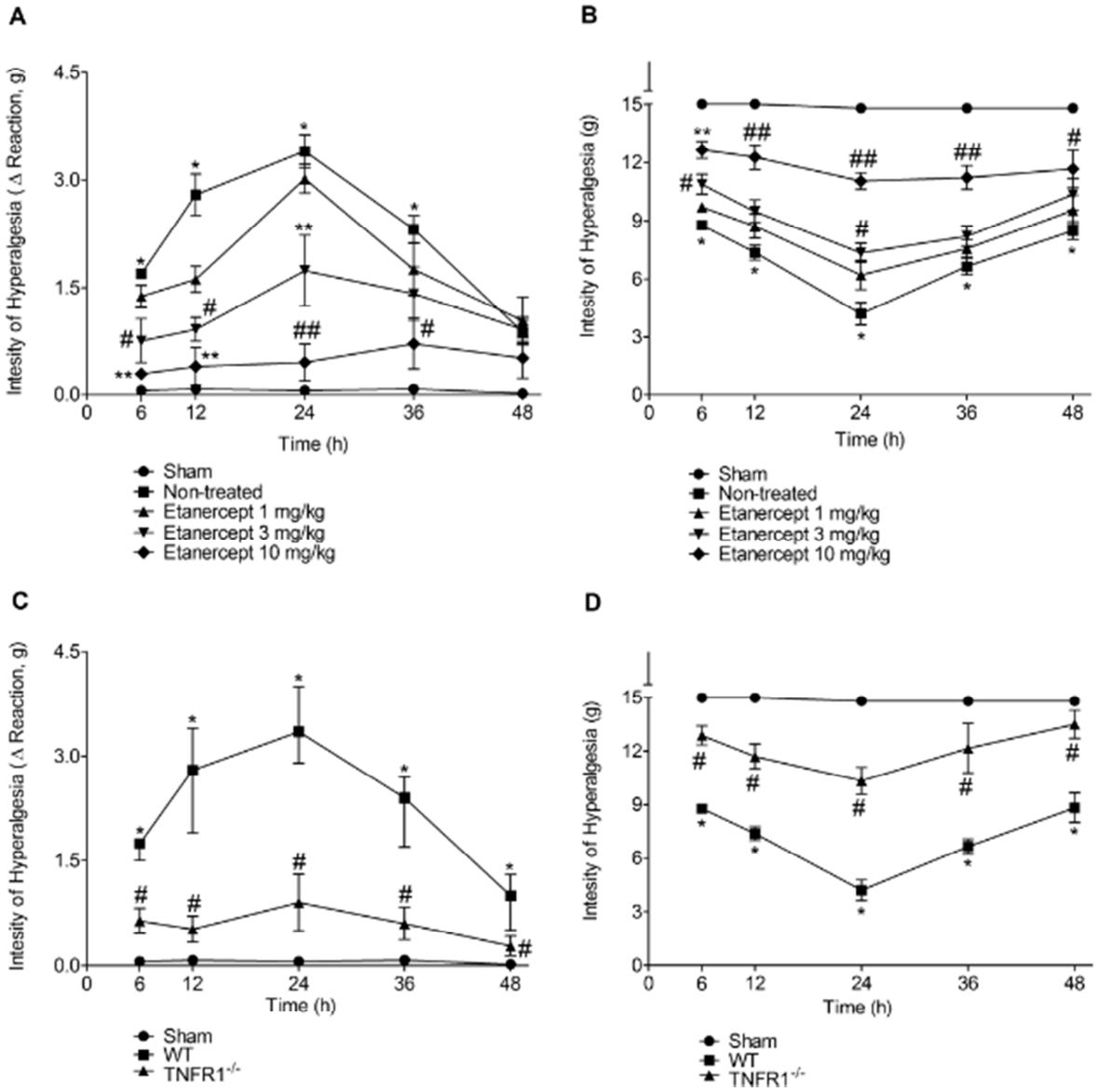

Fig. 2 - Reduction of intense acute swimming-induced muscle mechanical hyperalgesia in etanercept treated and TNFR1$^{-/-}$ mice. Mice were exposed to sham conditions (30 sec exposure to water) or intense acute swimming for 120 min. The intensity of mechanical hyperalgesia was evaluated 6-48 hours after the session in etanercept treated (1-10 mg/kg, i.p., 48 and 1 h before the session, Panels A and B) and TNFR1$^{-/-}$ mice (Panels C and D). Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. *P<0.05 compared with sham; #P<0.05 compared with controls [non-treated (Panels A and B) or WT (Panels C and D)]; **P<0.05 compared with vehicle control (saline) and 1 mg/kg dose of etanercept; ##P<0.05 compared with vehicle control (saline), 1 and 3 mg/kg doses of etanercept (One-way ANOVA followed by Tukey's t test).

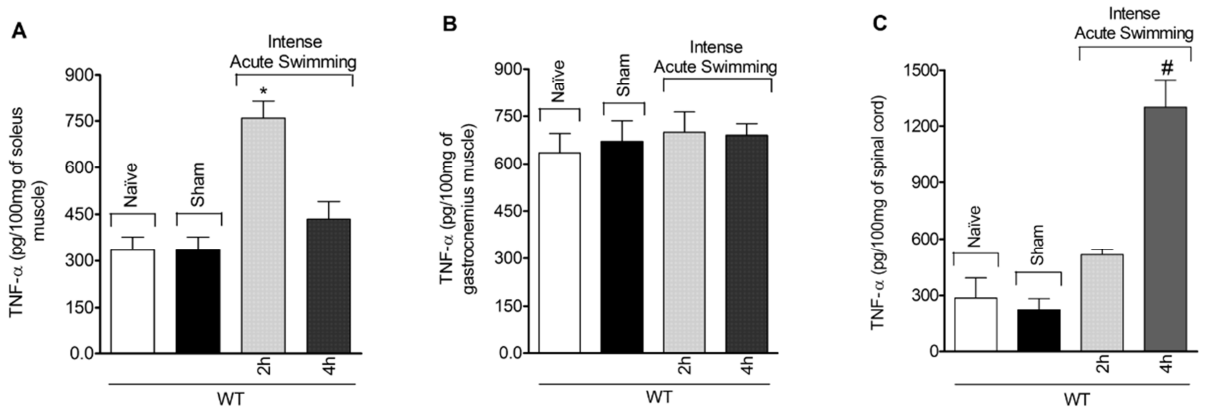

Fig. 3 – Intense acute swimming induces TNF-α production in the soleus muscle and spinal cord, but not in the gastrocnemius muscle. WT mice were exposed to sham conditions (30 sec exposure to water) or received intense acute swimming for 120 min, and the levels of TNF-α in soleus (Panel A) and gastrocnemius (Panel B) muscles, and spinal cord (L_4-L_6) (Panel C) were quantified, as determined by ELISA. The samples were collected immediately in the end (2 h) and 2 hours after the exercise session (4 h). Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. *P<0.05 compared with naive, sham and 4 h; #P<0.05 compared with naive, sham and 2h (One-way ANOVA followed by Tukey's t test).

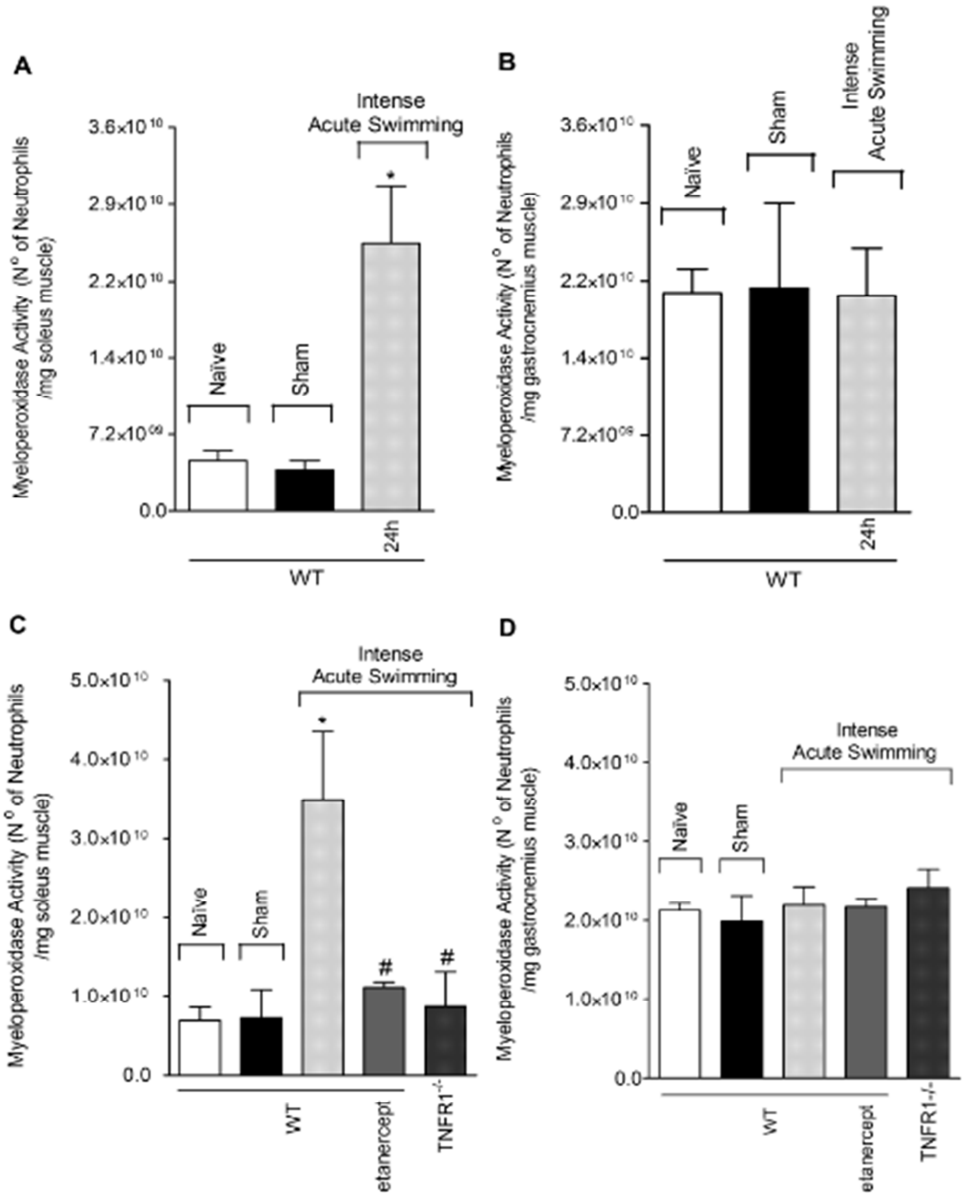

Fig. 4 – Intense acute swimming-induced increase in MPO activity was reduced by etanercept treatement and in TNFR1$^{-/-}$ mice in the soleus muscle and unaltered in the gastrocnemius muscle. Mice were exposed to sham conditions (30 sec exposure to water) or received acute swimming for 120 min. Samples were collected 24 h after the intense acute swimming and the myeloperoxidade (MPO) activity was determined in the soleus and gastrocnemius muscles of WT (Panels A and B), etanercept treated (10 mg/kg, i.p., 48 and 1 h before the session) and TNFR1$^{-/-}$ (Panels C and D) mice. Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. *P<0.05 compared with naive and sham; #P<0.05 compared with intense acute swimming positive control (One-way ANOVA followed by Tukey's t test).

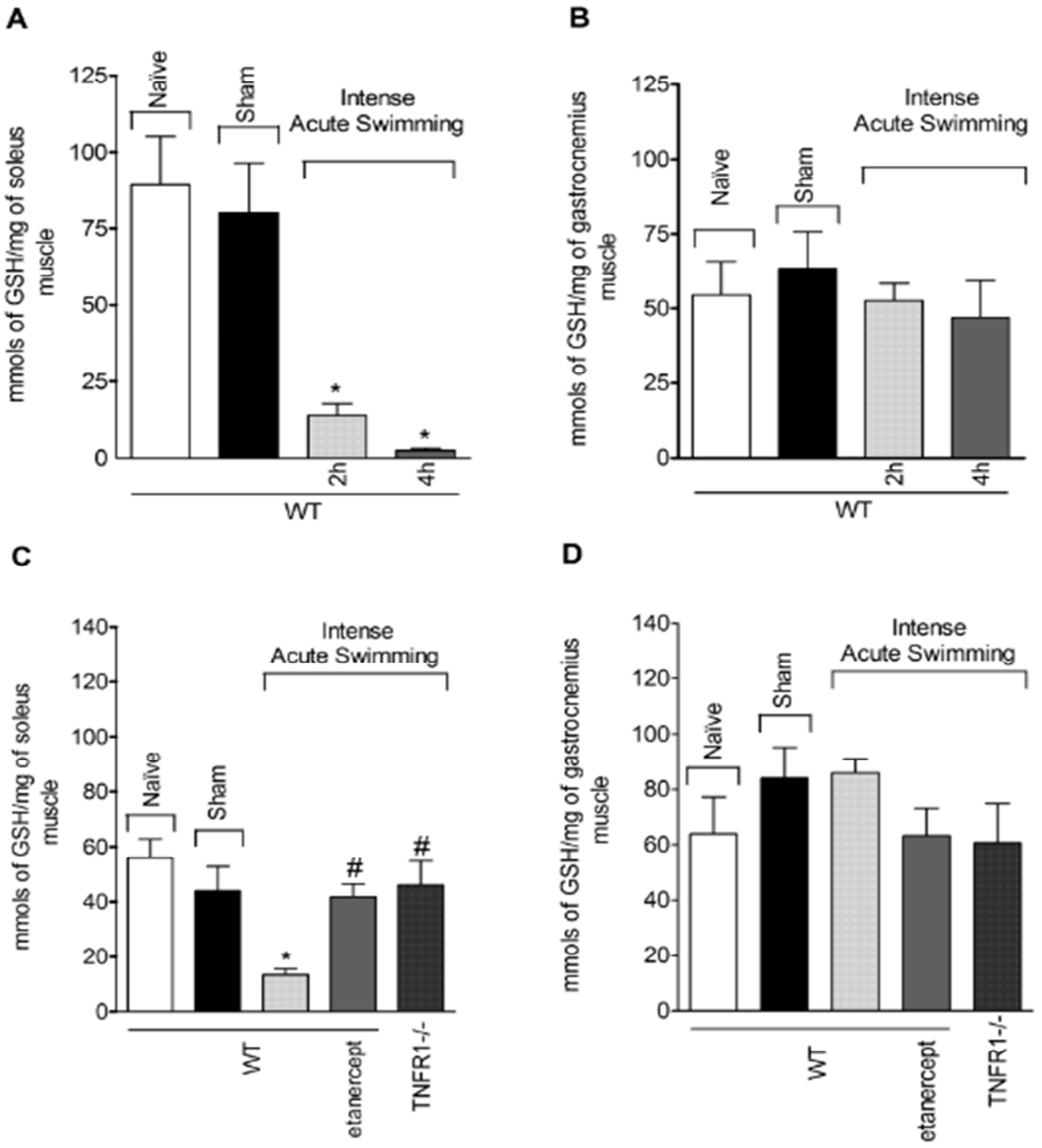

Fig. 5 – Intense acute swimming-induced depletion of endogenous GSH levels was reversed by etanercept treatement and in TNFR1$^{-/-}$ mice in the soleus muscle but, not in the gastrocnemius muscle. Mice were exposed to sham condition (30 sec expose to water) or received intense acute swimming for 120 min. The samples of soleus and gastrocnemius muscles were collected and endogenous reduced glutathione (GSH) levels was evaluated immediately after (2 h) and 2 hours after (4 h) intense acute swimming in WT (Panels A and B), etanercept treated (10 mg/kg, i.p., 48 and 1 h before the session) and TNFR1$^{-/-}$ (Panels C and D) mice. Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. *P<0.05 compared to naive and sham; #P<0.05 compared with intense acute swimming positive control (One-way ANOVA followed by Tukey's t test).

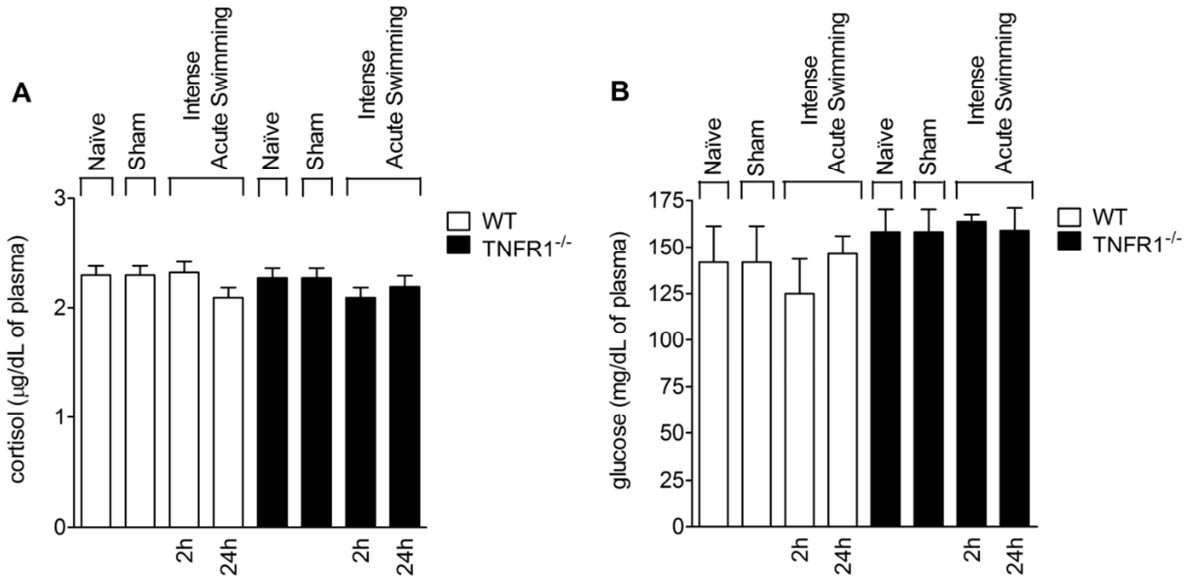

Fig. 6 - Intense acute swimming did not induce increases in plasmatic concentrations of cortisol and glucose in WT and TNFR1$^{-/-}$ mice. Mice were exposed to sham condition (30 sec expose to water) or received intense acute swimming for 120 min. Blood samples were collected immediately after (2 h) and 24 hours after intense acute swimming session for determination of plasma concentrations of cortisol (Panel A) and glucose (Panel B) in WT and TNFR1$^{-/-}$ mice. Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. P>0.05 (One-way ANOVA followed by Tukey's t test).

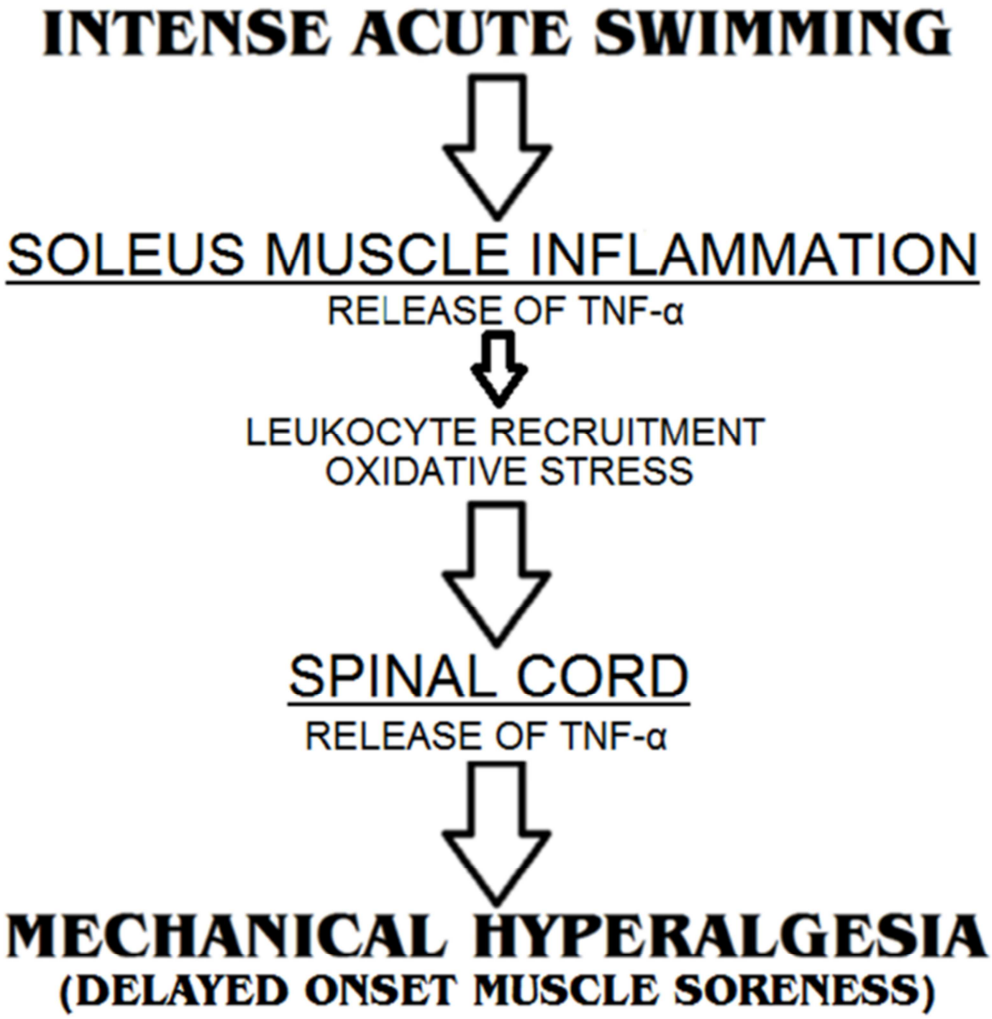

Fig. 7 - Schematic representation of the proposed mechanism for the role of TNF-α acting on TNFR1 receptor in intense acute swimming-induced mechanical hyperalgesia in mice, a model of delayed onset muscle soreness (DOMS). Intense acute swimming for 120 min induces the release of TNF-α in peripheral tissues (soleus, but not gastrocnemius muscle), resulting in a local inflammatory process, due to its chemoattractant and NADPH oxidase activating actions. This peripheral process leads to TNF-α production in the spinal cord. As a result of this peripheral and spinal production of TNF-α, there is an increase of movement-induced muscle mechanical hyperalgesia with characteristics of DOMS such as untrained subjects, high intensity single training session, peak of response between 24-48h and peripheral inflammation [71].

ANEXO 2

ARTIGO SUBMETIDO 2

Este trabalho foi realizado no laboratório de fisiopatologia e farmacologia da dor, neuropatia, câncer e inflamação, resultando na formação do artigo científico: Targeting interleukin-1β reduces intense acute swimming-induced delayed onset muscle soreness on mice, de autoria de Sergio M. Borghi, Ana C. Zarpelon, Felipe A. Pinho-Ribeiro, Renato D. R. Cardoso, Thiago M. Cunha, Jose C. Alves-Filho, Sergio H. Ferreira, Fernando Q. Cunha, Rubia Casagrande e Waldiceu A. Verri Jr.

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Targeting interleukin-1β reduces intense acute swimming-induced delayed onset muscle soreness in mice

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Running head: IL-1β mediates DOMS

Abstract

Objectives: The IL-1β release and signaling scenario during strenuous exercise needs to be more comprehensive. Herein, we investigated the role of IL-1β in intense acute swimming-induced delayed onset muscle soreness (DOMS) in mice.

Methods: Untrained mice were submitted to one session of intense acute swimming for 120 minutes or were submitted to sham conditions (30 sec exposure to water), and muscle mechanical hyperalgesia, IL-1β production (skeletal muscle and spinal cord), leukocyte recruitment (myeloperoxidase activity), reduced glutathione levels (GSH) (skeletal muscle and spinal cord) and cortisol, glucose, lactate and creatine kinase levels (CK) (plasma) were analyzed.

Key findings: Intense acute swimming-induced muscle mechanical hyperalgesia was dose-dependently inhibited by IL-1ra treatment. IL-1β levels were increased in soleus, but not gastrocnemius muscle and spinal cord 2 and 4 hours after the session, respectively. Intense acute swimming-induced increase of myeloperoxidase activity and reduced GSH levels in soleus muscle were reversed by IL-1ra treatment. In the spinal cord, exercise induced an increase of GSH levels, which was reduced by IL-1ra. Finally, IL-1ra treatment reduced plasma levels of creatine kinase, a reliable indicator of myocyte damage.

Conclusions: IL-1β mediates delayed onset muscle soreness-induced muscle mechanical hyperalgesia by peripheral and spinal mechanisms, and could be considered a potential target to treat exercise-induced muscle pain.

Key Words: delayed onset muscle soreness, pain, hyperalgesia, cytokine, oxidative stress and mice.

Introduction

Delayed onset muscle soreness (DOMS) is an uncomfortable feeling that occurs 1 to 2 days after exercise usually affecting untrained people who are submitted to moderate to intense exercise session. Muscle soreness usually is delayed, and develops in the first 24 hours following exercise,[1] and manifests as a dull, aching pain combined with tenderness and stiffness.[2] Nowadays, rehabilitation protocols of various diseases such as heart diseases, hypertension, obesity, type II diabetes and rheumatoid arthritis, include exercise sessions as part of reeducation program in lifestyle. This type of patient normally is extremely vulnerable to present DOMS symptoms since even low intensity exercise to a healthy subject may be considered as high intensity to patients with those chronic diseases, and this condition represents a negative point in the initiative of physical activity. Additionally, professional athletes returning to normal activities after rehabilitation period that included immobilization, and consequently debilitation of muscle fibers, may present reduced performance due to DOMS.[3-5]

The peak of the hyperalgesia of DOMS is associated with a peripheral inflammatory response. Eccentric contractions induce soreness and injuries in the muscle and consequently DOMS occurs after eccentric rather than concentric contractions.[3,6] Corroborating, eccentric physical exercise induces thermal hyperalgesia, and consequently chronic muscle pain.[7]

It is well established that chemical mediators, especially cytokines are involved in the mechanisms of pain development in several models.[8] IL-1β but not IL-1α induces mechanical hyperalgesia.[9] Interleukin (IL)-1β is produced by a variety of cells, including neutrophils, macrophages and fibroblasts, in response to cell damage and its products. IL-1β induces mechanical hyperalgesia by inducing

prostaglandin production during carrageenin-induced inflammation.[9] Furthermore, intramuscular administration of inflammatory stimulus such as carrageenan induces hyperalgesia with elevation of IL-1β levels in the muscle[10] and IL-1β levels increase in blood samples of horses after high intensity exercise.[11] Therefore, strenuous exercise promotes the increase of inflammatory cytokines including IL-1β. One possible cellular source of IL-1β is the local myocyte, which produces IL-1β upon mechanical damaged during intense exercise.[12]

IL-1β is also capable of inducing neutrophils recruitment to inflammatory tissues as well as superoxide anion production.[13,14] In this sense, elevated levels of oxygen reactive species could account for the contractile dysfunction that leads to fatigue and weakness.[15] Loss of muscle force has been observed immediately after the exercise in humans, however, in mice, a second period of strength loss has been reported 1 to 3 days in post-exercise.[3] Moreover, the fatigue experienced during the resistance exercise session influences cytokine response patterns, with high release of IL-1β.[16]

It is likely that muscle soreness post-exercise depends on acute inflammatory response upon muscle injury.[3,17] There is increased tension generated by individual cross bridge resulting in mechanical disruption of the ultrastructural elements in the muscle fibers, such as the Z-line and contractile filaments.[18] The actual proposed mechanism of DOMS begins at the time of exercise with damage of muscle function unit, the sarcomere. After functional unit breakdown, an intracellular accumulation of calcium contributes to further degradation of the sarcomere, and this cellular damage induces the activation of resident cells such as macrophages and recruitment of leukocytes including neutrophils by cytokine production.[19-21] In turn, cytokines and other molecules sensitize the nociceptors resulting in hyperalgesia.[22]

Taking into account evidence indicating IL-1β as a hyperalgesic cytokine and that intense acute exercise induces IL-1β production, the effect of targeting IL-1β with IL-1 receptor antagonist (IL-1ra) in intense acute swimming-induced muscle mechanical hyperalgesia was investigated in a model of delayed onset muscle soreness in mice.

Materials and Methods

Animals

The experiments were performed on male Swiss mice, 20-25g from State University of Londrina, Londrina, PR, Brazil. Mice were housed in standard clear plastic cages (6 per cage) with free access to water and food, light / dark cycle of 12 / 12h and controlled temperature. The mice were reared in the central vivarium of State University of Londrina, and kept in the vivarium of the Department of Pathology of the same University for at least two days before experiments. Mice were used only once and were acclimatized to the testing room at least 1 hour before the experiments, which was conducted during the light cycle. Animals' care and handling procedures was approved by the Institutional Ethics Committee for Animal Use of the State University of Londrina, process number 17550.2012.88 of September, 11, 2012, and were in accordance with the National Research Council's for laboratory animals and with International Association for Study of Pain (IASP) guidelines. All efforts were made to minimize the number of animals used and their suffering.

Drugs

Drugs were obtained from the following sources: Interleukin 1 receptor antagonist (IL-1ra) from National Institute for Biological Standards and Control (NIBSC, South Mimms, Hertfordshire, UK), saline solution (NaCl 0.9%) from Gaspar Viana S/A (Fortaleza, CE, Brazil). During the experiments IL-1ra was dissolved in saline solution immediately before use.

Experimental protocols

Mice were treated with IL-1 receptor antagonist (IL-1ra, 3-30 mg/kg, i.p.) 30 min before and 12 h after beginning of exercise section of 120 min. There were naïve and sham (30 s exposition to water) control groups. After beginning of exercise section the following parameters at indicated time points were evaluated: mechanical hyperalgesia between 6-48 h, myeloperoxidase (MPO) activity at 24 h, reduced glutathione (GSH) levels at 4 h, and cortisol, glucose, lactate and creatine kinase at 2 and 24 h. IL-1β levels were determined at 2 and 4 h in samples of soleus and gastrocnemius muscle and spinal cord (L4-L6).

Intense acute swimming

Mice were placed in a glass box (45×28×25 cm, divided in six compartments) with approximately 20 liters of water at 31° ± 1°C.[23] Each mouse was placed in one compartment and swam all at the same time. A drop of liquid soap was added to reduce the surface tension of water diminishing the “floating” behavior.[24] After the intense acute swimming session or sham conditions, animals were dried and placed in cages together with their respective group. Mice were randomized in sham and exercised groups. Sham animals were allowed to swim for just 30 seconds, and were immediately removed from the water after this period and dried. Mice in the swimming group were exposed to water for 1 session of 120 min. Naïve unstimulated mice were used as negative control group. The mechanical hyperalgesia was evaluated 24h after the swimming session. The experimental conditions of this model were standardized previously (Borghi et al., 2013, submitted for publication).

Evaluation of muscle mechanical hyperalgesia

Muscle mechanical hyperalgesia was tested in mice as previously reported.[25] Briefly, in a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15–30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic von Frey anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 contact area polypropylene tip (Borghi et al., 2013, submitted for publication). The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The applied pressure to hind paw surface induce an articular movement on ankle joint, evoking a strench on Aquilles tendon, which in turn, promotes muscle movement response (movement-induced hyperalgesia) when the latter is sensitized. The end point was characterized by the removal of the hindlimb followed by clear flinching movements. Muscle distention is sufficient to trigger muscle nociceptive responses, since the cutaneous tissue are unstimulated. Moreover, the decreased mechanical threshold observed after swimming sessions was related to muscle and not cutaneous hyperalgesia (Borghi et al., 2013, submitted for publication). Therefore, these data demonstrate a novel applicability for the electronic von Frey test. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were evaluated at baseline and 6-48 h after exercise. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements (indicated time points) after stimulus from the baseline measurements. The basal mechanical withdrawal threshold was 8.8±0.1 g (mean ± SEM of 6 groups, 6 mice per group) before intense acute swimming session. There was no difference of basal mechanical withdrawal thresholds between groups in the same experiment.

IL-1β production

Mice were killed 2 and 4 h after swimming session and samples of spinal cord (L4-L6), and gastrocnemius and soleus muscles were collected. The samples were homogenized in 300 µl (spinal cord pool of three mice) or 500 µl (skeletal muscles) of the appropriate buffer containing protease inhibitors and IL-1β levels were determined by enzyme-linked immunosorbent assay (ELISA).[26] The results were expressed as picograms (pg) of IL-1β per 100 mg of tissue. Naive and sham groups were used as control.

Leukocyte migration to the skeletal muscle tissue

The intense acute swimming-induced leukocyte recruitment to the soleus and gastrocnemius muscles of mice was evaluated using the myeloperoxidase (MPO) kinetic–colorimetric assay.[27] Samples of skeletal muscles were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at −86 °C until use. Samples were homogenized using a Polytron (PT3100), centrifuged at 16.100 *g* in 4 °C for 2 min and the resulting supernatant assayed spectrophotometrically for MPO activity determination at 450 nm (Spectra max), with 3 readings in 1 min. The MPO activity of samples was compared to a standard curve of neutrophils. Briefly, 10 µl of sample were mixed with 200 µl of 50 mM phosphate buffer pH 6.0, containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results were presented as MPO activity (number of total neutrophils x 10^{10}/ mg of muscle).

Reduced glutathione (GSH) assay

The levels of GSH in skeletal muscle samples were determined using a spectrophotometric method.[28] Samples of gastrocnemius (100 mg) or soleus (40 mg) (1:10 dilution) were homogenized (IKA T10) in 4 and 1.6 ml of EDTA 0,02M, respectively. Homogenates (2.5 ml) were treated with 2 ml H_20 Milli Q plus 0.5 ml of trichloroacetic acid 50%. After 15 minutes, the homogenates were centrifuged at 1500 *g* for 15 min, and 1 ml from supernatant was added to 2 ml of a solution containing Tris 0.4M (pH 8.9) plus 50 ml of DTNB. After 5 min, the measurements were performed in 412 nm against white control (UV - Visible spectrophotometer [UV-1650 PC] – SHIMADZU). The GSH levels were corrected according to the total protein concentration. The results were presented as mmols of GSH per gram of protein in skeletal muscles.

Cortisol, glucose, lactate and creatine kinase (CK) plasmatic concentrations.

Mice were anesthetized and blood samples of all groups were collected immediately after (2 hours) and 24 hours after intense acute swimming session, always in the afternoon (between 15:00h and 17:00h). Samples were centrifuged at 3.300 *g* in 4 °C for 5 min and the resultant plasma was assayed for cortisol, glucose, lactate and CK levels. Cortisol was evaluated by Architect System Kit, which is a chemiluminescent assay of microparticles for quantitative determination of serum cortisol. Glucose, Lactate and CK concentrations (Dimension® Clinical Chemistry System – SIEMENS) was quantified with spectrophotometer according to the manufacturer's instructions. Naïve and sham groups were used as control.

Statistical analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group per experiment, and are representative of two experiments. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves). The analyzed factors were treatments, time and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's t-test was performed for each time. On the other hand, when the nociceptive responses were presented as total values at indicated time period, the differences between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Statistical differences were considered to be significant at $P < 0.05$.

Results

Role of IL-1β in intense acute swimming-induced muscle mechanical hyperalgesia

Mice were treated with IL-1ra (3 or 30 mg/kg i.p.)[29] or vehicle (saline) 30 minutes before plus reinforcements 12 hours after swimming session and the intensity of muscle mechanical hyperalgesia was measured 6, 12, 24, 36 and 48 hours after the exercise beginning (Figure 1). Intense acute swimming induced significant muscle mechanical hyperalgesia in exercised mice compared to the sham group between 6-48 h (*n=6*, P<0.05) with gradual increase up to 24 hours which was the peak of hyperalgesia. From 24th to 48th h the intensity of muscle mechanical hyperalgesia decreased continuously maintaining the significant difference with the sham group. Intense acute swimming-induced muscle mechanical hyperalgesia was diminished at 12th and 24th h by the 3 mg/kg dose of IL-1ra (*n=6*, P<0.05), without differences at 6th, 36th and 48th h (*n=6*, P>0.05), whereas 30 mg/kg dose of IL-1ra abolished muscle mechanical hyperalgesia between 6-48 h compared to exercised non-treated animals (0.74 ± 0.30 versus 4.26 ± 0.19 in the peak of hyperalgesia at 24th h, respectively, *n=6*, P<0.05) (Figure 1). IL-1ra (30 mg/kg, i.p.) did not affect the nociceptive responses of sham group. Therefore, 30 mg/kg dose was chosen for the subsequent experiments. These results indicate that IL-1 is an important hyperalgesic cytokine in intense acute swimming-induced muscle pain in mice. **(Include figure 1 here).**

Intense acute swimming induces the production of IL-1β in the peripheral (soleus, but not in the gastrocnemius muscle) and spinal cord sites

The levels of IL-1β were measured in the soleus and gastrocnemius skeletal muscles, and spinal cord (L4-L6) samples 2 and 4 hours after exercise session

(Figure 2). There was a significant increase of IL-1β levels in the soleus muscle immediately after (2 h) intense acute swimming when compared to sham group (13.05 ± 0.84 versus 5.74 ± 0.67, respectively, *n=6*, P<0.05), but basal levels were detected 4 h (*n=6*, P>0.05) after exercise session (Figure 2a). On the other hand, in the gastrocnemius muscle, IL-1β levels present a tendency to increase, but there was no significant difference between naïve, sham and intense acute swimming groups (*n=6*, P>0.05, Figure 2b). Furthermore, in spinal cord samples, IL-1β levels increased only at 4 h compared to the sham group (11.56 ± 1.65 versus 2.23 ± 0.59, respectively, *n=6*, P<0.05) without alteration at 2 h (*n=6*, P>0.05, Figure 2c). These results evidenced that IL-1β production follows a temporal profile from periphery to spinal sites after intense acute swimming protocol. **(Include figure 2 here).**

Intense acute swimming induces IL-1-dependent increase of myeloperoxidase (MPO) activity in the soleus, but not in the gastrocnemius muscle

Mice were treated with IL-1ra (30 mg/kg, 30 min before and 12 h after exercise) and samples of the soleus and gastrocnemius skeletal muscle were collected 24 hours after the exercise (Figure 3). Figure 3a shows that intense acute swimming induced significant increase of MPO activity compared to naïve and sham groups in the soleus muscle (*n=6*, P<0.05), which was inhibited by IL-1ra treatment ($29.64x10^9$ versus $8.348x10^9$, *n=6*, P<0.05). There was no significant alteration of MPO activity in the gastrocnemius muscle (*n=6*, P>0.05, Figure 3b). **(Include figure 3 here).**

Intense acute swimming induces IL-1-dependent modulation of reduced glutathione (GSH) levels in peripheral (soleus, but not gastrocnemius muscle) and spinal cord sites

Mice were treated with IL-1ra (30 mg/kg, i.p.) 30 min before intense acute swimming session and samples of the soleus and gastrocnemius muscle, and spinal cord were collected 2 and 4 h after the beginning of the exercise session (Figure 4). Intense acute swimming induced the reduction of GSH levels at 2 (data not shown) and more intensely at 4 h after exercise session in the soleus (*n=6*, P<0.05, Figure 4a) but not in the gastrocnemius muscle (*n=6*, P>0.05, Figure 4b). Figure 4a shows that intense acute swimming-induced GSH depletion at 4 h was prevented in the soleus muscle by IL-1ra treatment (0.013 ± 0.002 versus 0.050 ± 0.011, *n=6*, P<0.05), while all groups presented similar results to sham group in the gastrocnemius muscle (*n=6*, P>0.05, Figure 4b). On the other hand, in spinal cord samples, the intense acute swimming session induced increase in GSH levels at 4h when compared to the naïve and sham groups (*n=6*, P<0.05), which was prevented by IL-1ra treatment (2.872 ± 0.197 versus 1.918 ± 0.153, *n=6*, P<0.05, Figure 1c). **(Include figure 4 here).**

Evaluation of serum cortisol, glucose, lactate and CK levels

Stress is a confounding factor that can directly interfere in pain measurement.[30] To determine whether the present model is a stress-induced hyperalgesia model or not, plasma cortisol and glucose levels were assessed (Figures 5a and b, respectively). Two time points were chosen, 2 h representing the immediately time point after swimming session end and 24 h representing the peak of hyperalgesia. Intense acute swimming did not modify cortisol and glucose levels in exercised mice at 2 and 24 h compared to naïve and sham groups (*n=6*, P>0.05). IL-1ra treatment did not induce changes in the plasmatic concentrations of cortisol and glucose (Figures 5a and b). The levels of lactate and CK, which are biomarkers of myocyte overload, were also determined (Figures 5c and d). Intense acute swimming induced significant

increase of lactate and CK levels when compared to sham group at 2 h (36.5% and 101.7% respectively, *n*=*6*, P<0.05), suggesting myocyte overload. There was tendency of reduction of lactate levels by IL-1ra treatment (8.52 ± 0.59 versus 7.02 ± 0.14, *n*=*6*, P>0.05) while intense acute swimming-induced CK plasma elevation at 2 h was significantly reduced by IL-1ra treatment (11982.5 ± 1187 versus 473.5 ± 59.7, *n*=*6*, P<0.05). **(Include figure 5 here).**

Discussion

IL-1β was the first cytokine reported to be hyperalgesic, and on the other hand, the hyperalgesic effect of IL-1α was disproved.[9] IL-1β enhances nociceptive responses, and its administration in the peripheral tissue or in central nervous system induces hyperalgesia that is prevented by pretreatment with IL-1ra.[31,32] The role of IL-1β in the induction of inflammatory nociception and efficacy of IL-1 targeting therapies were also demonstrated in models of inflammatory hyperalgesia such as carrageenan, LPS, antigen-induced arthritis[9,26,33] and neuropathic pain.[34,35]

Regular exercise in a program of swimming training did not induce increases in plasma levels of IL-1β.[36] On the other hand, high levels of IL-1β was detected in the trapezius muscle of individuals with myofascial trigger points, eccentric exercise induces IL-1 receptor expression in human muscle, [37,38] one session of acute exercise of long duration and high intensity induce cytokine release immediately after running in humans,[39] and acute resistance exercise increases IL-1β levels in skeletal muscle.[40] Thus, these data demonstrate an important correlation between IL-1β levels and muscle painful conditions in humans as well as the present pharmacological and behavioral data add to the correlation of IL-1β and muscle pain.

Intense acute swimming induced significant production of IL-1β, which is in accordance with the reduction of hyperalgesia, MPO activity and oxidative stress by the treatment with IL-1ra. IL-1β is a chemotactic cytokine[41] recruiting cells involved in the beginning of response to tissue lesion such as neutrophils that express MPO. Neutrophils contribute to inflammatory hyperalgesia by further producing nociceptive molecules.[42,43] In agreement with the IL-1β-dependent increase of MPO activity, eccentric cycling exercise induces concomitant detection of neutrophils and IL-1β in human skeletal muscle[44] and others have also detected increase of MPO activity 24

h after high intensity exercise.[45] Macrophages also express MPO and produce many hyperalgesic cytokines including IL-1β.[8,29,46] Therefore, the increase of MPO activity may be related to neutrophils and macrophages.

IL-1ra treatment also reduced the peripheral oxidative stress in DOMS, which was observed in the soleus, but not gastrocnemius muscle. In agreement, slow fibers (type I and IIa) present in the soleus muscle are highly oxidative.[15,47,48] The slow fibers type I and IIa are preferentially recruited during exercise session of long duration and high intensity such as intense acute swimming. In contrast, the gastrocnemius muscle presents predominantly fast twitch fibers, susceptible to fatigue and consequently low oxidative potential.[15,47,48] Therefore, the characteristic fibers in each muscle explain the prominent and selective oxidative response in the soleus muscle compared to the gastrocnemius muscle.

IL-1β activates NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidade) to produce superoxide anion, which in turn, is a substrate to form additional reactive oxygen species.[14,49] Superoxide anion can also activate transcription factors such as NFκB[50,51] that mediates IL-1β-induced expression and activation of cyclooxygenase-2, which is responsible for the production of prostaglandin E_2, a nociceptor sensitizing molecule.[8] Superoxide anion also activates mytogen-activated protein kinases including p38 that in turn, activates sodium channels resulting in nociceptor sensitization;[52,53] and in fact, inhibition of oxidative stress reduces inflammatory pain.[28,50,51] The IL-1β-induced oxidative stress was represented by the decrease of GSH levels in the soleus muscle reversed by IL-1ra and might account for the analgesic effect of IL-1ra.

On the other hand, intense acute swimming induced an increase of GSH levels in the spinal cord. In models of neuropathic pain there is also increase rather

than reduction of GSH spinal levels.[54,55] This is likely an adaptive response to the peripheral injury-induced oxidative stress evidencing the integrative mechanism between peripheral and spinal sites.[55] It is important to point out that IL-1ra reversed the increase of GSH levels in the spinal cord evidencing the key role of IL-1β in modulating oxidative imbalance caused by intense acute swimming.

The present study used a model of exercise that avoids stress or analgesia, and focused in exercise-induced hyperalgesia. Protocols involving stress frequently use temperatures of 25°C or lower,[56-58] and higher temperatures like 37°C are used to induce hypoalgesia.[24,59] We used an intermediate temperature (31°C) to focus in exercise-induced hyperalgesia. Reinforcing this concept, cortisol and glucose levels of all groups immediately after (2 h) and 24 h after (peak of hyperalgesia) swimming session were similar to that observed in basal levels (naïve and sham groups), indicating that mice were not under stress.

Lactate levels were increased by exercise at 2 h evidencing that this is a high intensity exercise protocol. The lactate levels declined at 24 h without significant difference when compared to the control group. Furthermore, exercise induced increase of creatine kinase levels at 2 h. CK is considered a reliable indicator of sarcolemma permeability as this enzyme is found within myocyte and is released by disruption/damage of muscle sarcomere (z-lines).[4] Moreover, circulating CK levels have been shown to rise up to 400 times in DOMS.[3,4] Together, the increase of lactate and CK blood levels indicate muscle exercise overload. The treatment with IL-1ra significantly reduced CK levels and induced tendency of reduction of lactate levels compared to exercise control group immediately after the session (2 h). In agreement, human myogenic cells constitutively produce IL-1β and IL-1 receptors, and treatment with exogenous IL-1β induced myogenic cell apoptosis.[60] Therefore,

myocyte derived IL-1β could act in an autocrine manner inducing tissue damage/cell death[60] with the contribution of reactive oxygen species.[61] Thus, IL-1ra treatment might avoid excessive damage of the myocyte structure, reducing the release of biomarkers from injured cells such as creatine kinase.

It is noteworthy to mention that there is a lack of models to evaluate movement-induced muscular mechanical hyperalgesia (tenderness and movement-related pain) during DOMS[62] while we provide a reliable model to study DOMS in this regard.

Conclusions

IL-1β has an important endogenous role in muscle mechanical hyperalgesia in DOMS induced by intense acute swimming in mice. In the periphery, intense acute swimming affected mainly the soleus muscle compared to the gastrocnemius muscle since there was increase of IL-1β levels, MPO activity and reduction of GSH in the soleus muscle and not in the gastrocnemius. In the spinal cord, there was increase of IL-1β production and GSH levels. The treatment with IL-1ra prevented all biochemical modifications and also the behavioral nociceptive response induced by DOMS. There is a temporal coordination of the IL-1β-dependent events in DOMS since IL-1β levels increased significantly at 2 h in the soleus muscle and at 4 h in the spinal cord corroborating the notion that the peripheral stimulus was responsible for triggering spinal signaling events. Fig. 6 shows the schematic proposed role of IL-1β in delayed onset muscle soreness.

Declarations

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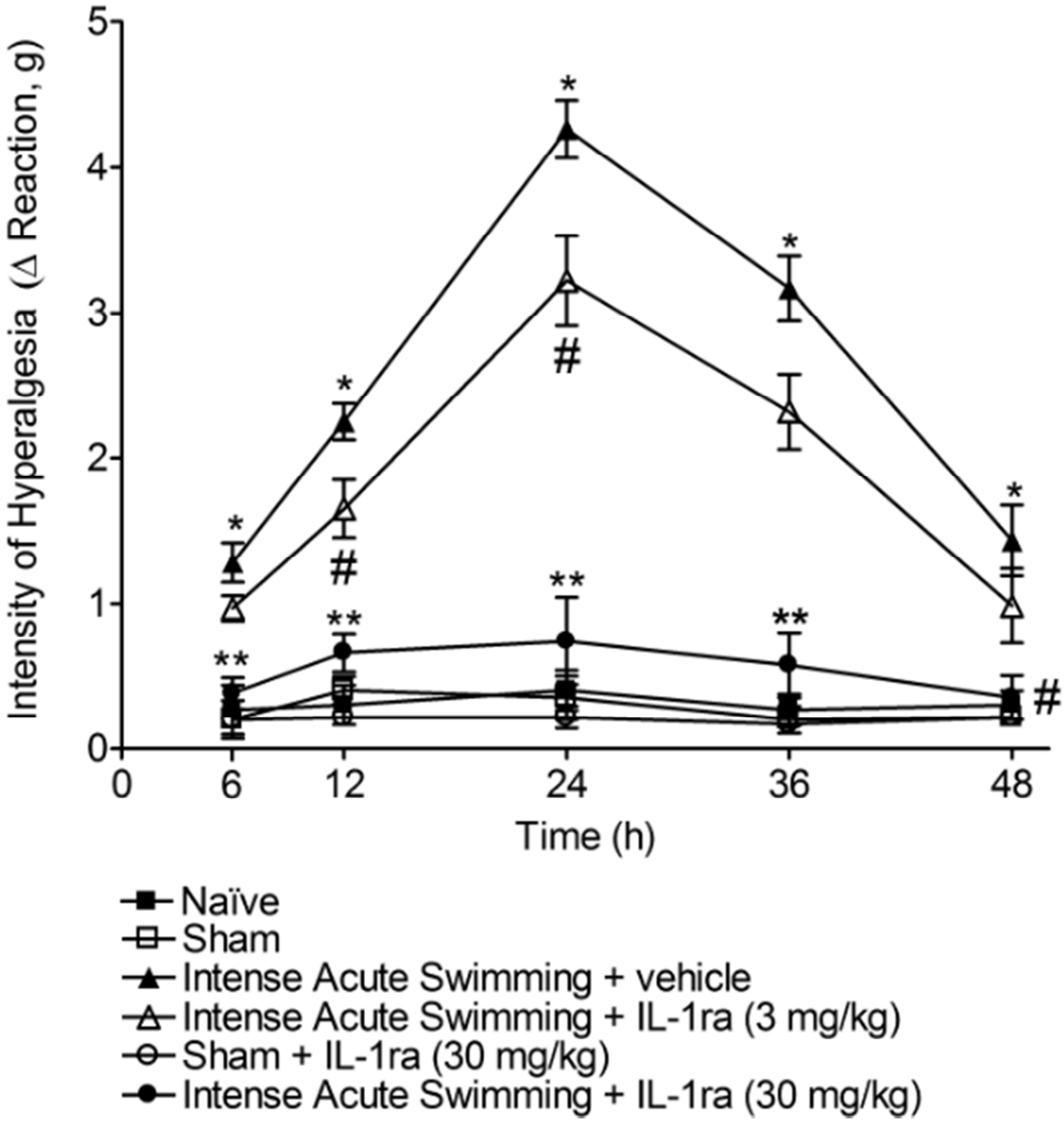

Figure 1 IL-1 receptor antagonist (IL-1ra) treatment reduces intense acute swimming-induced muscle mechanical hyperalgesia. Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. *P < 0.05 versus naive and sham groups; #P < 0.05 versus intense acute swimming group; and ** P < 0.05 versus intense acute swimming + IL-1ra (3 mg/kg) group.

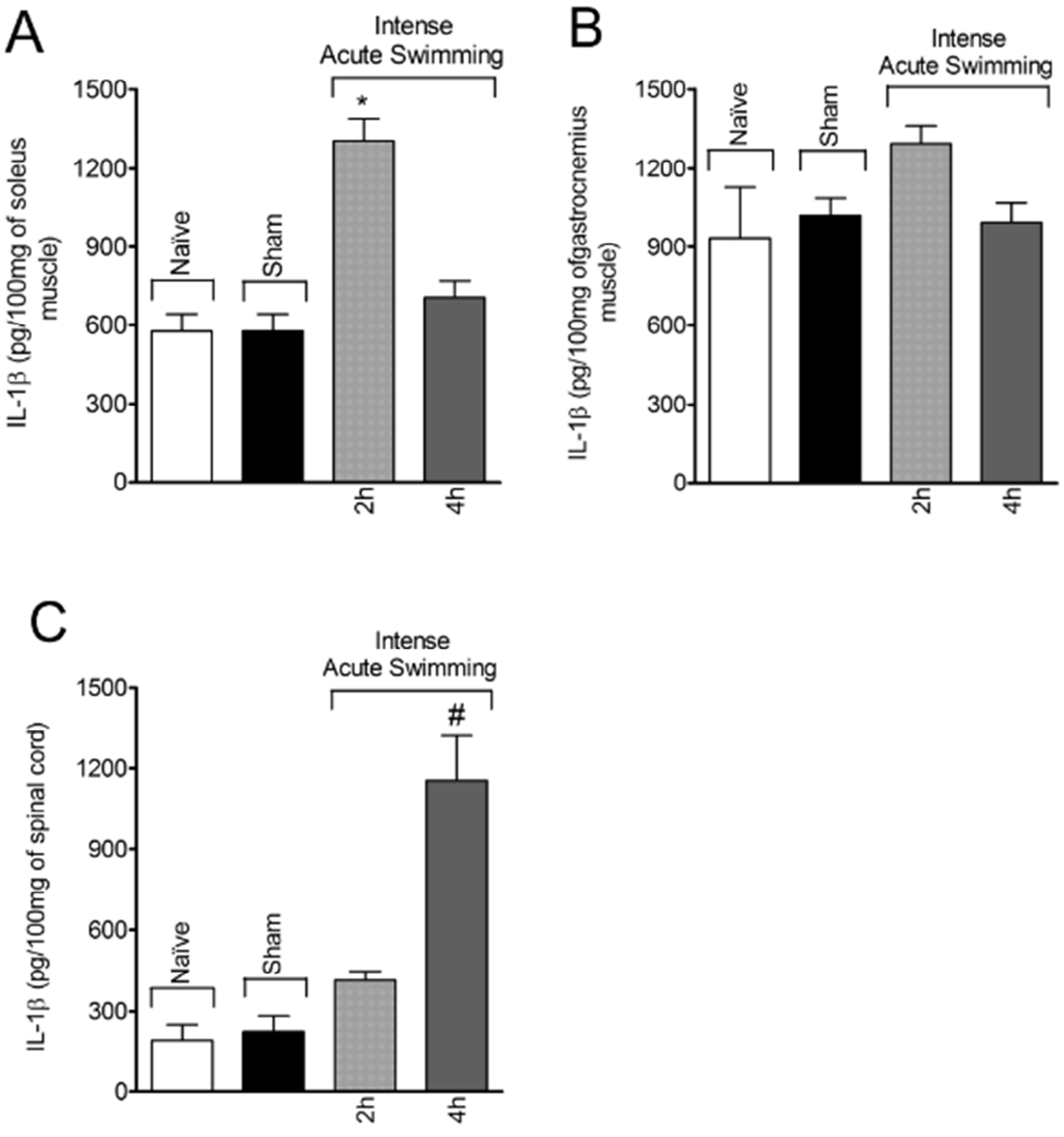

Figure 2 Intense acute swimming induces IL-1β production in the soleus muscle (a) and spinal cord (c), but not in the gastrocnemius muscle (b). Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. * $P < 0.05$ versus naïve and sham groups; and $^{\#}$ $P< 0.05$ versus naive, sham and 2 h groups.

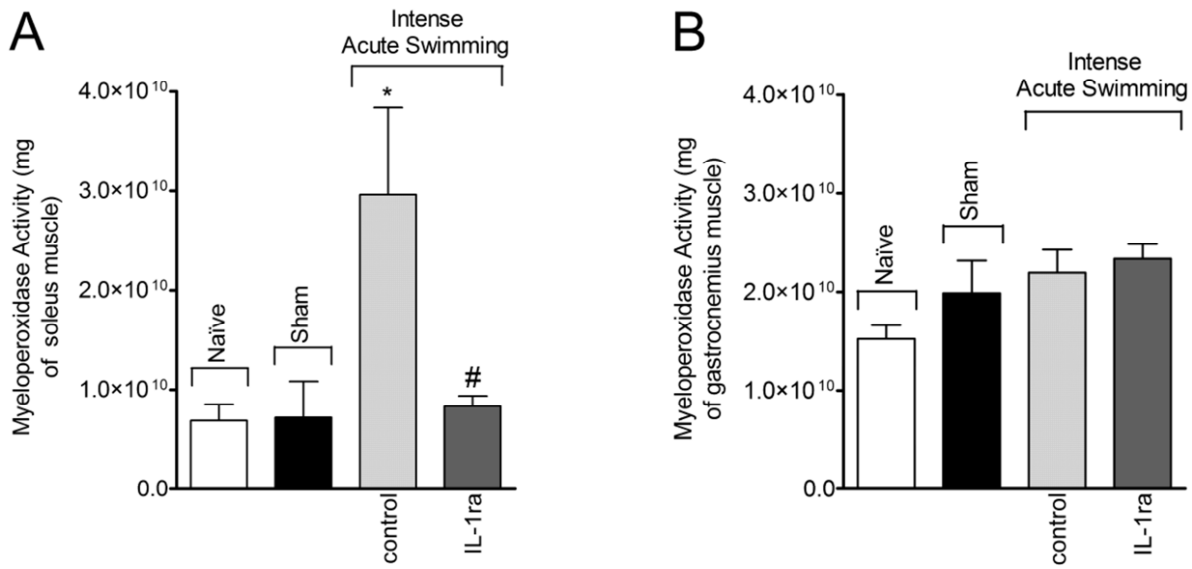

Figure 3 IL-1ra treatment reduces intense acute swimming-induced myeloperoxidase (MPO) activity in the soleus (a), but not in the gastrocnemius muscle (b). Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. *P < 0.05 versus naive and sham groups; and $^{\#}$P < 0.05 versus control group.

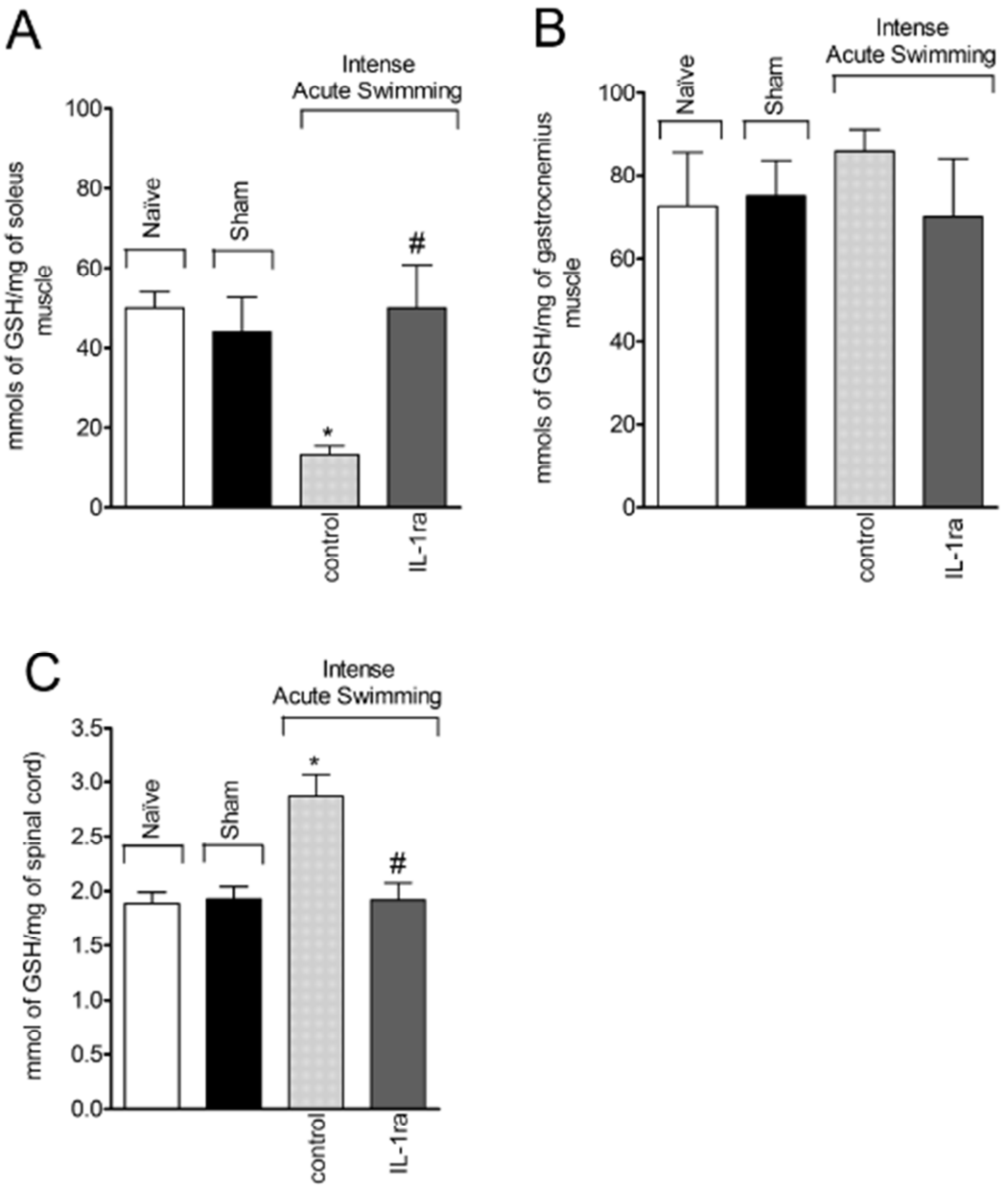

Figure 4 IL-1ra treatment reverses the intense acute swimming-induced modulation of reduced glutathione (GSH) levels in the soleus muscle (a) and spinal cord (c), but not in the gastrocnemius muscle (b). Results are presented as means ± S.E.M. of 6 mice per group for muscle samples or 6 pools of two mice per group for spinal cord samples, and are representative of 2 separated experiments. *P < 0.05 versus naive and sham groups; and $^{\#}$P < 0.05 versus control group.

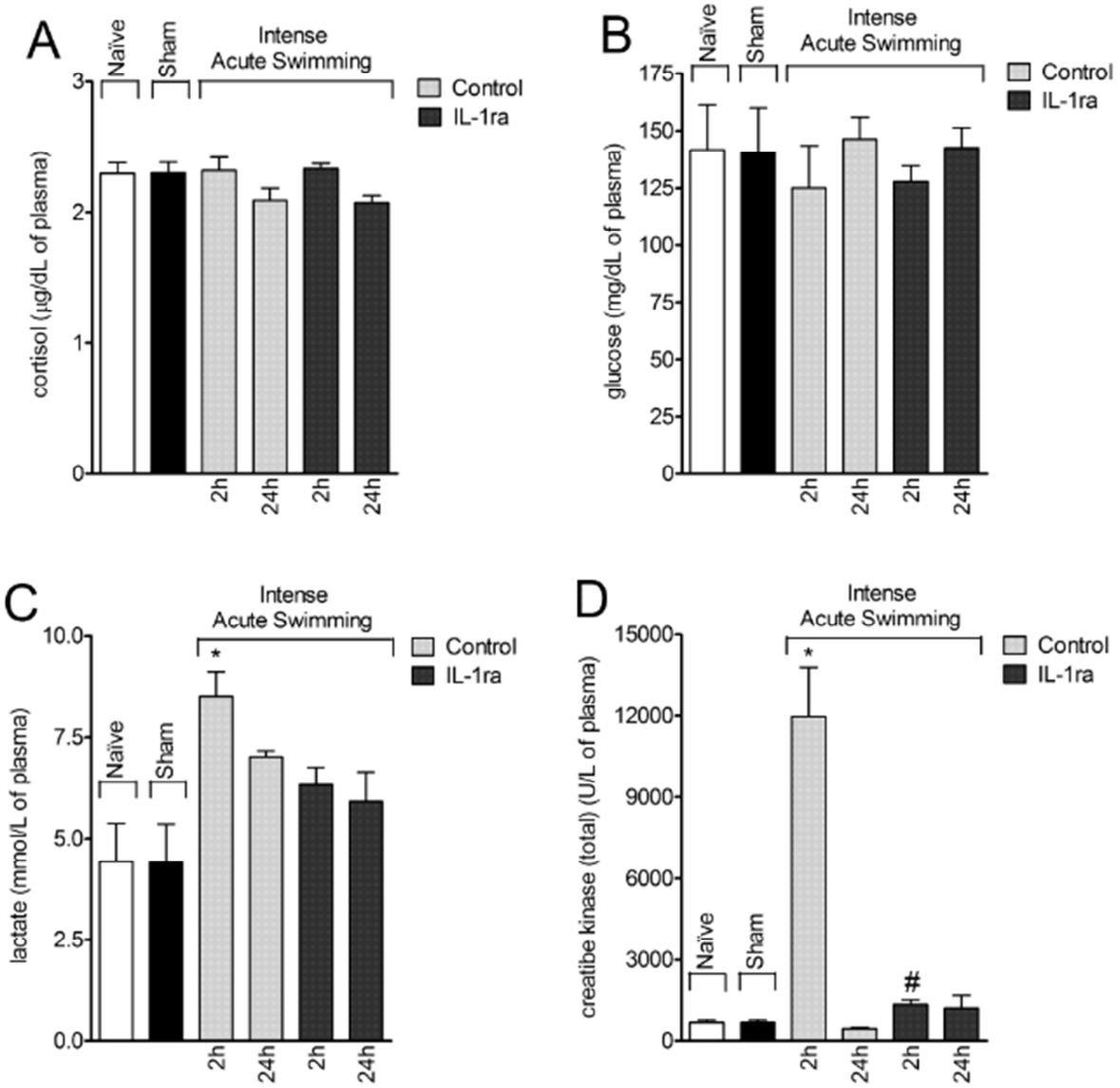

Figure 5 IL-1ra treatment did not induce alterations in plasma cortisol (a) and glucose (b) levels and reduced intense acute swimming-induced lactate (c) and creatine kinase (d) plasma concentrations increase. Results are presented as means ± S.E.M. of 6 mice per group, and are representative of 2 separated experiments. *P < 0.05 versus naive and sham groups; and $^{\#}$P < 0.05 versus control group.

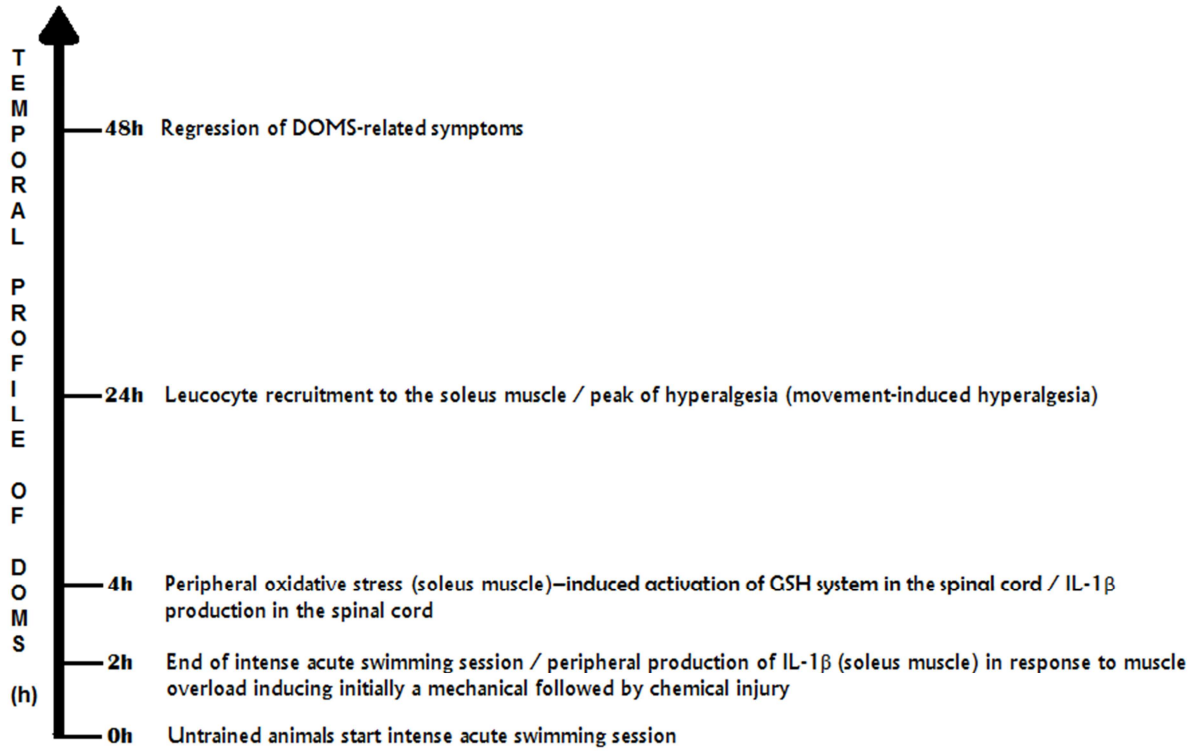

Figure 6 Chronological schematic representation of the proposed mechanisms for IL-1β participation in delayed onset muscle soreness in mice. Intense acute swimming session in untrained mice induces a) myocyte injury with resultant IL-1β production by resident activated cells in highly oxidative muscles such as soleus muscle; b) IL-1β chemoattracts neutrophils that once activated produce additional pro-nociceptive molecules including reactive oxygen species in peripheral sites; c) there is production of IL-1β in the spinal cord induced by the peripheral stimulation and the activation of GSH system to prevent further oxidative damages; d) the peak of mechanical hyperalgesia (DOMS) is achieved at 24th h; e) after 48 h there is regression of DOMS-related symptoms.

ANEXO 3

ARTIGO SUBMETIDO 3

Este trabalho foi realizado no laboratório de fisiopatologia e farmacologia da dor, neuropatia, câncer e inflamação, resultando na formação do artigo científico: Interleukin-10 limits intense acute swimming-induced muscle mechanical hyperalgesia in mice, de autoria de Sergio M. Borghi, Felipe A. Pinho-Ribeiro, Ana C. Zarpelon, Thiago M. Cunha, Sergio H. Ferreira, Fernando Q. Cunha, Rubia Casagrande e Waldiceu A. Verri Jr.

As formatações do artigo seguem as normas da revista *Experimental Physiology*.

Interleukin-10 limits intense acute swimming-induced muscle mechanical hyperalgesia in mice

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Running title: Interleukin-10 as a key mediator in DOMS control.

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Bullet Points

1. What is the central question of this study?

This study investigated the role of endogenous IL-10 in intense acute-swimming-induced muscle mechanical hyperalgesia.

2. What is the main finding and its importance?

Endogenous IL-10 limits intense acute swimming-induced muscle mechanical hyperalgesia since its genetic deletion resulted in increased hyperalgesia, leukocyte recruitment, hyperalgesic cytokine production and oxidative stress.

Abstract

Interleukin-10 (IL-10) is an anti-hyperalgesic cytokine. In the present study, IL-10 deficient$^{-/-}$ mice were used to investigate the endogenous role of IL-10 in intense acute swimming-induced DOMS (delayed onset muscle soreness). Intense acute swimming session of 1 or 2 h induced significant muscle mechanical hyperalgesia in WT mice when compared to sham group 24 hours after the session (2.53±0.23 and 4.65±0.43, respectively vs. 0.25±0.17, $p < 0.05$), which was further increased in IL-10$^{-/-}$ mice (3.25±0.38 and 5.98±0.22, respectively, $p < 0.05$ for 2 h). Using the 2 h of swimming protocol, it was observed that IL-10$^{-/-}$ mice presented an increase of TNFα and IL-1β production in the soleus muscle overt time (2-6 h), which normally is restricted to 2 h in WT mice. There was no statistical difference in the levels of IL-4, IL-5, IL-13 and TGF-β between WT and IL-10$^{-/-}$ mice ($p > 0.05$). IL-10 deficiency also resulted in increased myeloperoxidase activity ($2.0x10^{10}$±$1.8x10^{9}$ vs. $1.3x10^{10}$±$1.8 x10^{9}$, $p < 0.05$), greater depletion of reduced glutathione levels (0.039±0.003 vs. 0.071±0.010, $p < 0.05$) and increased superoxide anion production (0.567±0.069 vs. 0.368±0.064, $p < 0.05$) in soleus muscle than in WT mice. These results demonstrate that endogenous IL-10 presents an anti-hyperalgesic role to control excessive intense acute swimming-induced DOMS, which merits further pre-clinical and clinical investigation as a therapeutic strategy.

Introduction

Delayed onset muscle soreness (DOMS) is a common experience for athletes and any people who experience some kind of unaccustomed exercise, and the symptoms can range from muscle tenderness to severe debilitating pain. Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. The process of pain includes two main conditions: hyperalgesia (an increased response to a stimulus that is normally painful) or allodynia (pain due to a stimulus that does not normally provoke pain) (Verri *et al*. 2006). For instance, in inflammatory process, released cytokines such as tumor necrosis alpha (TNFα) that via interleukin (IL)-1β stimulates the production of directly acting hyperalgesic mediators such as prostaglandin E_2 (PGE_2) that sensitizes the primary nociceptive neurons resulting in hyperalgesia (Verri *et al*. 2006).

Cytokines are potent intercellular signaling molecules that regulate inflammatory, neuropathic, cancer and muscle pain (Schafers *et al*. 2003; Cunha *et al*. 2005; Verri *et al*. 2006; Loram *et al*. 2007; Shah *et al*. 2008; Hu *et al*. 2012; Heitzer *et al*. 2012; Ellis and Bennett, 2013). IL-10 was the first anti-hyperalgesic cytokine to be described (Poole *et al*. 1995). IL-10 inhibits the production of hyperalgesic cytokines such as IL-1β, IL-6, IL-8 and TNFα (Poole *et al*. 1995, Vale *et al*. 2003; Verri *et al*. 2006). Additional anti-hyperalgesic cytokines include IL-4, IL-5, IL-13 and tumor growth factor-beta (TGF-β) (Standiford *et al*. 1990; Fiorentino *et al*. 1991).

Intense and prolonged exercise induces myocyte oxidative stress. Free radicals modulate a number of cell signaling pathways and the expression of multiple genes in eukaryotic cells. These free radicals change gene expression at

transcriptional, mRNA stability and signal transduction levels. Moreover, numerous products related to oxidative stress-responsive genes were identified in myocytes, which include antioxidants enzymes, stress proteins, DNA repair proteins and mitochondrial electron transports proteins (Powers and Jackson, 2008). In this sense, evidences show that IL-10 can suppress superoxide anion production, accompanied by down-regulation of the genes for proteic subunits of NADPH oxidase (Kuga *et al*. 2006). Additionally, IL-10 could inhibit the NADPH oxidase in human and mouse epithelial colonic cells, protects from LPS-induced neurotoxicity in primary midbrain cultures and improves microvascular endothelial functions by inhibiting NADPH oxidase function, thus acting indirectly as an antioxidant (Qian *et al*. 2006; Kamizato *et al*. 2009; Kassan *et al*. 2011).

Delayed onset muscle soreness, one of the most relevant clinical symptoms related to unaccustomed acute exercises presents the peak of hyperalgesia around 24-48 h after the session, accompanied by peripheral inflammatory character, with enhanced cytokine levels (Graven-Nielsen and Arendt-Nielsen, 2003; Borghi *et al*. 2013a, submitted). Corroborating, acute physical exercise has been linked with cytokine signaling pathways in post-exercise (Davis *et al*. 2007; Cleto *et al*. 2011, Borghi *et al*. 2013a, submitted; Borghi *et al*. 2013b, submitted). Reinforcing this concept, Nieman *et al*. (2005) found elevated levels of systemic (plasma) cytokines in humans, including IL-10 after 160 Km race. In this sense, according to the Lewis *et al*. (2012) DOMS is initiated at the time of exercise with strains on the sarcomere, resulting in an intracellular accumulation of calcium, leading to extra degradation of this functional unit. The subsequent inflammatory response depends on cytokines acting as hyperalgesic mediators and the recruitment of inflammatory cells.

The influence of pro-inflammatory cytokines in DOMS is well established (MacIntyre *et al*. 2001; Nieman *et al*. 2005; Nieman *et al*. 2006; Chatzinikolaou *et al*. 2010; Borghi *et al*. 2013a; Borghi *et al*. 2013b), however, the role of IL-10 in DOMS remains to be investigated. In the present study, it was evaluated the role of endogenous IL-10 using a mice model of intense acute swimming-induced hyperalgesic DOMS.

Methods

Ethical Approval

Animals' care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Institutional Ethics Committee for Animal Research of the Universidade Estadual de Londrina, process number 17550.2012.88. All efforts were made to minimize the number of animals used and their suffering.

Animals

The experiments were performed on male C57 BL/6 (wild type – WT) and IL-10 deficient ($^{-/-}$) mice, weighing between 20-25g from University of São Paulo, Ribeirão Preto, SP, Brazil. Mice were housed in standard clear plastic cages (5-6 per cage) with free access to water and food, light / dark cycle of 12 / 12h and controlled temperature. Mice were maintained in the vivarium of the Department of Pathology of Universidade Estadual de Londrina for at least two days before experiments. Mice were used only once and were acclimatized to the testing room at least 1 hour before the experiments, which was conducted during the light cycle. A total of 180 animals were used during this study.

Intense Acute Swimming Session

Mice were placed in a glass box (45×28×25 cm, divided in six compartments) with approximately 20 liters of water at 31° ± 1°C. Each mouse was placed in one compartment and swam all the same time. A drop of liquid soap was added to reduce the surface tension of water diminishing the “floating” behavior. Mice were

randomized in sham and exercised groups. Sham animals were allowed to swim for just 30 seconds, and were immediately removed from the water after this period. Mice in the swimming group were exposed to water for 1 session of 60 or 120 min. The muscle mechanical hyperalgesia was evaluated 6-48 h after the swimming session. The present study used a model of exercise avoiding stress or hypoalgesia, and focusing in exercise-induced hyperalgesia. The experimental conditions of this model were standardized previously (Borghi *et al*. 2013a, submitted for publication).

Evaluation of Mechanical Hyperalgesia

Mechanical hyperalgesia was tested in mice as previously reported (Cunha *et al*. 2004). The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic von Frey anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 contact area polypropylene tip. The applied pressure to hind paw surface induce an articular movement on ankle joint, evoking a strench on Aquilles tendon, which in turn, promotes a muscle movement response (movement-induced hyperalgesia) when the latter is sensitized (Borghi *et al*. 2013a, submitted). The end point was characterized by the removal of the paw followed by clear flinching movements. Muscle distention is sufficient to trigger muscle nociceptive responses. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements (indicated time points) after stimulus from the baseline measurements. The basal mechanical withdrawal threshold was 8.8±0.1 g (mean ± SEM of 5 groups, 6 mice per group) before intense

acute swimming session. There was no difference of basal mechanical withdrawal thresholds between groups in the same experiment.

Cytokine Production

Mice were euthanized 2, 4 and 6 h after the intense acute swimming session and samples of the soleus and gastrocnemius muscles were collected. The samples of the skeletal muscles were homogenized in 500 (soleus) and 700 (gastrocnemius) µl of the appropriate buffer containing protease inhibitors. Cytokine levels were determined as described previously (Verri *et al*. 2008) by enzyme-linked immunosorbent assay (ELISA). The results were expressed as picograms (pg) of cytokine per 100 mg of tissue. Naïve and sham groups were used as control.

Leukocyte Migration to the Skeletal Muscle Tissue

The intense acute swimming-induced leukocyte recruitment to the soleus and gastrocnemius muscles of mice was evaluated using the myeloperoxidase (MPO) kinetic–colorimetric assay (Casagrande *et al*. 2006). Samples of skeletal muscles were collected 24 hours after the intense acute swimming (peak of hyperlagesia) and assayed spectrophotometrically for MPO activity determination. The results were presented as MPO activity (number of total neutrophils x 10^{10}/ mg of muscle).

Reduced Glutathione (GSH) Assay

The levels of skeletal muscle GSH were determined using a spectrophotometric method. Samples of gastrocnemius (100 mg) or soleus (40 mg) (1:10 dilution) were collected and followed the previously described protocol (Borghi *et al*. 2013c). The GSH levels were corrected according to the total protein

concentration. The results were presented as mmols of GSH per gram of protein in skeletal muscles.

Superoxide Anion Production

Samples of the soleus and gastrocnemius muscles from mice were collected in 300 µl and 500 µl, respectively of nitro blue tetrazolium (NBT, Sigma) 4 hours after the session (Borghi *et al*. 2013a, submitted) and maintained at 37°C in warm bath for 5 minutes, to intense acute swimming-induced oxidative stress assessment. Samples were homogenized using a Polytron (PT3100), centrifuged at 3300 g in 4 °C for 10 min and the resulting supernatant assayed spectrophotometrically for superoxide anion production determination at 600 nm (Spectra max). The results were presented as total superoxide anion production per mg of tissue.

Statistical Analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group per experiment, and are representative of two experiments. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves). The analyzed factors were treatments, time and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's t-test was performed for each time. On the other hand, when the nociceptive responses were presented as total values at indicated time period, the differences between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Statistical differences were considered to be significant at $P < 0.05$.

Results

IL-10 limits intense acute swimming-induced muscle mechanical hyperalgesia

WT and IL-10 deficient$^{-/-}$ mice were submitted to one session of intense acute swimming of 1 or 2 h (Fig. 1). Sham group was used as negative control. One h of intense acute swimming session induced muscle mechanical hyperalgesia in WT mice compared to the sham group from the 6th to 36th h ($p < 0.05$), and IL-10$^{-/-}$ mice presented an even greater muscle mechanical hyperalgesia, which was different from swimming group in the 6th, 12th and 48th h ($p < 0.05$) (Fig. 1A). Two h of swimming induced muscle mechanical hyperalgesia in WT mice compared to sham group from the 6th to 48th h ($p < 0.05$) while 2 h swimming IL-10$^{-/-}$ mice presented further increase of the muscle mechanical hyperalgesia compared to swimming WT group between 24-48h ($p < 0.05$) (Fig. 1B). Thus, the swimming time of 2 h was chosen for the next experiments.

Intense acute swimming induces the production of IL-10 in the soleus, but not in the gastrocnemius muscle

WT mice underwent 2 h of intense acute swimming session or sham conditions and samples of the soleus and gastrocnemius muscles were collected for IL-10 measurement by ELISA (Fig. 2). IL-10 levels increased in the soleus muscle immediately after ending the session (2 h) ($p < 0.05$), and returning to the baseline values at 4 h (Fig. 2A). There was no alteration in the IL-10 levels in the gastrocnemius muscle ($p > 0.05$) (Fig. 2B).

IL-10 deficiency increases the production of hyperalgesic cytokines induced by intense acute swimming

It was evaluated the effect of IL-10 deficiency over intense acute swimming-induced cytokine production (Fig. 3). Intense acute swimming-induced significant increase of TNFα (Fig. 3A), IL-1β (Fig. 3C), IL-4 (Fig. 3E), IL-5 (Fig. 3G), IL-13 (Fig. 3I) and TGF-β (Fig. 3K) levels in the soleus muscle at 2 h ($p < 0.05$) and control levels were observed at 4 and 6 h in WT mice ($p > 0.05$). There was no alteration of the levels of those cytokines in the gastrocnemius muscle at all time points evaluated ($p > 0.05$) in WT mice (Figs. 3B, D, F, H, J and L). In the soleus muscle, the TNFα (Fig. 3A) and IL-1β (Fig. 3C) levels were significantly increased ($p < 0.05$) in IL-$10^{-/-}$ mice from 2^{th} to 6^{th} h when compared to the sham group, been greater including in comparison with the WT control mice at the same times. There was no statistical difference ($p > 0.05$) between WT and IL-$10^{-/-}$ mice regarding the production of IL-4 (Fig. 3E), IL-5 (Fig. 3G), IL-13 (Fig. 3I) and TGF-β (Fig. 3K) in the soleus muscle. In the gastrocnemius muscle of IL-$10^{-/-}$ mice was detected only significantly increased production ($p < 0.05$) of IL-1β from 2^{th} to 6^{th} h (Fig. 3D) in comparison with the gastrocnemius WT mice at the same times, without difference ($p > 0.05$) in other cytokines evaluated (Figs. 3B, F, H, J and L).

Intense acute swimming induced-myeloperoxidase (MPO) activity was increased in the soleus, but not in the gastrocnemius muscle of IL-$10^{-/-}$ mice

Intense acute swimming induced significant increase ($p < 0.05$) of MPO activity at 24 h in WT compared to naïve and sham groups in the soleus muscle (Fig. 4A) without differences ($p > 0.05$) in the gastrocnemius muscle (Fig. 4B). The deficiency on IL-10 resulted in an increase of MPO activity in the soleus muscle compared to WT mice ($p < 0.05$) (Fig. 4A), again without difference in the gastrocnemius muscle ($p > 0.05$) (Fig. 4B).

Increase of intense acute swimming-induced oxidative stress in the soleus but not in the gastrocnemius muscle of IL-10$^{-/-}$ mice

Samples of the soleus and gastrocnemius muscles from WT and IL-10$^{-/-}$ mice were collected 4 h after the intense acute swimming session (Fig. 5). Intense acute swimming induced the reduction of GSH levels in WT mice and significantly greater reduction was observed in IL-10$^{-/-}$ mice in the soleus muscle ($p < 0.05$) (Fig. 5A). Superoxide anion production was also increased by intense acute swimming and again, this increase was greater in IL-10$^{-/-}$ mice compared to WT mice ($p < 0.05$) (Fig. 5C). There was no difference ($p > 0.05$) of GSH (Fig. 5 B) and superoxide anion (Fig. 5D) levels among groups in the gastrocnemius muscle.

Discussion

The biological roles of endogenous IL-10 have not been explored on a model of DOMS. The present study is, to our knowledge, the first evidence that IL-10 deficiency increases the muscle mechanical hyperalgesia induced by intense acute swimming session. Furthermore, this event depends on increased production of TNFα and IL-1β, leukocyte recruitment (MPO assay), and oxidative stress (demonstrated by NBT and GSH assays) in the soleus, but not gastrocnemius muscle. Therefore, IL-10 is a key anti-hyperalgesic molecule in intense acute swimming-induced DOMS.

After acute downhill running there is significant increase of IL-10 plasma levels in health young men (van de Vyver *et al*. 2012). Evidence suggests that IL-10 production depends on STAT-3-induced activation of mTOR (mammalian target of rapamycin) (Foldenauer *et al*. 2013). IL-10 is produced by various types of cells including monocytes, macrophages, mast cells and regulatory T cells (Verri *et al*. 2006; Akdis *et al*. 2011). The anti-hyperalgesic effect of IL-10 was firstly demonstrated in a model of carrageenan-induced inflammation, in which a single local injection of IL-10 abolished the mechanical hyperalgesia induced by the administration of carrageenan in the hind paw of rats (Poole *et al*. 1995). Furthermore, PGE_2-induced hyperalgesia was also inhibited by IL-10, and IL-10 abolished the production of prostaglandins by peripheral blood mononuclear cells stimulated with IL-1β (Poole *et al*. 1995). Moreover, focal micro-injections of IL-10 attenuate masseter hyperalgesia and cutaneous inflammation induced by complete Freund's adjuvant in a nociceptive orofacial model (Shimizu *et al*. 2009). Corroborating, the analgesic effect of endogenous and exogenous IL-10 was showed in neuropathic pain models (Milligan *et al*. 2005; Wang *et al*. 2012). In agreement,

altered local and systemic cytokine profile was observed in patients with several different pain disorders, with reduced expression of analgesic cytokines such as IL-10 (Üçeyler *et al*. 2006; Üçeyler *et al*. 2007a; Üçeyler *et al*. 2007b).

DOMS is associated with pain, peripheral cytokine production, leukocyte muscle infiltration and oxidative stress (MacIntyre *et al*. 1995; Graven-Nielsen and Arendt-Nielsen, 2003; Connelly *et al*. 2003; Lewis *et al*. 2012). Consistent with the physiopathological mechanisms of DOMS and with a promising therapeutic effect of targeting endogenous IL-10, it was observed that IL-10 deficiency caused an increase of intense acute swimming-induced muscle mechanical hyperalgesia, TNFα and IL-1β production, MPO activity and oxidative stress in the soleus muscle.

It has been consistently shown that IL-10 presents anti-hyperalgesic effects in various inflammatory and neuropathic diseases. There are some possible mechanisms of IL-10 to reduce hyperalgesia. IL-10 blocks the activation of the mytogen-activated protein kinases p38 and JNK, and up-regulates the suppressor of cytokine signaling-1 and -3 (SOCS-1 and 3) resulting in reduced cytokine signaling and immune response (Donnelly *et al*. 1999; Akdis *et al*. 2011; Yin *et al*. 2013), and as a consequence, reduced neuronal nociceptive sensitization and neurotransmission is expected (Poole *et al*. 1995; Vale *et al*. 2003; Verri *et al*. 2006; Shen *et al*. 2013; Sacerdote *et al*. 2013). Furthermore, IL-10 reduces the activation of the transcription factor NFκB resulting in the reduction of TNFα and IL-1β production in a model of running activity (Hoffman *et al*. 2010). Considering that TNFα and IL-1β are hyperalgesic cytokines in DOMS, inhibition of their excessive production might account for the anti-hyperalgesic effect of IL-10. In addition to IL-10 mechanisms described above, it is noteworthy to mention that TNFα is chemotactic to neutrophils, and macrophages are activated and produce TNFα, (Verri *et al*. 2010; Akdis *et al*.

2011), and TNFα induces the activation of NADPH oxidase in a MAP kinase dependent manner with the consequent production of superoxide anion (Kilpatrik *et al*. 2010). Oxidative stress is related to nociceptive behaviors since their inhibition reduces inflammatory pain (Borghi *et al*. 2013c; Navarro *et al*. 2013). Therefore, the described mechanisms of IL-10 and the effects of inhibiting the production of cytokines such as TNFα lines up well with the increase of intense acute swimming-induced muscle mechanical hyperalgesia, TNFα and IL-1β production, MPO activity and oxidative stress by IL-10 deficiency.

In previous study of neuropathic pain that used a chronic constriction injury (CCI) model of the sciatic nerve in IL-$4^{-/-}$ mice, increased IL-10 gene expression were detected in sciatic nerve and ipsilateral spinal cord of the IL-$4^{-/-}$ mice (Uçeyler et al. 2011). This hyperexpression of endogenous IL-10 may be considered as an adaptive response to the necessity of extra protection of IL-$4^{-/-}$ mice, whereas IL-4 is an analgesic and anti-inflammatory cytokine and its deletion by genetic tolls could represent greater nociceptive responses (Cunha *et al*. 1999; Vale *et al.* 2003; Uçeyler et al. 2011). This statement emphasizes the critical modulation of IL-10 in controlling pain behaviors. In the present study, it was observed that intense acute swimming induces an increase of IL-4, IL-5, IL-13 and TGF-β in the soleus, but not gastrocnemius muscle. However, IL-$10^{-/-}$ mice presented similar levels of these cytokines in comparison with WT mice, which indicates that modulation of IL-4, IL-5, IL-13 and TGF-β levels by IL-10 deficiency could not be considered in the present model. This difference in results might be related to the difference in the nociceptive model used in each study. It remains to be determined the function of IL-4, IL-5, IL-13 and TGF-β in intense acute swimming-induced muscle hyperalgesia.

Conclusion

IL-10 presents an endogenous role as an anti-hyperalgesic cytokine in intense acute swimming by regulating cytokine production, leukocyte recruitment and oxidative stress in the soleus muscle. Therefore, it is envisaged that modulating IL-10 activity might be a promising approach to control DOMS.

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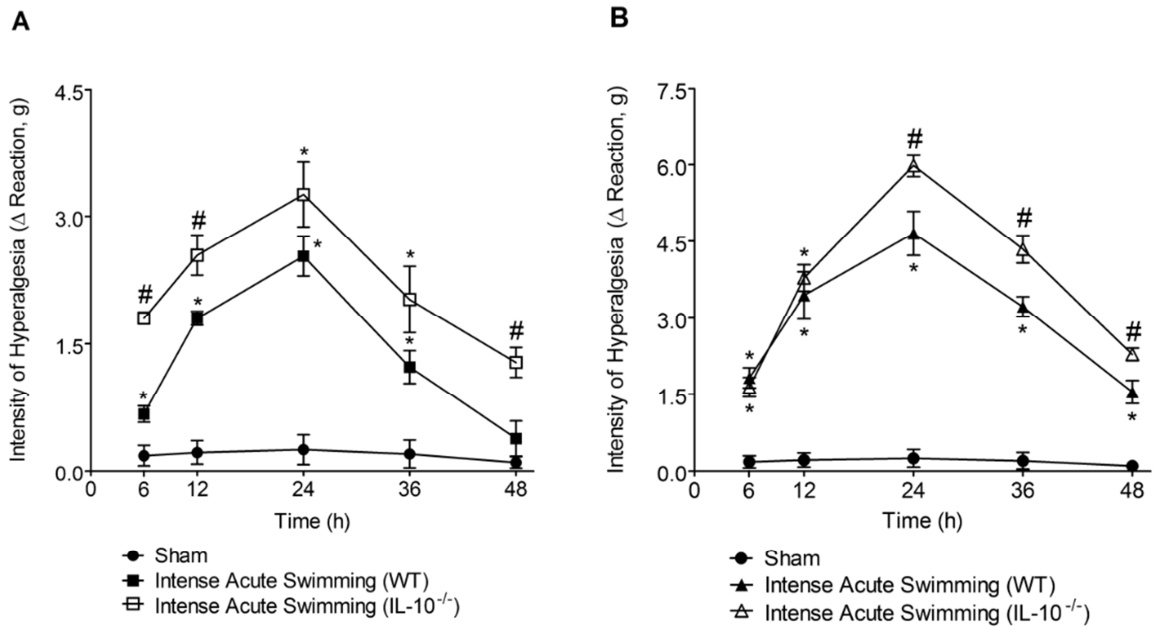

Figure 1. Increase of intense acute swimming-induced muscle mechanical hyperalgesia in IL-10$^{-/-}$ mice. WT and IL-10$^{-/-}$ mice were submitted to one session of intense acute swimming of 1 (A) or 2 (B) h. Sham group was used as negative control. Results are presented as means ± S.E.M. of 6 mice per group per experiment and are representative of two separated experiments. *P < 0.05 versus sham group; #P < 0.05 versus sham and WT groups. (One-way ANOVA followed by Tukey's t test).

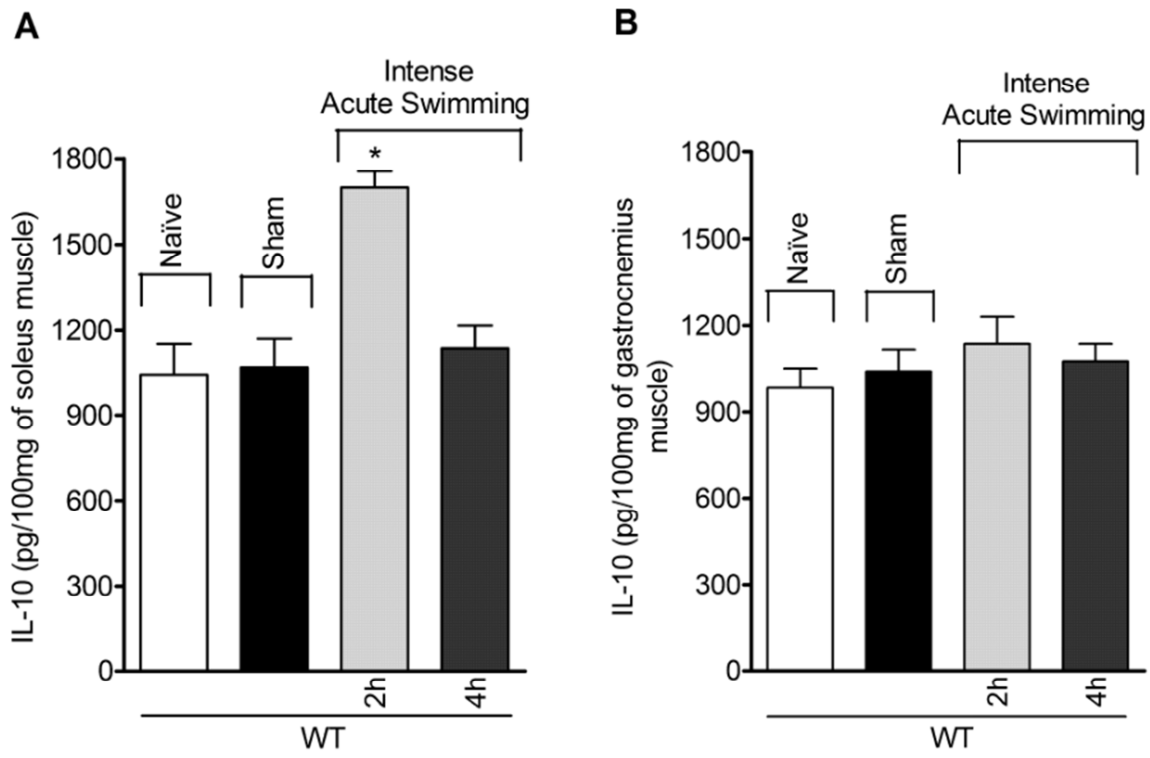

Figure 2. Intense acute swimming induces the production of IL-10 in the soleus muscle and had no effect in the gastrocnemius muscle. WT mice were submitted to one session of intense acute swimming of 2 h. Sham group was used as negative control. Samples of soleus (A) and gastrocnemius (B) muscles were collected at indicated time points. Results are presented as means ± S.E.M. of 6 mice per group per experiment and are representative of two separated experiments. *P < 0.05 versus naïve and sham groups. (One-way ANOVA followed by Tukey's t test).

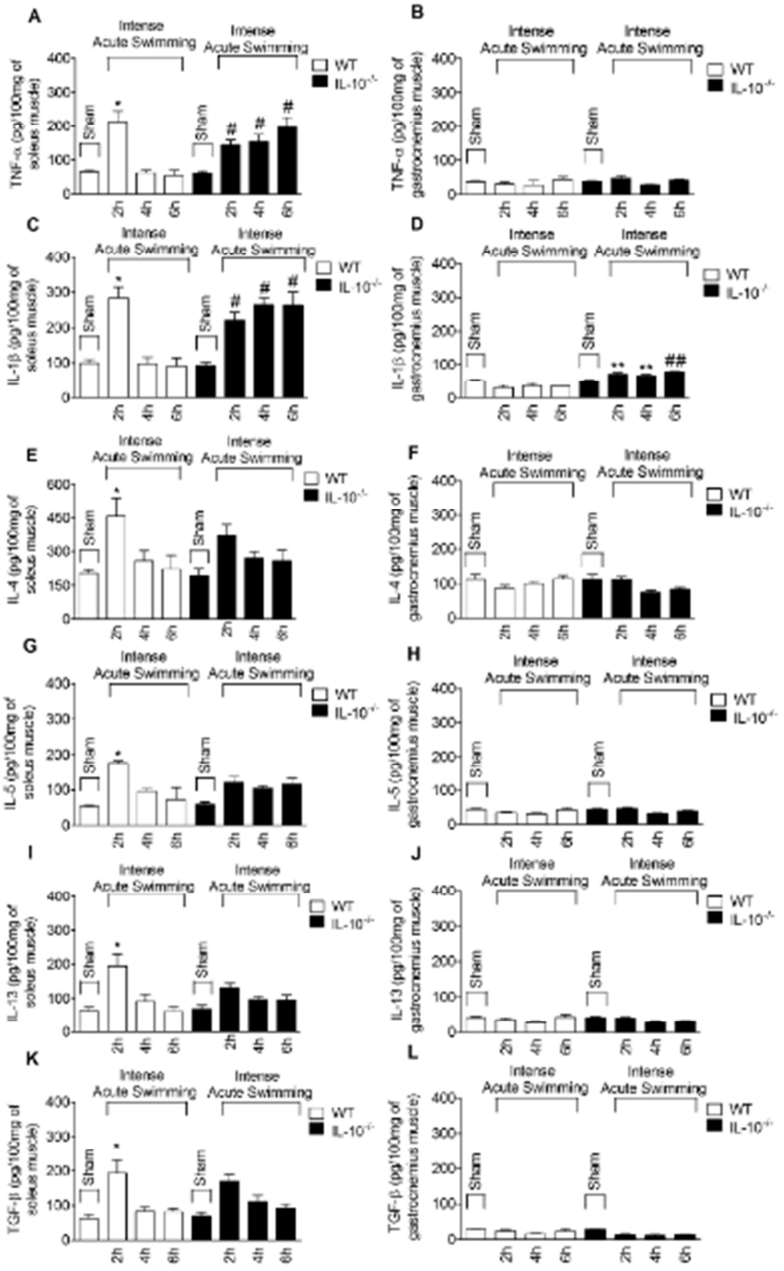

Figure 3. Effect of IL-10 deficiency in intense acute swimming-induced cytokine production. WT and IL-10$^{-/-}$ mice were submitted to one session of intense acute swimming of 2 h. Sham group was used as negative control. At indicated time points, samples of soleus muscle were collected for TNFα (A), IL-1β (C), IL-4 (E), IL-5 (G), IL-13 (I) and TGF-β (K), and samples of gastrocnemius muscle were collected for TNFα (B), IL-1β (D), IL-4 (F), IL-5 (H), IL-13 (J) and TGF-β (L) measurement by ELISA. Results are presented as means ± S.E.M. of 6 mice per group per experiment

and are representative of two separated experiments. *P < 0.05 versus sham WT group; and #P < 0.05 versus sham IL-$10^{-/-}$ group; and **P < 0.05 versus 2, 4 and 6 h WT group; and ##P < 0.05 versus 2, 4 and 6 h WT and sham IL-$10^{-/-}$ groups. (One-way ANOVA followed by Tukey's t test).

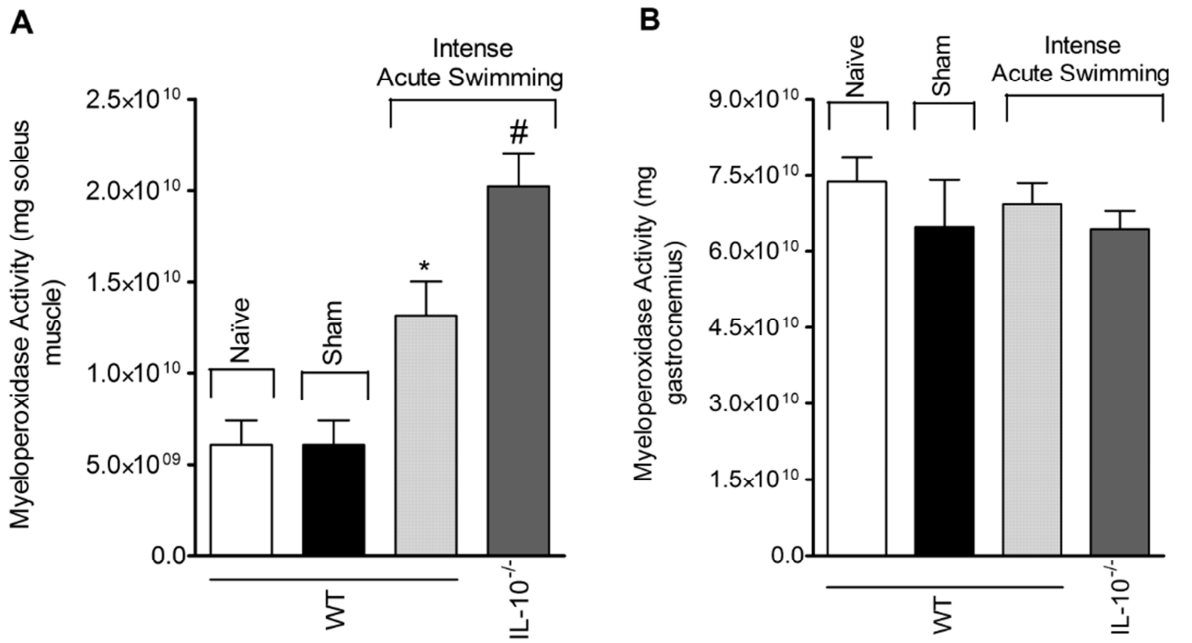

Figure 4. Increase of intense acute swimming-induced MPO activity in L-10$^{-/-}$ mice. WT and IL-10$^{-/-}$ mice were submitted to one session of intense acute swimming of 2 h. Sham group was used as negative control. Twenty four h after intense acute swimming, samples of soleus (A) and gastrocnemius (B) muscles were collected for myeloperoxidase activity assay. Results are presented as means ± S.E.M. of 6 mice per group per experiment and are representative of two separated experiments. *P < 0.05 versus naive and sham groups; and #P < 0.05 versus control WT group.

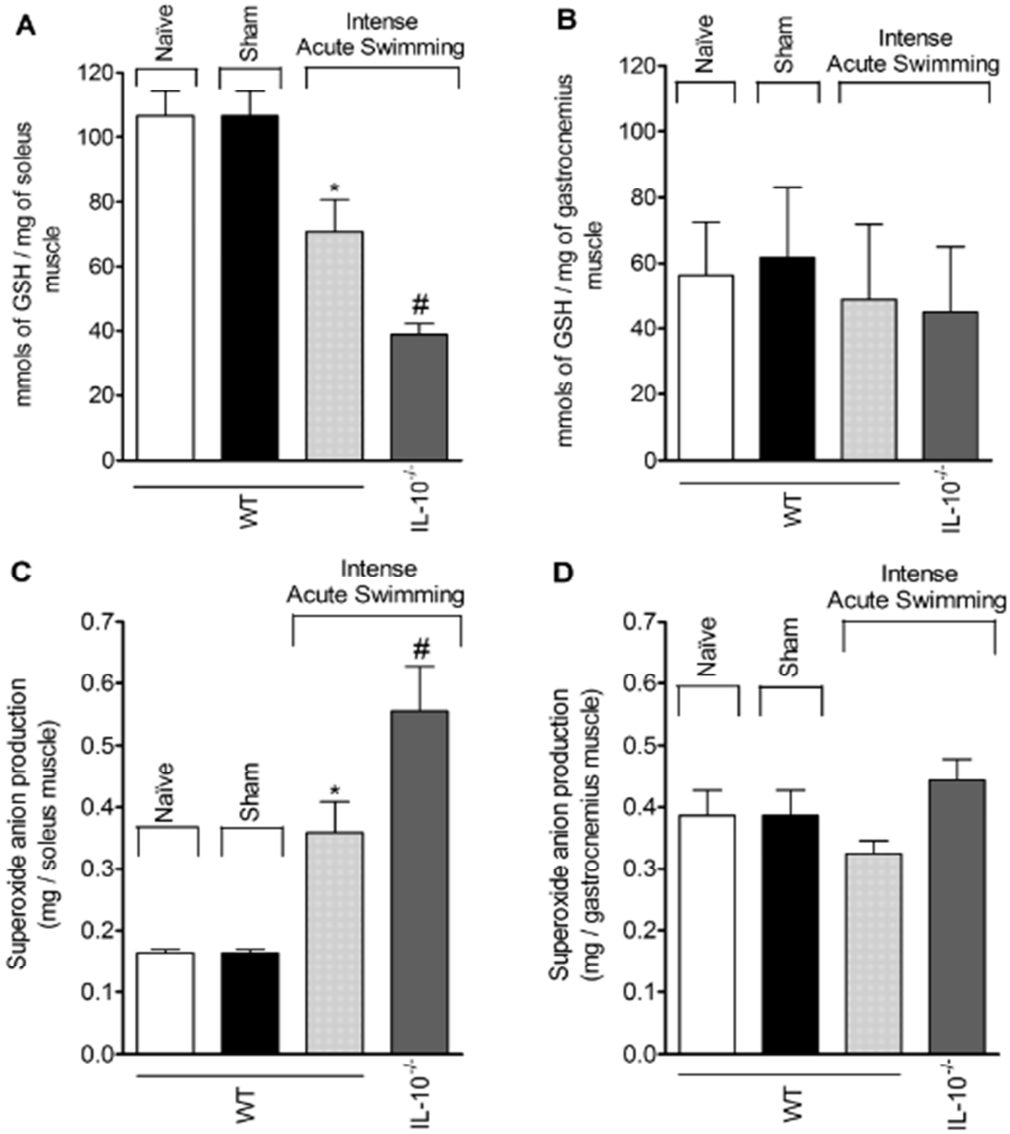

Figure 5. Increase of intense acute swimming-induced oxidative stress in L-10$^{-/-}$ mice. WT and IL-10$^{-/-}$ mice were submitted to one session of intense acute swimming of 2 h. Sham group was used as negative control. At 4 h after intense acute swimming session, samples of soleus and gastrocnemius muscles were collected for GSH (A and B) and superoxide anion (C and D) assay. Results are presented as means ± S.E.M. of 6 mice per group per experiment and are representative of two separated experiments. *P < 0.05 versus naive and sham groups; and #P < 0.05 versus control WT group. (One-way ANOVA followed by Tukey's t test).

ANEXO 4

ARTIGO SUBMETIDO 4

Este trabalho foi realizado no laboratório de fisiopatologia e farmacologia da dor, neuropatia, câncer e inflamação, resultando na formação do artigo científico: Pentoxifylline inhibits delayed onset mucle soreness by targeting cytokines, leukocyte recruitment, and oxidative stress, de autoria de Sergio M. Borghi, Felipe A. Pinho-Ribeiro, Ana C. Zarpelon, Thiago M. Cunha, Sergio H. Ferreira, Fernando Q. Cunha, Rubia Casagrande e Waldiceu A. Verri Jr.

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Pentoxifylline inhibits delayed onset muscle soreness by targeting cytokines, leukocyte recruitment, and oxidative stress in mice.

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Abstract

The growing recognition of the benefits of exercise in lifestyle and quality of life is unquestionable. On the other hand, the deleterious impact of delayed onset muscle soreness (DOMS) symptoms could represent a real negative issue in inclusion of patients in physical activity programs related to rehabilitation protocols of chronic inflammatory diseases. Although some studies evidenced effective results in prophylaxis of DOMS symptoms, consistent data about pharmacological approaches in human and animal models of DOMS reported inefficiency of nonsteriodal anti-inflammatory drugs use. We have previously demonstrated the participation of TNFα, IL-1β and IL-10 in DOMS. In this line, the effect of pentoxifylline (1.5-13.5 mg/kg) in intense exercise-induced nociceptive and biochemical alterations was addressed. In a resistance exercise protocol, untrained mice were submitted to a single uninterrupted intense acute swimming session of 120 min. Mechanical hyperalgesia, cytokines production, myeloperoxidase activity and oxidative stress were analyzed. Pentoxifylline reduced DOMS-induced mechanical hyperalgesia in a dose-dependent manner, and inhibited both pro- and anti-inflammatory cytokine (TNFα, IL-1β and IL-10) productions in the soleus muscle and spinal cord. Pentoxifylline also reduced the increase of myeloperoxidase activity and reduction of GSH in the soleus muscle. DOMS did not affect cytokine, MPO or oxidative stress in the gastrocnemius muscle. Concluding, the treatment with pentoxifylline might represent a conceivable approach to reduce DOMS symptoms.

Key Words: Pentoxifylline, cytokine, hyperalgesia, muscle pain, oxidative stress and mice.

Introduction

The main pharmacological treatment modality used to improve muscle function and pain in delayed onset muscle soreness (DOMS) is the use of nonsteroidal anti-inflammatory drugs (NSAIDs). However, the effectiveness of NSAIDs is equivocal since the majority of studies show no effect despite continuous use of this class of drugs (Connelly et al. 2003).

Recently, it has been demonstrated the role of TNFα, IL-1β and IL-10 in intense acute swimming-induced muscle hyperalgesia (Borghi et al. 2013a; Borghi et al. 2013b, Borghi et al. 2013c). Both TNFα and IL-1β act by inducing oxidative stress and increasing myeloperoxidase activity. These effects are tissue specific since occur in the soleus, but not in the gastrocnemius muscle. Furthermore, TNFα and IL-1β are produced at 2h in the soleus muscle and at 4h in the spinal cord, which shows a temporal profile of these cytokines suggesting that the spinal effects depend on peripheral initiation (Borghi et al. 2013a; Borghi et al. 2013b). On the other hand, IL-10 has an anti-hyperalgesic role in intense acute swimming since IL-10 deficient mice present an increase of muscle hyperalgesia, myeloperoxidase activity, TNFα and IL-1β production, and increased oxidative stress as determined by reduced glutathione (GSH) levels and increased superoxide anion production (Borghi et al. 2013c). Therefore, targeting hyperalgesic cytokines TNFα and IL-1β or treating with anti-hyperalgesic IL-10 seem promising approaches to reduce the pain observed in DOMS.

In this sense, the main mechanism of action of pentoxifylline, a methylxanthine derivative, is the reduction of TNFα production by inhibiting its mRNA expression by more than 50% (Strieter et al. 1988; Doherty et al. 1991; Sampaio et al. 1998).

Corroborating, the local injection of pentoxifylline reduced inflammatory pain by down-regulating TNFα production (Dorazil-Dudzik et al. 2004; Vale et al. 2004). Furthermore, pentoxifylline was also described to attenuate mechanical and thermal hyperalgesia in a neurophatic pain model by inhibiting nuclear factor Kappa B (NFκB) activation and modulation of pro- and anti-inflammatory cytokines production (Liu et al. 2007). In fact, it has been described that pentoxifylline inhibits the production of TNFα, IL-1β, IL-8, IL-6 and endothelin-1 as well as increased IL-10 production has been observed (Neuner et al. 1994; Lundblad et al. 1995; Liu et al. 2007; Verri et al. 2006; Mika et al. 2008; Wei et al. 2009).

Therefore, the aim of the present study was to evaluate the effect of the treatment with pentoxifylline in intense acute swimming-induced muscle hyperalgesia and whether its mechanism of action depends on the modulation of cytokine production (TNFα, IL-1β and IL-10), leukocyte recruitment (MPO activity) and oxidative stress (GSH levels).

Materials and methods

Animals

The experiments were performed on male Swiss mice, 20-25g from State University of Londrina, Londrina, PR, Brazil. Mice were housed in standard clear plastic cages (5-6 per cage) with free access to water and food, light/dark cycle of 12/12h, controlled temperature (22 ±1 °C), with exhaust air and humidity 50-60%. The mice were reared in the central vivarium of State University of Londrina, and kept in the vivarium of the Department of Pathology of the same University for at least two days before experiments. Mice (n = 6 animals per group) were used only once and were acclimatized to the testing room at least 1 hour before the experiments, which was conducted during the light cycle. Animals' care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Institutional Ethics Committee for Animal Research of the Universidade Estadual de Londrina, process number 2066.2011. All efforts were made to minimize the number of animals used and their suffering.

Drugs

Drugs were obtained from the following sources: pentoxifylline (Trental®) was purchased from Hoecht (São Paulo, Brazil) and saline solution 0,9% from Gaspar Viana S/A (Fortaleza, CE, Brazil). Pentoxifylline were dissolved in DMSO (20%) and isotonic saline solution (NaCl 0,9%) (80%) immediately before use.

Experimental Protocols

Mice were treated with pentoxifylline 1.5-13.5 mg/kg, i.p. 30 min before and 12 h after beginning of exercise section of 120 min. Naïve and sham control groups were exposed for 30 s to water. After the beginning of exercise section the following parameters at indicated time points were evaluated: muscle mechanical hyperalgesia between 6-48 h, myeloperoxidase (MPO) activity at 24 h and reduced glutathione (GSH) levels at 4 h. Furthermore, TNF-α, IL-1β and IL-10 levels were also determined in samples of soleus and gastrocnemius muscle and spinal cord (L4-L6), at 2 and 4 h respectively. Time points were based on previous studies (Borghi et al. 2013a; Borghi et al. 2013b; Borghi et al. 2013c).

Intense Acute Swimming Protocol

Mice were placed in a glass box (45×28×25 cm, divided in six compartments) with approximately 20 liters of water at 31° ± 1°C as described previously (Borghi et al., 2013a). Briefly, each mouse was placed in one compartment and swam all the same time. After the intense acute swimming session or sham conditions, animals were dried and placed in cages together with their respective group. Mice were randomized in sham and exercised groups. Sham animals were allowed to swim for just 30 seconds, and were immediately removed from the water after this period and dried. Mice in the swimming group were exposed to water for 1 session of 120 min. The mechanical hyperalgesia was evaluated between 6-48 h after the swimming session. The results were expressed as the mean ± S.E.M. of swimming time.

Evaluation of Mechanical Hyperalgesia

Muscle mechanical hyperalgesia was tested in mice as previously reported (Cunha et al. 2004). Briefly, in a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15–30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic von Frey anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 contact area polypropylene tip. The investigator was trained to apply the probes perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the withdrawal response, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were evaluated at baseline and between 6-48h after exercise. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements (indicated time points) after stimulus from the baseline measurements. The basal mechanical withdrawal threshold was 8.6 ± 0.3 g (mean ± S.E.M. of 5 groups, 6 mice per group) before intense acute swimming session. There was no difference of basal mechanical withdrawal thresholds between groups in the same experiment.

ELISA Tests

Mice were euthanized 2 and 4 h after swimming session and samples of the gastrocnemius and soleus muscles and spinal cord (L4-L6) were collected. The

samples were homogenized in 300 µl (spinal cord pool of three mice) or 500 µl (skeletal muscles) of the appropriate buffer containing protease inhibitors. TNF-α, IL-1β and IL-10 level was determined as described previously (Verri et al. 2008) by enzyme-linked immunosorbent assay (ELISA). The results were expressed as picograms (pg) of cytokine per 100 mg of tissue. Naïve and sham groups were used as control.

Leukocyte Migration to the Skeletal Muscle Tissue

The intense acute swimming-induced leukocyte recruitment to the soleus and gastrocnemius muscles of mice was evaluated using the myeloperoxidase (MPO) kinetic–colorimetric assay (Casagrande et al. 2006). Samples of skeletal muscles were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at −86 °C until use. Samples were homogenized using a Polytron (PT3100), centrifuged at 16.100 *g* in 4 °C for 2 min and the resulting supernatant assayed spectrophotometrically for MPO activity determination at 450 nm (Spectra max), with 3 readings in 1 min. The MPO activity of samples was compared to a standard curve of neutrophils. Briefly, 10 µl of sample were mixed with 200 µl of 50 mM phosphate buffer pH 6.0, containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results were presented as MPO activity (number of total neutrophils x 10^{10}/ mg of muscle).

Reduced Glutathione (GSH) Assay

The levels of skeletal muscle GSH were determined using a spectrophotometric method (Borghi et al., 2013d). Samples of gastrocnemius (100 mg) or soleus (40 mg) (1:10 dilution) were homogenized (IKA T10) in 4 and 1.6 ml of EDTA 0,02M, respectively. Homogenates (2.5 ml) were treated with 2 ml H_20 Milli Q plus 0.5 ml of trichloroacetic acid 50%. After 15 minutes, the homogenates were centrifuged at 1500 *g* for 15 min, and 1 ml from supernatant was added to 2 ml of a solution containing Tris 0.4M (pH 8.9) plus 50 ml of DTNB. After 5 min, the measurements were performed in 412 nm against white control (UV - Visible spectrophotometer [UV-1650 PC] – SHIMADZU). The GSH levels were corrected according to the total protein concentration. The results were presented as mmols of GSH per gram of protein in skeletal muscles.

Statistical Analysis

Results are presented as means ± S.E.M. of measurements made on 6 mice in each group. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The analyzed factors were treatments, time and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's t-test was performed for each time. On the other hand, when the hyperalgesic response were measured once after the administration or enforcement of the stimuli, the difference between responses were evaluated by one-way ANOVA followed by Tukey's t-test.

Results

Pentoxifylline inhibited muscle mechanical hyperalgesia induced by intense acute swimming

Figure 1 shows that sham group (30 sec exposure to water) did not present muscle mechanical hyperalgesia. On the other hand, positive control swimming group submitted to 120 min of intense acute swimming presented an increase in muscle mechanical hyperalgesia from 6^{th} to 48^{th} h after exercise section. Significant differences were detected comparing sham and positive control groups. The dose of 1.5 mg/kg of pentoxifylline (30 min before plus reinforcements 12 h after the session, i.p.) did not affect the intense acute swimming-induced muscle mechanical hyperalgesia. On the other hand, the doses of 4.5 and 13.5 mg/kg of pentoxifylline abolished intense acute swimming-induced muscle mechanical hyperalgesia from 12-48h and significant differences were achieved when compared to the lower dose of pentoxifylline tested (Fig. 1). Considering that the dose of 4.5 mg/kg of pentoxifylline achieved maximal response, being practically equivalent to the higher dose of 13.5 mg/kg, the dose of 4.5 mg/kg of pentoxifylline was selected for next experiments.

Pentoxifylline reduced intense acute swimming-induced TNF-α, IL-1β and IL-10 production in the soleus muscle, but not in the gastrocnemius muscle

Mice were pre-treated with pentoxifylline (4.5 mg/kg, 30 min before, i.p.) and underwent intense acute swimming for 120 minutes or were exposed to sham

conditions (30 sec exposure to water) and the concentrations of TNFα, IL-1β and IL-10 were measured immediately after (2 hours) after the swimming session in the soleus and gastrocnemius muscles (Fig. 2, a-f). This time point was selected in previous studies (Borghi et al. 2013a; Borghi et al. 2013b; Borghi et al. 2013c). The levels of TNFα (Fig. 2a), IL-1β (Fig. 2c) and IL-10 (Fig. 2e) increased significantly in positive swimming control animals when compared to naive and sham groups in the soleus muscle. Pentoxifylline pre-treatment was efficient in down-regulating significantly the levels of TNFα (Fig. 2a), IL-1β (Fig. 2c) and IL-10 (Fig. 2e) in the soleus muscle. In the gastrocnemius muscle no difference was observed between the experimental groups (Figs. 2b, d and f).

Pentoxifylline reduced intense acute swimming-induced TNF-α, IL-1β and IL-10 concentrations increases in spinal cord

Mice were submitted to the same protocol as in Fig. 2 and samples of the spinal cord samples (L4-L6) were collected after 4 h for determination of TNFα, IL-1β and IL-10 levels (Fig. 3). There was significant increase of TNFα, IL-1β and IL-10 production in the positive swimming group compared to the naïve and sham groups (Figs. 3, a-c). In turn, pentoxifylline treatment inhibited intense acute swimming-induced production of TNFα, IL-1β and IL-10 (Figs. 3, a-c, respectively).

Intense acute swimming-induced increase in myeloperoxidase (MPO) activity was reduced by pentoxifylline treatment in the soleus, but not in the gastrocnemius muscle

Mice were submitted to the same protocol as in Fig. 2 and samples of soleus and gastrocnemius muscles were collected for MPO activity assay at 24 h (Fig. 4). Intense acute swimming induced a significant increase in MPO activity in positive control swimming group in the soleus muscle compared to naïve and sham groups, and the treatment with pentoxifylline (as in Fig. 2) inhibited the MPO activity increase in the soleus muscle (Fig. 4a). None of the groups showed differences in the MPO activity in the gastrocnemius muscle (Fig. 4b).

Intense acute swimming-induced depletion of endogenous reduced glutathione (GSH) levels was prevented by pentoxifylline pre-treatment in the soleus, but not in gastrocnemius muscle

Mice were submitted to the same protocol as in Fig. 2 and samples of soleus and gastrocnemius muscles were collected for GSH assay (Fig. 5). Intense acute swimming induced a significant decrease in endogenous GSH levels when compared to the naïve and sham groups in the soleus muscle (Fig. 5a). In turn, pentoxifylline treatment prevented the depletion of GSH (Fig. 5a). In the gastrocnemius muscle, as in the other biochemical measurements, the levels of endogenous GSH did not differ between groups (Fig. 5b).

Discussion

In the present study it was addressed the effect and mechanisms of action of pentoxifylline in intense acute swimming-induced delayed onset muscle soreness (DOMS) in mice. It was observed that pentoxifylline inhibited intense acute swimming-induced muscle mechanical hyperalgesia, cytokine production, myeloperoxidase (MPO) activity and oxidative stress.

Pentoxifylline inhibits inflammatory pain in experimental models suggesting its potential as an analgesic. For instance, pentoxifylline inhibits acetic acid- and zymozan-induced writings response, mechanical hyperalgesia induced by TNFα and bradykinin and zymozan-induced arthritis (Vale et al. 2004). In a model of complex regional pain syndrome (CRPS) induced by tibia fracture, pentoxifylline reduces nociceptive sensitization by a mechanism related to reducing mRNA expression / protein levels of the cytokines TNFα, IL-1β and IL-6 in mice (Wei et al. 2009). In humans, pentoxifylline reduces the intermittent claudication and pain resulting in an increase of maximal walking distance concomitantly with the reduction of C-reactive protein (CRP) (de Albuquerque et al. 2008). Pentoxifylline also reduces disease development by modulating cytokine production in other models or in clinical settings (Schwarz et al. 1993; Huizinga et al. 1996; Sampaio et al. 1998; Liu et al. 2007; Melo et al. 2008; Fernandes et al. 2008; Le Campion et al. 2008). Therefore, inhibition of cytokine production is a consistent mechanisms of action of pentoxifylline. In this line, pentoxifylline does not affect the cytokine-independent hyperalgesia induced by prostaglandin E2 (PGE_2) or prostacyclin analog iloprost (Vale et al. 2004; Verri et al. 2006).

TNFα and IL-1β are well described hyperalgesic cytokines in inflammatory pain (Cunha et al. 1992; Verri et al. 2006), neuropathic pain (Ellis and Bennett, 2013) and muscle pain (Schäfers et al. 2003; Loram et al. 2007; Shah et al. 2008; Meotti et al. 2012). In the present model of DOMS, targeting TNFα using TNFR1 deficient mice and etanercept or IL-1 with IL-1 receptor antagonist (IL-1ra) reduced intense acute swimming-induced hyperalgesia, MPO activity and oxidative stress in the soleus muscle (Borghi et al. 2013a; Borghi et al. 2013b). On the other hand, IL-10 is an anti-hyperalgesic cytokine in inflammatory pain (Poole et al. 1995; Verri et al. 2006; Borghi et al. 2013d), neuropathic pain (Milligan et al. 2012; Wang et al. 2012; Shen et al. 2013) and muscle pain (Shimizu et al. 2009; Borghi et al. 2013d). IL-10 has an endogenous role in painful conditions since it is co-released with hyperalgesic cytokines and limits the pain development. In fact, the actual pain results from the balance between hyperalgesic and anti-hyperalgesic cytokines (Poole et al. 1995; Oberholzer et al. 2002; Vale et al., 2003; Verri et al. 2006). In the intense acute swimming-induced muscle hyperalgesia, IL-10 deficient mice present increase hyperalgesia, MPO activity, TNFα and IL-1β production and oxidative stress in the soleus muscle as well as increase TNFα and IL-1β production in the spinal cord, which further corroborates the notion that IL-10 is an anti-hyperalgesic cytokine. Pentoxifylline inhibited intense acute swimming-induced production of TNFα, IL-1β and IL-10 in the soleus muscle and spinal cord, and as a result there is reduction of hyperalgesia. In this condition, it is likely that the inhibition of TNFα and IL-1β production by pentoxifylline also prevented the endogenous release of IL-10, which would counteract TNFα and IL-1β functions.

TNFα and IL-1β are also chemoattractant molecules for neutrophils and activate macrophages (Ming et al. 1987; Atkinson et al. 1988; Faccioli et al. 1990;

Verri et al. 2006; Verri et al. 2010; Borghi et al. 2013a; Borghi et al. 2013b). MPO activity is an indirect marker of neutrophil and macrophages counts (Bradley et al. 1982; Casagrande et al. 2006; Henrique de Araujo et al. 2013), indicating that the reduction of MPO activity by pentoxifylline treatment reduced the number of inflammatory cells in the soleus muscle.

There is an interaction between cytokines and oxidative stress considering that, for instance, cytokines induce NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase)-dependent superoxide anion production (Meier et al. 1989; Jiménez-Altayó et al. 2006; Kilpatrick et al. 2010). In turn, superoxide anion activates NFκB to induce cytokine production (Wang et al., 2004; Verri et al. 2012). Pentoxifylline decreases malondialdehyde formation and increase GSH levels in an acute radiation enteritis rat model (Hepgül et al. 2010) as well as inhibits NFκB activation (Liu et al. 2007; Hernandez-Florez et al. 2011; Mahmoud et al. 2012). Lining up well with this rationale, pentoxifylline inhibited intense acute swimming-induced oxidative stress and cytokine production. Furthermore, the antioxidant action of pentoxifylline might account for its anti-hyperalgesic effect since antioxidant molecules reduce nociceptive behaviors (Wang et al. 2004; Valerio et al. 2009; Navarro et al. 2013; Borghi et al. 2013).

Conclusion

Concluding, the efficient analgesic and anti-inflammatory action of pentoxifylline in exercise-induced muscle pain and damage was demonstrated, making it possible its use as a novel promising therapy to reduce the unpleasant symptoms of DOMS. We suggest that pentoxifylline may be used in a prophylactic

manner in the case of sedentary patients vulnerable to DOMS, about to enter in rehabilitation protocols that involves exercise, because normally, people tend to medicate just after the onset of the DOMS symptoms (Cheung et al. 2003), thus compromising the efficacy of therapy. Another positive point that makes the use of pentoxifylline interesting is the low cost of this therapy, considering that other anti-cytokines therapy, like etanercept, infliximab or anakinra besides presenting elevated costs, are associate to the need of better pharmacokinetics, due to their daily and / or weekly use, apart from the possibility of occurring antibody induction against the immunobiological that may compromise their effectiveness.

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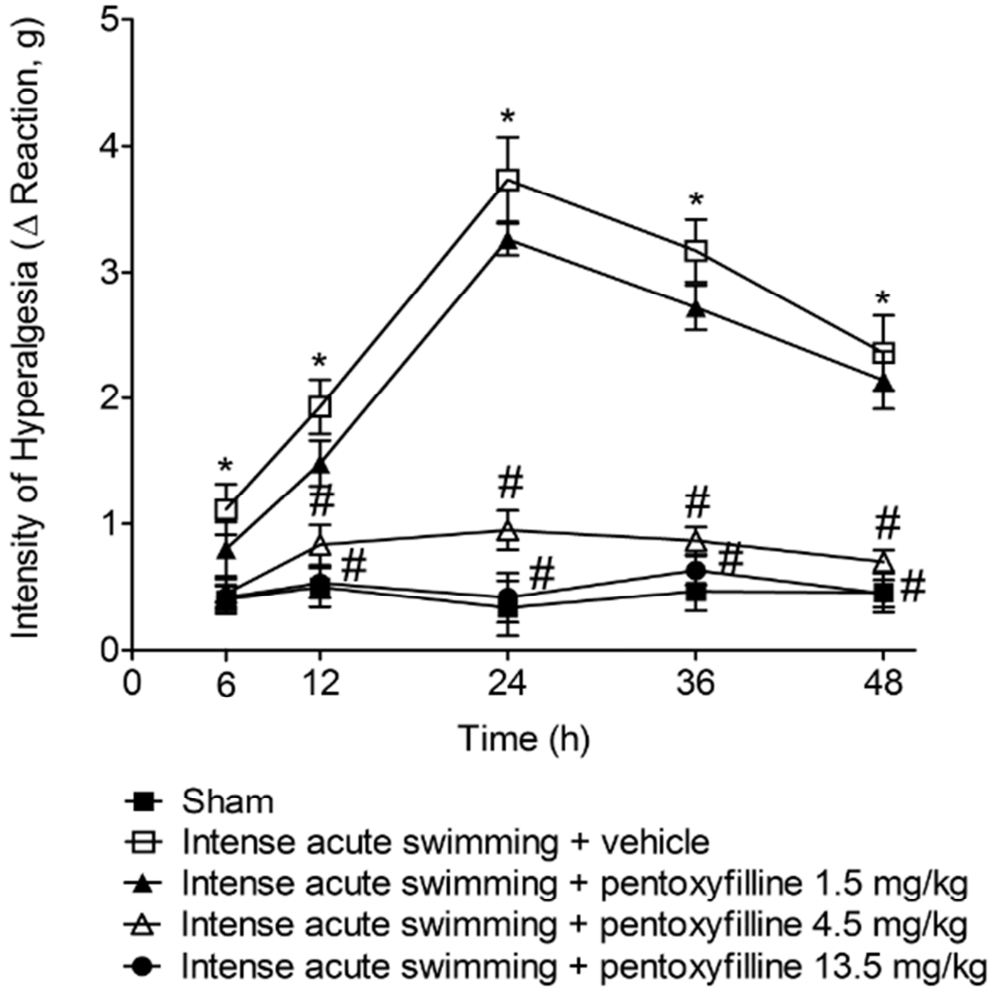

Figure 1. Pentoxifylline reduced intense acute swimming-induced muscle mechanical hyperalgesia in mice. Mice underwent intense acute swimming for 120 min or were exposed to sham conditions (30 sec exposure to water), and were treated with pentoxifylline (1.5- 13.5 mg/kg, i.p., diluted in saline) or vehicle (saline) 30 min before and 12 h after the intense acute swimming session. The intensity of muscle mechanical hyperalgesia was evaluated 6-48 hours after intense acute swimming session. Results are presented as intensity of hyperalgesia (Δ reaction, in grams). *n* = 6 mice per group per experiment, representative of two separated experiments. *P<0.05 compared with sham group, #P<0.05 compared with control non-treated and 1.5 mg/kg dose of pentoxifylline groups (One-way ANOVA followed by Tukey's t test).

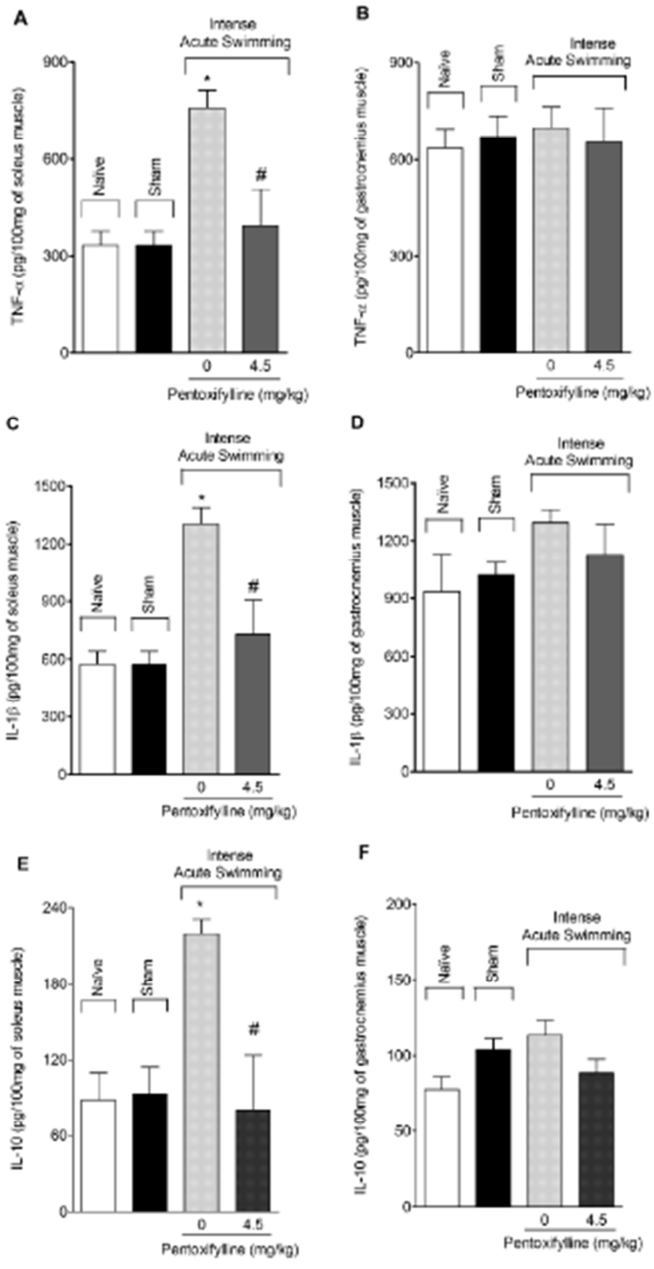

Figure 2. Pentoxifylline inhibited intense acute swimming-induced TNF-α, IL-1β and IL-10 production in the soleus muscle, but not in the gastrocnemius muscle. Mice were treated with pentoxifylline (4.5 mg/kg, i.p., diluted in saline) 30 minutes before intense acute swimming session. The levels of TNF-α, IL-1β and IL-10 in soleus (Panels A, C and E) and gastrocnemius (panels B, D and F) muscles were determined immediately after the end of the swimming section (2 h) by ELISA. Results are presented as picograms per 100 mg of the soleus and gastrocnemius muscles. *n* = 6 mice per group per experiment, representative of two separated

experiments. *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).

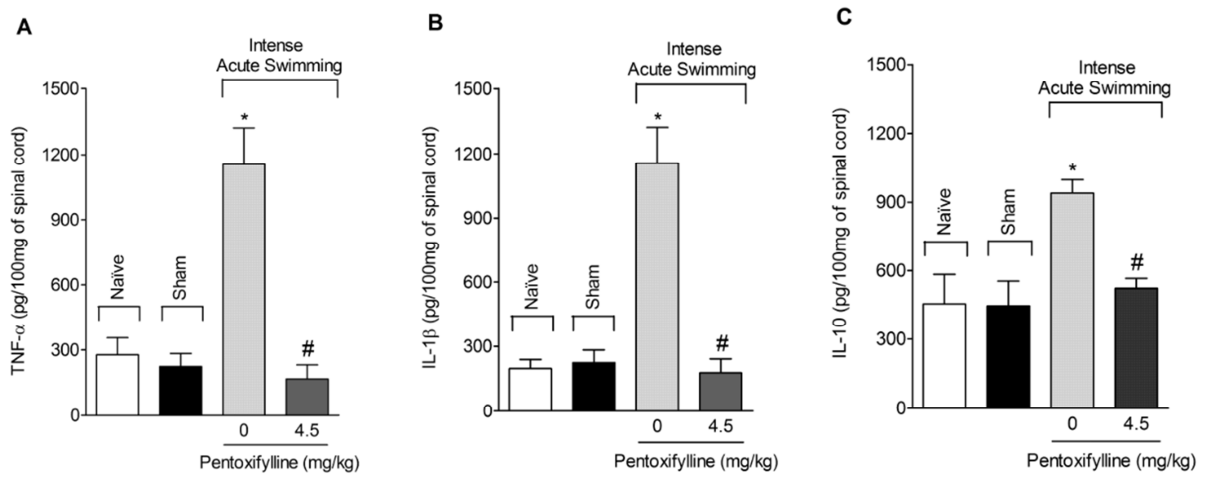

Figure 3. Pentoxifylline inhibited intense acute swimming-induced TNF-α, IL-1β and IL-10 production in the spinal cord. Mice were treated with pentoxifylline (4.5 mg/kg, i.p., diluted in saline) 30 minutes before intense acute swimming session. The samples of spinal cord (L_4-L_6) were collected 4 h after the beginning of exercise session. TNF-α (Panel A), IL-1β (Panel B) and IL-10 (Panel C) concentrations (picograms/100 mg of spinal cord) were quantified by ELISA. *n* = 6 mice per group per experiment, representative of two separated experiments. *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).

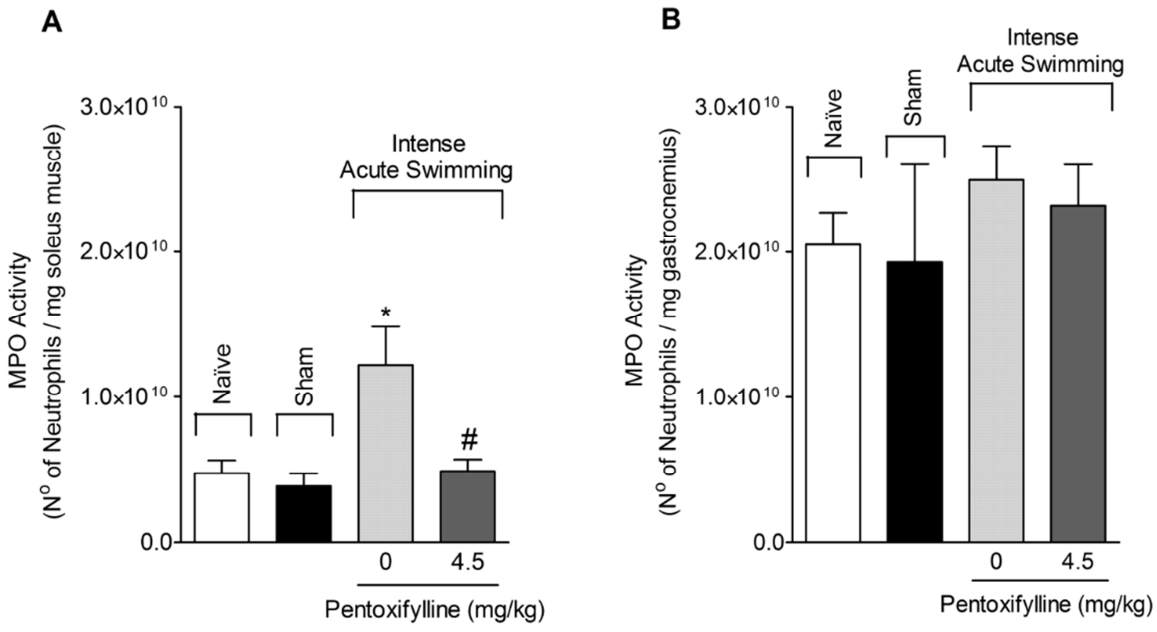

Figure 4. Pentoxifylline inhibited intense acute swimming-induced increase of myeloperoxidase (MPO) activity in the soleus muscle, but not in the gastrocnemius muscle. Mice were treated with pentoxifylline (4.5 mg/kg, i.p., diluted in saline) 30 minutes before and 12 h after intense acute swimming session. The samples of the soleus (Panel A) and gastrocnemius (Panel B) muscles were collected 24 h after the intense acute swimming session and the MPO activity was determinate by an enzymatic assay. Results are presented as myeloperoxidase activity per milligram of the soleus and gastrocnemius muscles. *n* = 6 mice per group per experiment, representative of two separated experiments. *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).

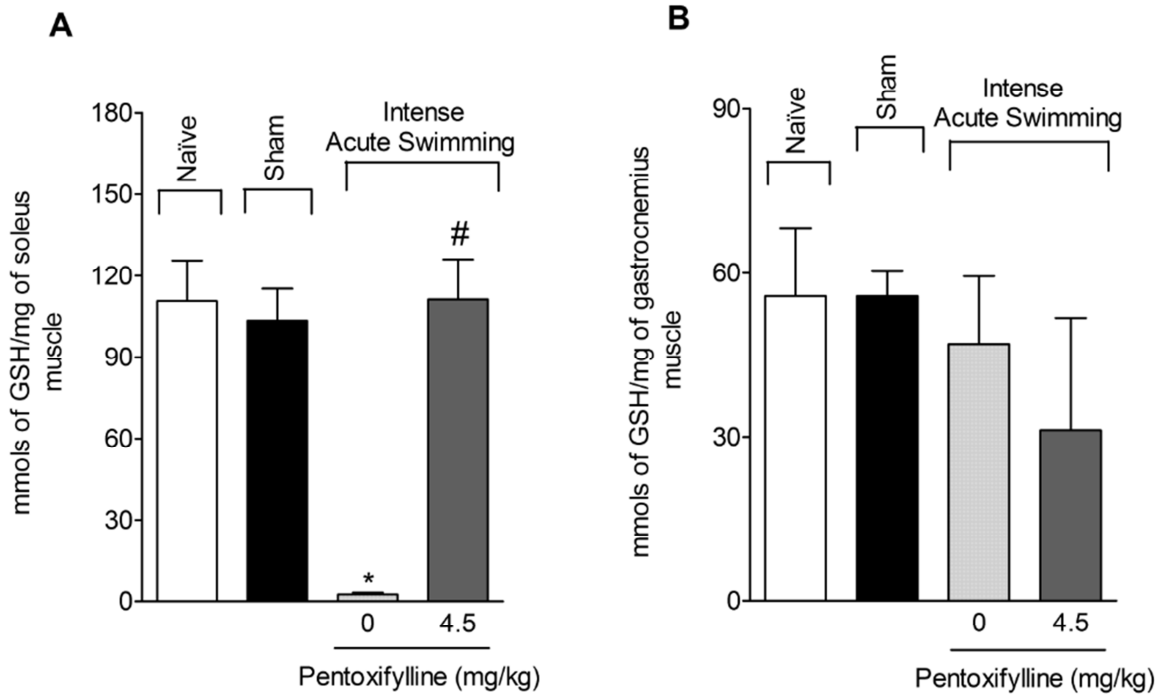

Figure 5. Pentoxifylline inhibited intense acute swimming-induced reduced glutathione (GSH) depletion in the soleus, but not gastrocnemius muscles. Mice were treated with pentoxifylline (4.5 mg/kg, i.p., diluted in saline) 30 minutes before intense acute swimming session. Samples of the soleus (Panel A) and gastrocnemius (Panel B) muscles were collected 4 h after the beginning of the intense acute swimming session and endogenous GSH levels were evaluated by kinetic-colorimeric assay. Results are presented as mmols per milligram of the soleus and gastrocnemius muscles. *n* = 6 mice per group per experiment, representative of two separated experiments. *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).

ANEXO 5

ARTIGO SUBMETIDO 5

Este trabalho foi realizado no laboratório de fisiopatologia e farmacologia da dor, neuropatia, câncer e inflamação, resultando na formação do artigo científico: Thalidomide inhibits intense acute swimming-induced delayed onset muscle soreness in mice, de autoria de Sergio M. Borghi, Felipe A. Pinho-Ribeiro, Ana C. Zarpelon, Thiago M. Cunha, Sergio H. Ferreira, Fernando Q. Cunha, Rubia Casagrande e Waldiceu A. Verri Jr.

As formatações do artigo seguem as normas da revista *European Journal of Pharmacology*.

Thalidomide Inhibits Intense Acute Swimming-induced Delayed Onset Muscle Soreness in Mice

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Abstract

Muscle pain occurs after intense exercise in untrained people but also represents a health problem that reduces life quality of patients presenting metabolic myopathies. Recently, our group developed an animal model of delayed-onset muscle soreness (DOMS) induced by intense acute-swimming in order to investigate the mechanisms of exercise-induced muscle soreness and compounds with therapeutic potential. The antihyperalgesic, anti-inflammatory and antioxidant effects of thalidomide were investigated in intense acute swimming-induced delayed-onset muscle soreness (DOMS) model in mice. The treatment with thalidomide (5-45 mg/kg, i.p.) inhibited the increased nociceptive response at the peak of hyperalgesia (24 hours after the exercise, characteristc of DOMS) caused by the exercise session in a dose- and time-dependent manner. Additionally, thalidomide treatment significantly reduced the increased levels of the cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-10 induced by intense acute swimming in both soleus muscle and spinal cord, and reduced inflammatory parameters such as muscle edema and leukocyte recruitment in the soleus muscle of exercised animals, evaluated by distal hindlimb circumference / muscle weight and MPO (myeloperoxidase) activity / histological analysis, respectively. Moreover, the peripheral oxidative stress observed by the reduced glutathione (GSH) depletion in soleus muscle after intense acute swimming was reversed by thalidomide pre-treatment. No change in all parameters was observed in the gastrocnemius muscle. In conclusion, thalidomide may represent a novel pharmacological approach to reduce DOMS.

Key Words: Thalidomide, cytokine, hyperalgesia, muscle pain, oxidative stress and mice.

1. Introduction

The intense acute exercise can produce long-lasting muscle pain (myalgia) in untrained people and reaches its peak 24 to 48 h after exercise. The inflammatory response to tissue injury is the principal cause of movement-induced muscle pain experienced in this situation. In fact, in untrained patients with chronic diseases, skeletal muscle tissue is usually susceptible to exercise-induced muscle damage and pain, with this condition being frequently treated with anti-inflammatory drugs such as corticosteroids (Shelton, 2004). In a previous study, our group has standardized a model of exercise induced-DOMS (delayed onset muscle soreness), in which untrained mice are exposed to one session of intense swimming during 120 min (Borghi et al., 2013a). In this model, the strenuous exercise is able to induce early increases in the production of TNF-α and IL-1β in soleus muscle and subsequently in the spinal cord, but not in the gastrocnemius muscle. We also showed that the session of intense exercise lead to increased myeloperoxidase (MPO) activity and oxidative stress in these two foci.

Furthermore, IL-10 presents an analgesic effect in DOMS since its genetic deletion resulted in increased intense acute swimming-induced hyperalgesia, TNFα and IL-1β production and oxidative stress (Borghi et al., 2013c). Therefore, the endogenous cytokines TNF-α and IL-1β have a key role in the mechanisms that lead to muscle pain and consequently to mechanical hyperalgesia as well as IL-10 has an important role in limiting DOMS (Borghi et al., 2013a; Borghi et al., 2013b; Borghi et al., 2013c). In fact, cytokines are biological targets or treatments for a variety of diseases considering their broad role in pathophysiology of diseases. In this sense, modulation of cytokine production is a promising therapeutic approach. Nevertheless,

biological targeting cytokines are expensive and overtime induce antibodies shortening the possible time of treatment with biological therapies. Therefore, the modulations of cytokine production using drugs that interfere with cytokine production or action are conceivable therapeutic approaches.

Thalidomide or α-N-phthalimidoglutarimide, is a synthetic glutamic acid derivative which is capable of inhibiting TNF-α production by enhancing the degradation of its messenger RNA (Moreira et al., 1993; Sampaio et al., 1991). The immunomodulatory and anti-inflammatory effects of thalidomide suggests its possible use in treatment of inflammatory pain due to inhibition of cytokine production. Thalidomide may be useful for the treatment of a wide range of painful conditions including bone cancer pain, inflammatory pain and diabetes-induced neuropathic pain (Cata et al., 2004, 2008; Chauhan et al., 2012; Gu et al., 2010; Ribeiro et al., 2000). Moreover, there is an increase of MPO activity in DOMS and thalidomide reduces the activity of MPO in a model of intestinal mucositis (Melo et al., 2008).

Taking into account the evidence described above, in the present study it was evaluated the effects of thalidomide in muscle mechanical hyperalgesia, cytokine production, edema, leukocyte recruitment and oxidative stress induced by intense acute swimming in mice.

2. Materials and methods

2.1. Animals

Male Swiss mice (25−30 g), from the Universidade Estadual de Londrina, Londrina, Parana, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, with a light/dark cycle of 12:12 h, at 21 °C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (protocol number 2066.2011). Every effort was made to minimize the number of animals used and their suffering.

2.2. Drugs

Drugs were obtained from the following sources: thalidomide was purchased from FUNED (Fundação Ezequiel Dias, Belo Horizonte, Brazil), saline solution 0.9% from Gaspar Viana S/A (Fortaleza, Ceará, Brazil). Thalidomide was dissolved in dimethyl sulfoxide (DMSO, 20% v/v in saline) immediately before use.

2.3. Experimental Protocols

Mice received intraperitoneal treatment with thalidomide (i.p, 5-45 mg/kg) or vehicle (DMSO 20% v/v in saline) 30 min before and 12 h after beginning of the tests. After the swimming session, the following parameters were evaluated at the indicated

time points: muscle mechanical hyperalgesia (between 6-48 h) and edema (between 2-48 h), MPO activity and histological analysis of muscle injury (at 24 h), GSH (at 4 h) and cytokines levels (TNF-α, IL-1β and IL-10 at 2 and 4 h for muscle [soleus and gastrocnemius] and spinal cord [L4-L6], respectively).

2.4. Intense Acute Swimming Protocol

The test apparatus consisted of a glass box (45×28×25 cm) containing 20 liters of water at 31 ± 1°C and divided in six individual compartments. In order to reduce the surface tension of water and the “floating” behavior, a drop of liquid soap was added to water. Mice from sham or exercised groups were randomly and individually placed in the compartments at the same time and exposed to 1 session of 120 min or 30 seconds respectively, as described previously (Borghi et al., 2013a; Borghi et al., 2013b). After the intense acute exercise session, all animals were carefully dried and placed in cages together with their respective group.

2.5. Evaluation of Muscle Mechanical Hyperalgesia by the Eletronic von Frey Test

The movement-induced muscle hyperalgesia was evaluated using a previous method (Guerrero et al., 2006) with modification. In a quiet room, mice were placed in acrylic cages (12×10×17 cm) with a wire grid floor 15–30 min before testing for environmental adaptation. Stimulations were performed only when animals were quiet, did not display exploratory movements or defecation, and were not resting on their paws. In these experiments, an electronic pressure-meter was used. It consists of a hand-held force transducer fitted with a polypropylene tip (IITC Inc., Life Science

Instruments, Woodland Hills, CA, USA). For this model, a large tip (4.15 mm^2) was adapted to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce articular movement on ankle joint. This muscle movement is followed by paw withdrawal and subsequent flinching movements, which characterize muscle hyperalgesia induced by exercise (Borghi et al., 2013a; Borghi et al., 2013b). After the paw withdrawal, the intensity of the pressure was recorded automatically. The animals were evaluated at baseline, and during the two subsequent days to acute swim session (6°, 12°, 24°, 36° and 48° hours). The test was repeated until three subsequently consistent measurements (i.e. the variation among these measurements was less than 1 g) were obtained, and the results expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements of indicated time points after swim session. The basal mechanical withdrawal threshold was 8.8±0.1 g (mean±SEM of 5 groups, 6 mice per group). There was no difference of basal mechanical withdrawal thresholds between groups in the same experiment.

2.6. Cytokine Measurement

Mice were terminally anesthetized 2 or 4 h after exercise session and samples from spinal cord (L4-L6), gastrocnemius and soleus muscles were collected. The samples were homogenized in 300 µl (spinal cord, pool of 3 animals) or 500 µl (soleus or gastrocnemius muscle) of buffer containing protease inhibitors, and TNF-α, IL-1β and IL-10 levels were determined as described previously (Verri et al., 2008; Verri et al., 2010) by enzyme-linked immunosorbent assay (ELISA) using

eBioscience kits. The results were expressed as picograms (pg) of each cytokine per 100 mg of tissue. Naïve and sham groups were used as control groups.

2.7. Muscle edema assessment

First, edema of the hind limb was evaluated by measuring the transverse diameters (anteroposterior and lateral), using a digital paquimeter (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). The thickness values of hind limb were expressed by the differences between the diameters measured before (basal) and after the intense acute swimming (2, 4, 6, 12, 24, 36 and 48 hours after the session) in millimeters (mm) (Conte et al., 2008). After all measurements with the paquimeter, in another phase of the experiments, mice were terminally anesthetized and soleus and gastrocnemius muscles weight was assessed, proportionally to the individual weight of each animal of the group, in grams (g).

2.8. Leukocyte Migration to the Skeletal Muscle Tissue

The intense acute swimming-induced leukocyte recruitment to soleus and gastrocnemius muscles of mice was evaluated using the myeloperoxidase (MPO) kinetic–colorimetric assay (Casagrande et al., 2006). Samples of skeletal muscles were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at −86 °C until use. Samples were homogenized using an ultra turrax (IKA T10), centrifuged at 14.000 rpm in 4°C for 2 min and the resulting supernatant assayed spectrophotometrically for MPO activity determination at 450 nm (Spectra max), with 3 readings in 1 min. The MPO activity of

samples was compared to a standard curve of neutrophils. Briefly, 10 µl of sample was mixed with 200 µl of 50 mM phosphate buffer pH 6.0, containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results were presented as MPO activity (number of total neutrophils x 10^{10}/ mg of muscle).

2.9. Histological Analysis

Soleus and gastrocnemius muscles were collected from mice at 24 h after intense acute swimming session. The samples were obtained from transverse cuts (5 µm) made in the belly of muscles and fixed in 10% buffered formaldehyde. Tissue sections were processed for paraffin embedding followed by hematoxylin-eosin (HE) staining for examination under optic microscope (Olympus OX31, Tokyo, Japan), coupled with a digital camera (Lumenera Infinity 1, Ottawa, Canada). Criteria for histopathological alterations assessment of damage in muscle tissue sections was graded semiquantitatively and examined independently of each other, in a double-blind manner by the evaluator who was unaware of which group samples belonged to. The alterations assessed were: the degree of (1) loss of myofibrillar architecture, (2) splitting areas, (3) vacuoles formation and (4) sarcolemmal disruption / necrotic sites, which were expressed as the mean of 5 fields chosen at random and were classified on a scale of 0-3 according to the number fibers affected by the above described lesions (0 fiber – 0; 1-4 fibers – 1; 4-6 fibers – 2; 6 or more fibers – 6). Finally, (5) cellular infiltrate was expressed as the mean of 5 fields chosen at random and has classified on a scale of 0-3, according to the number cells present on the muscle sections (0 cell – 0; 1-4 cells – 1; 4-6 cells – 2; 6 or more cells – 6). The final score was determinate by summing the scores above for each of the samples.

2.10. Reduced Glutathione (GSH) Assay

The levels of GSH in skeletal muscle tissue were determined using a spectrofotometric method (Borghi et al., 2013d). Samples of gastrocnemius (100 mg) or soleus (40 mg) muscle homogenates (1:10 dilution) were obtained in 4 and 1.6 ml of EDTA 0.02M, respectively, using a homogenizer (IKA T10). Homogenates (2.5 ml) were treated with 2 ml H_20 Milli Q plus 0.5 ml of trichloroacetic acid 50%. After 15 minutes, the homogenates were centrifuged at 1500 rcf for 15 min, and 1 ml from supernatant was added to 2 ml of a solution containing Tris buffer 0.4M (pH 8.9) plus 50 ml of DTNB. After 5 min, the measurements were performed in 412 nm against blank control [UV - Visible spectrophotometer (UV-1650 PC) – SHIMADZU]. The GSH levels were corrected according to the total protein concentration. The results were presented as mmols of GSH per gram of protein in skeletal muscles.

2.11. Statistical Analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The analyzed factors were treatments, time and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's t-test was performed for each time. On the other hand, when the hyperalgesic response was measured once after the administration or enforcement

of the stimuli, the difference between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Statistical differences were considered to be significant at $P<0.05$.

3. Results

3.1. Thalidomide reduces muscle mechanical hyperalgesia induced by intense acute swimming in a time- and dose-dependent manner

Untrained mice received one session of intense acute swimming of 120 min or were exposed to sham conditions (30 sec exposure to water). Treatment with thalidomide (5, 15 and 45 mg/kg) or saline (200µl) was performed i.p. (intraperitoneal) 30 min before plus reinforcement 12 h after the session. The intensity of hyperalgesia was evaluated 6, 12, 24, 36 and 48 hours after the stimulus of intense acute swimming. Intense acute swimming induced significant muscle mechanical hyperalgesia compared to the sham group at 6, 12, 24 and 36 h, with the peak of hyperalgesia occurring at 24 h (Fig. 1). The i.p. treatment with the dose of 5 mg/kg of thalidomide shows a biological tendency of reduction of muscle mechanical hyperalgesia between 24-36 h, although it was not significant. The dose of 15 mg/kg of thalidomide reduced muscle mechanical hyperalgesia 24 and 36 h after the session. On the other hand, the dose of 45 mg/kg of thalidomide abolished the intense acute swimming-induced muscle mechanical hyperalgesia between 12 - 36 h. In the 48^{th} h no difference was observed between the experimental groups. Therefore, the dose of 45 mg/kg of thalidomide was chosen for the next experiments.

3.2. Effect of thalidomide in intense acute swimming-induced TNF-α, IL-1β and IL-10 production in the soleus and gastrocnemius muscle

Mice were treated with thalidomide (45 mg/kg i.p., 30 min before) and underwent intense acute swimming for 120 minutes or were exposed to sham conditions (30 sec exposure to water) and the levels of TNF-α, IL-1β and IL-10 were measured immediately after (2 h) the swimming session in soleus and gastrocnemius muscles (Fig. 2, A-F). The levels of TNF-α, IL-1β and IL-10 increased significantly in control animals when compared to the baseline values (naive and sham animals) in soleus muscle (Figs. 1A, C and E, respectively). Thalidomide pre-treatment was able to reduce significantly the levels of TNF-α (Fig. 2A), IL-1β (Fig. 2C) and presented a biological tendency to reduce the levels of IL-10 (Fig. 2E) in soleus muscle. In the gastrocnemius muscle no difference was observed between the experimental groups (Figs. 2, B, D and F).

3.3. Effect of thalidomide in intense acute swimming-induced TNF-α, IL-1β and IL-10 production in the spinal cord

Intense acute swimming induces an increase of TNF-α, IL-1β and IL-10 production in the spinal cord. Therefore, it was evaluated the effect of thalidomide over the production of those cytokines following the same protocol as for Fig. 2 except that samples were collected 4 h after intense acute swimming. Thalidomide inhibited intense acute swimming-induced production of TNF-α (Fig. 3A), IL-1β (Fig. 3B) and IL-10 (Fig. 3B).

3.4. Thalidomide inhibits intense acute swimming-induced distal hind limb edema

Taking into account that the inflammatory process and the edema formation in skeletal muscle following tissue injury can contribute significantly to the development of DOMS, and that the augmented intra-muscular pressure caused by the edema leads to an increase of pain perception (Cheung et al., 2003; Hume et al., 2004), the circumference and weight of soleus and gastrocnemius muscles were evaluated to determine if the intense acute swimming session induces edema formation in skeletal muscle tissue. Mice were treated with thalidomide (45 mg/kg i.p., 30 min before plus reinforcements 12 hours after) and received intense acute swimming for 120 minutes or were exposed to sham conditions (30 sec exposure to water) and the transverse diameters (2-48 hours after the session) of the distal hind limb were assessed (Fig. 4A). There was a biphasic increase of distal hind limb edema in which significant difference was observed at 2 h with a decrease at 6 h and progressive increase at 12 h peaking at 24 h. At the 48^{th} h, no difference was detected between all experimental groups. Therefore, 24 h was selected to evaluate the weight of dissected muscles. There was no difference in the total weight of the soleus/gastrocnemius muscles together (Fig. 4B) and isolated gastrocnemius muscle (Fig. 4C) comparing all groups. On the other hand, there was significant increase of soleus weight at 24 h in the intense acute swimming group compared to sham group while thalidomide inhibited intense acute swimming-induced soleus muscle edema (Fig. 4D).

3.5. Thalidomide inhibits intense acute swimming-induced increase in myeloperoxidase (MPO) activity

Following the protocol as in Fig. 2, samples were collected at 24 h after intense acute swimming. Thalidomide inhibited intense acute swimming-induced

increase of MPO activity in the soleus muscle (Fig. 5A). There was no alteration of MPO activity in the gastrocnemius muscle (Fig. 5B).

3.6. Thalidomide inhibits intense acute swimming-induced histological changes in the soleus muscle.

To confirm that the soleus muscle presents structural dysfunctions resulting from mechanical stress, cellular infiltrate and consequently biomechanical damage in their myofibers induced by intense acute swimming, histological samples were investigated (Fig. 6). Mice received intense acute swimming session for 120 min or were exposed to sham conditions (30 sec exposure to water) and were treated with thalidomide (as in Fig. 5) before the exercise session. The soleus muscle was analyzed 24 h after the intense acute swimming session. Animals that experienced the intense acute swimming session show increased cellular infiltrate and changes that suggest myofibrillar disruption, necrosis and functional overload reflected by splitting areas with the vacuoles formation when compared to the naïve and sham animals (Fig. 6 A-D). Continuous high intracellular calcium levels, elevated calpain associated by ubiquitin-proteasome activity lead to increased lysossomal digestion of the cellular debris, leading to the lysossomal congestion and even breakdown of this organelle (vacuoles) (Paulsen et al., 2012). On the other hand, thalidomide treatment was capable to reduce especially cellular infiltrate (Fig. 6E and F), acting predominantly in biochemical phase of injury of the muscle fibers, probably by inhibiting chemoattractant action of TNF-α for leukocytes. Although myocytes from animals that received thalidomide treatment show some integrity (Panel F, white arrow), they still presented injuries in most of its architecture. This may be explained

by the reason that thalidomide apparently does not interfere with the mechanical injury of the sarcomere and extrasarcomeric cytoskeleton, take into account that this tissue damage is due mainly to the excessive mechanical stress imposed to myocytes during the exercise (Armstrong et al., 1984; Armstrong and Warren, 1993; MacIntyre et al., 1995; Lewis et al., 2012; Cheung et al., 2003). Fig. 6G shows a microscopic graphical score used to determinate the final score of the morphological changes between experimental groups.

Soleus muscle from naïve (Fig. 6A) and sham (Fig. 6B) groups are shown under hematoxylin and eosin staining demonstrating normal muscle section. Using the same protocol as in Fig. 2, it was observed at 24 h that intense acute swimming induced as shown by black arrows: (1) loss of myofibrillar architecture, (2) splitting areas, (3) vacuoles formation, (4) sarcolemmal disruption / necrotic sites and (5) cellular infiltration (Fig. 6C and 6D). Thalidomide treated group presented reduced morphological changes as shown by black arrows 1 (Fig. 6E), 2 and 3 as well as presented intact myocyte as indicated by white arrow (fig. 6F). The microscopic score showed an increase of muscle damage in intense acute swimming compared to the naïve and sham groups, which was inhibited by thalidomide treatment (Fig. 6G).

3.7. Intense acute swimming-induced depletion of endogenous reduced glutathione (GSH) levels was prevented by thalidomide treatment in the soleus, but not in gastrocnemius muscle

Following the same protocol as in Fig. 2, it was observed at 2 h after the session (4 h) that intense acute swimming induced significant decrease of GSH levels in the soleus muscle compared to the naïve and sham groups, which was

inhibited by thalidomide treatment (Fig. 7A). There was no change in the GSH levels among all groups in the gastrocnemius muscle (Fig. 7B).

4. Discussion

DOMS is classified as type I muscle strain injury accompanied by tenderness or stiffness to palpation or movement (Isabell et al., 1992; Gulik and Kimura, 1996; Sharkey, 1995; Rodenburg et al., 1994). The symptoms of DOMS may be subclinical since the perception of pain varies from slight muscle stiffness, which rapidly disappear during daily routine activities, to severe debilitating pain which restrict movements (Gulik and Kimura, 1996; Cheung et al., 2003). Tenderness is concentrated in the distal portion of the muscle and becomes progressively diffuse by 24-48 post-exercise. This specific localization of pain is due to a high concentration of muscle nociceptors in connective tissue of the myotendionous region (Armstrong et al., 1984; MacIntyre et al. 1995; Armstrong et al.; 1993, Garret, 1990; 1996; Noonan and Garret, 1992).

Nowadays, there is a consensus that myocyte injury depends on an early mechanical injury followed by a biochemical injury. Initially, a high tensile force during the eccentric activity of the muscle induces diffuse disruptions in the structure of muscle fibers, particularly in the z-lines (Smith et al., 1991; Cleak and Easton, 1992; MacIntyre et al., 1995; Proske and Morgan, 2001). Subsequent damage to the sarcolemma and t-tubules results in calcium accumulation and reduction of ATP production as a consequence of inhibition of cellular respiration. Calcium is an important molecule to activate photolytic enzymes that perpetuate the injury. Specific intracellular biomarkers such as creatine kinase (CK) are released to extracellular sites due to the muscle damage (Takehura et al., 2001; Cheung et al., 2003; Hume et al. 2004). Resident cells release chemical mediators such as cytokines that in turn chemoattract leukocytes such as neutrophils and monocytes. Both resident cells and

recruited cells produce prostaglandin E2 (PGE_2), a mediator that sensitizes type III and IV nociceptors in the muscles (Connelly et al., 2003; Lewis et al., 2012). The great amount of hyperalgesic molecules associated with edema, myocyte necrosis and increased local temperature activate sensorial neurons leading to DOMS symptoms (Tegeder et al., 2002). Furthermore, there is a contribution of inflammation to exercise-induced DOMS as observed by the analgesic effect of non-steroidal anti-inflammatory drugs in varied DOMS protocols (O'Grady et al., 2000; Ito & Kawakite, 2002; Hasson et al., 1993; Tokmakidis et al., 2003).

Previous data show the antinociceptice effect of thalidomide, which prevents carrageenan-induced mechanical hyperalgesia and acetic acid-induced abdominal contortions in mice (Verri et al., 2006). Thalidomide does not affect the mechanical hyperalgesia induced by local administration of PGE_2, which corroborates the evidence that thalidomide enhances the degradation of TNF-α mRNA expression (Moreira et al., 1993), and therefore, does not affect the hyperalgesia of a mediator that sensitizes nociceptors without further production of nociceptive molecules in acute administration such as PGE_2 (Verri et al., 2006). The potential use of thalidomide in pain conditions was also demonstrated in constriction chronic injury (CCI)-induced hyperalgesia in rats (Sommer et al., 1998a, 1998b) and carrageenin-induced chronic hyperalgesic priming that involves participation of TNF-α (Parada et al., 2003). Furthermore, thalidomide could also be effective in chronic pain states, such as complex regional pain syndrome (CRPS) (Asher and Furnish, 2013; Mackey and Feinberg, 2007). Thus, these data reflect the potential use of thalidomide to control inflammatory pain states related to cytokines, notably TNF-α.

It is noteworthy to mention that there is no alteration of the evaluated parameters in the gastrocnemius muscle due to its fiber characteristics (Borghi et al.,

2013a). The present study shows that thalidomide treatment reduces DOMS by inhibiting intense acute swimming-induced TNF-α production. Additionally, we show that thalidomide could also reduce intense acute swimming-induced IL-1β production. It has been demonstrated that administration of TNF-α induces hyperalgesia via IL-1β production as well as inhibition of TNF-α using antibody or genetically targeting TNFR1 reduces IL-1β production (Cunha et al., 1992; Cunha et al., 2005; Verri et al., 2008). Therefore, it is possible that the inhibition of IL-1β production is dependent on enhanced degradation of TNF-α mRNA expression as previously demonstrated (Moreira et al., 1993).

TNF-α and IL-1β are known for their activation of endothelial cells and chemoattractant effect over neutrophils (Verri et al., 2010). Neutrophils contribute to hyperalgesia by further producing hyperalgesic mediators (Cunha et al., 2008; Guerrero et al., 2008; Verri et al., 2009). Thus, inhibition of TNF-α and IL-1β production lines up well with the inhibition of hyperalgesia and MPO activity in the soleus muscle. In response to intense exercise, massive leukocyte infiltration and cellular accumulation inside myofibers were demonstrated (Child et al., 1999; O'Grady et al., 2000; Round et al., 1987). It is likely that in the late phase of DOMS, leukocytes have a role to remove cellular debris and prepare for regeneration of necrotic segments of myofibers (Paulsen et al., 2012). In accordance, in our study, intense acute swimming induced signs of ultra-structural damages reflected by splitting areas, vacuoles, necrotic sites, and cellular infiltration in the soleus muscle while thalidomide treatment inhibited such damage. Thalidomide also inhibited the edema in the soleus muscle, which might be related to inhibition of TNF-α and IL-1β production and their edematogenic effect (Joosten et al., 2006).

There is no evidence that thalidomide exerts direct antioxidant effects. TNF-α and IL-1β activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, inducing superoxide anion production and consequently oxidative stress (Meier et al., 1989; Jiménez-Altayó et al., 2006; Kilpatrick et al., 2010). Therefore, it is likely that the antioxidant effect of thalidomide treatment as observed by increased levels of GSH, was related with the inhibition of TNF-α and IL-1β production.

In intense acute swimming-induced DOMS, IL-10 acts as an endogenous down-regulator of pro-hyperalgesic factors such as TNF-α, IL-1β, MPO activity and oxidative stress. In this sense, inhibiting IL-10 increases all factors mentioned as well as muscle hyperalgesia. Thus, increasing the levels of IL-10 could be an anti-hyperalgesic therapeutic approach (Borghi et al., 2013c). Thalidomide did not induce an elevation of the IL-10 after intense acute swimming suggesting this is not its mechanism of action. This result is in agreement with previous report, in which analgesic effects of thalidomide were not related to increase of anti-hyperalgesic cytokines such as IL-4 and IL-10 (Verri et al., 2006).

The depolarization of primary afferent nociceptors induces the release of the chemokine CX3XL1 by neurons in the spinal cord, which in turn, activates CX3CR1 receptors in microglia resulting in TNF-α and IL-1β production. TNF-α and IL-1β activates second order neurons in the spinal cord to induce hyperalgesia (Gao and Ji, 2010; Gustafson-Vickers et al., 2008). Another possibility is the retrograde axonal transport of TNF-α and TNFR1 from the peripheral site to the dorsal root ganglia and spinal cord (Shubayev and Myers, 2001). An anterograde transport has also been described from the dorsal root ganglia to the nerve injury site and innervated muscle, but not skin (Schäfers et al., 2002). These results corroborate a peripheral and central (spinal cord) integrated nociceptive mechanism in the pathophysiology of

intense acute swimming-induced muscle mechanical hyperalgesia and suggests that the peripheral inhibition of TNF-α and IL-1β production might be responsible for the spinal inhibition of TNF-α, IL-1β and IL-10 production. Nevertheless, it was not disproved a spinal action of thalidomide.

6. Conclusions

Concluding, the present study provides evidence that thalidomide can be used as an effective drug to inhibit DOMS-related symptoms such as hyperalgesia. Thalidomide was able to reduce TNF-α and IL-1β production in the soleus muscle and spinal cord and as a consequence, there was reduction of muscle hyperalgesia, MPO activity, muscle lesions and oxidative stress. Although it has teratogenic effects, thalidomide could be used respecting safety standards, considering that it does not present the side effects of non-steroidal anti-inflammatory drugs and corticosteroids, and presents great potential as a substitute to patients in which anti-inflammatory drugs should be avoided (Connelly et al., 2003; Cheung et al., 2003). Thalidomide also presents cost benefits compared to immune-biological therapies targeting TNF-α and IL-1β (Verri et al., 2006).

7. Conflict of interest

No conflict of interest to disclosure.

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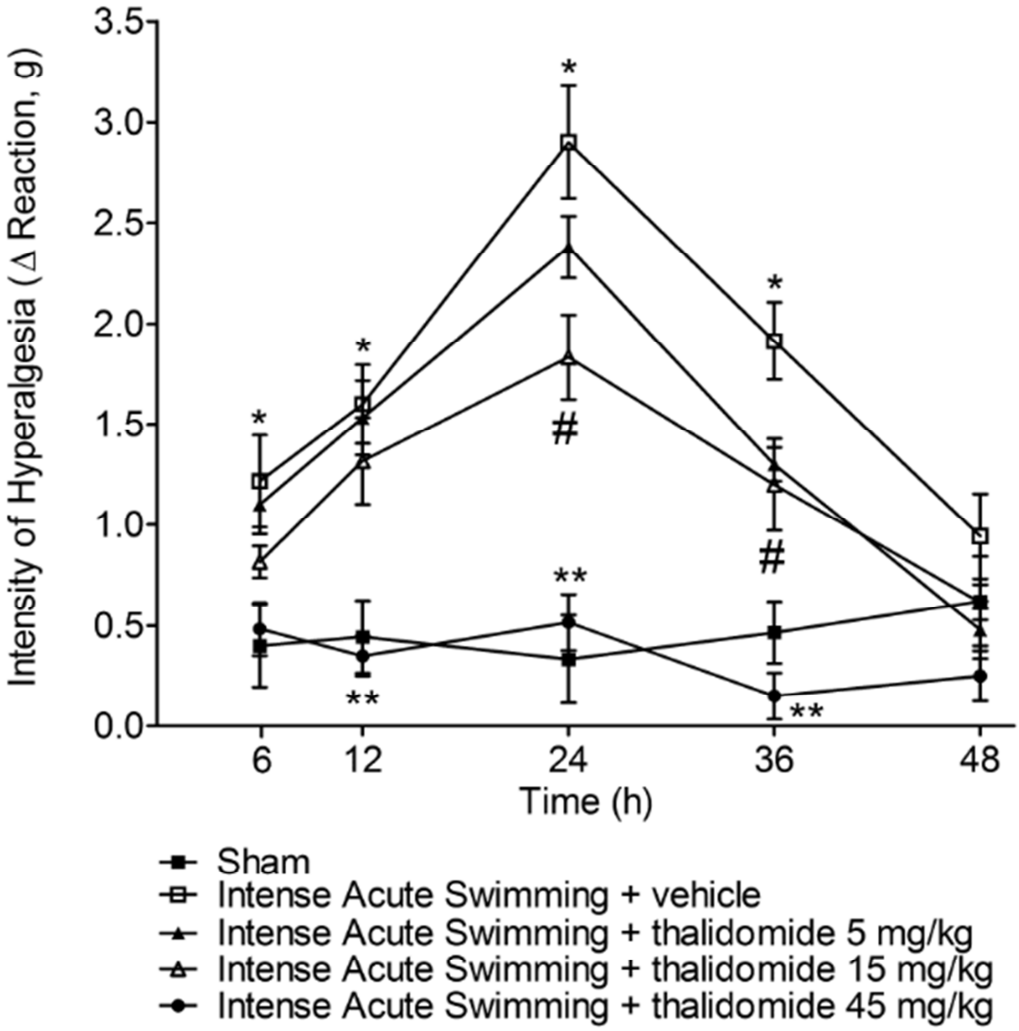


Fig. 1. Thalidomide reduced intense acute swimming-induced muscle mechanical hyperalgesia in mice. Mice received intense acute swimming for 120 min or were exposed to sham conditions (30 sec exposure to water), and were treated with thalidomide (5- 45 mg/kg i.p., diluted in DMSO/saline) or vehicle (saline) 30 minutes before and 12 hours after intense acute swimming session. The intensity of muscle mechanical hyperalgesia was evaluated 6-48 hours after intense acute swimming session. Results are presented as intensity oh hyperalgesia (Δ reaction, in grams) (*n* = 6 mice per group per experiment, representative of two separated experiments). *P<0.05 compared with sham group, #P<0.05 compared with control non-treated group, **P<0.05 compared with control non-treated and 5 and 15 mg/kg doses of thalidomide groups (One-way ANOVA followed by Tukey's t test).

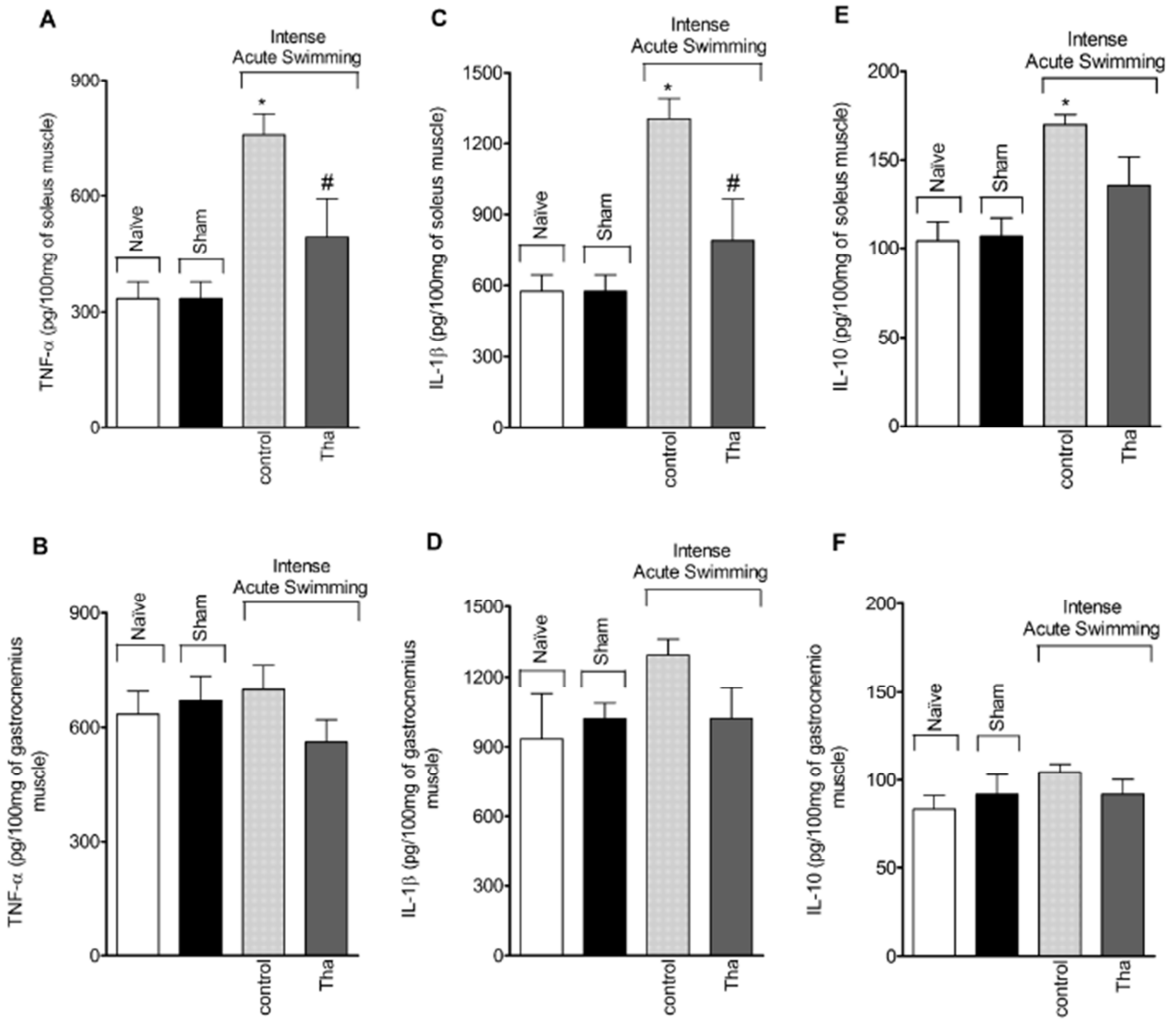


Fig. 2. Thalidomide inhibited intense acute swimming-induced TNF-α, IL-1β and IL-10 production in the soleus, but not in the gastrocnemius muscle. Mice were treated with thalidomide (45 mg/kg i.p., diluted in DMSO/saline) 30 minutes before intense acute swimming session. The levels of TNF-α, IL-1β and IL-10 in soleus (Panels A, C and E) and gastrocnemius (Panels B, D and F) muscles were quantified immediately after the end of swimming section (2 h) by ELISA. Results are presented as picograms per 100 mg of soleus and gastrocnemius muscles (*n* = 6 mice per group per experiment, representative of two separated experiments). *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).

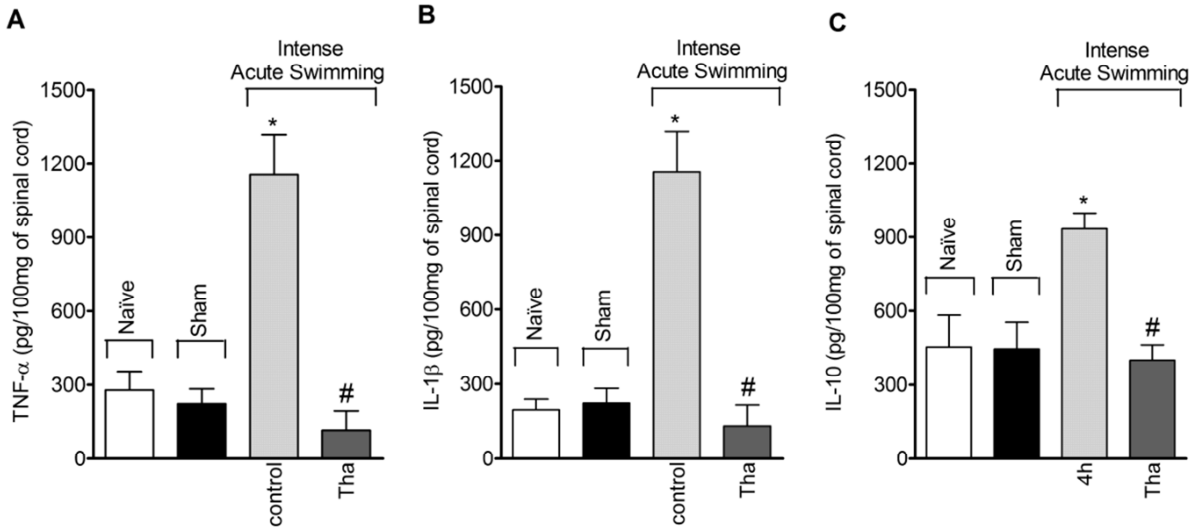


Fig. 3. Thalidomide inhibited intense acute swimming-induced TNF-α, IL-1β and IL-10 production in the spinal cord. Mice were treated with thalidomide (45 mg/kg i.p., diluted in DMSO/saline) 30 minutes before intense acute swimming. The samples of spinal cord (L4-L6) were collected 4 hours after the beginning of the exercise session. TNF-α (Panel A), IL-1β (Panel B) and IL-10 (Panel C) concentrations (picograms/100 mg of spinal cord) were quantified, as determined by ELISA. (*n* = 6 mice per group per experiment, representative of two separated experiments). *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).

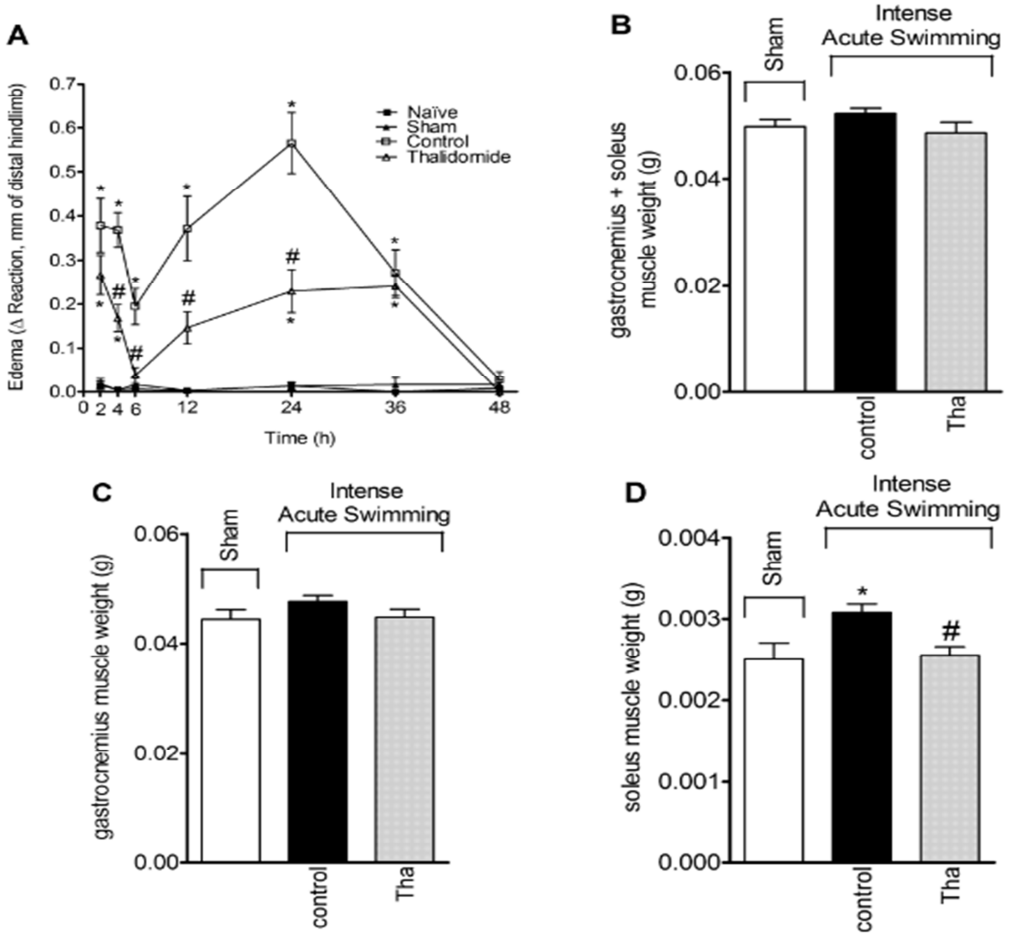


Fig. 4. Thalidomide inhibits intense acute swimming-induced edema in distal hind limb in the soleus, but not in the gastrocnemius muscle. Mice were treated with thalidomide (45 mg/kg i.p., diluted in DMSO/saline) 30 minutes before and 12 hours after intense acute swimming session. The anteroposterior and lateral-lateral diameters of distal hind limb were assessed 6-48 after the session (Panel A). 24 h after the intense acute swimming session, soleus and gastrocnemius muscles were removed and weighed together (Panel B) and individually (Panels C and D). Results are presented as edema (Δ reaction, mm of distal hind limb) and muscle weight in grams, respectively (*n* = 6 mice per group per experiment, representative of two separated experiments). *P<0.05 compared to the naïve and sham groups (Panel A) and with sham group (Panels B-D), #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).

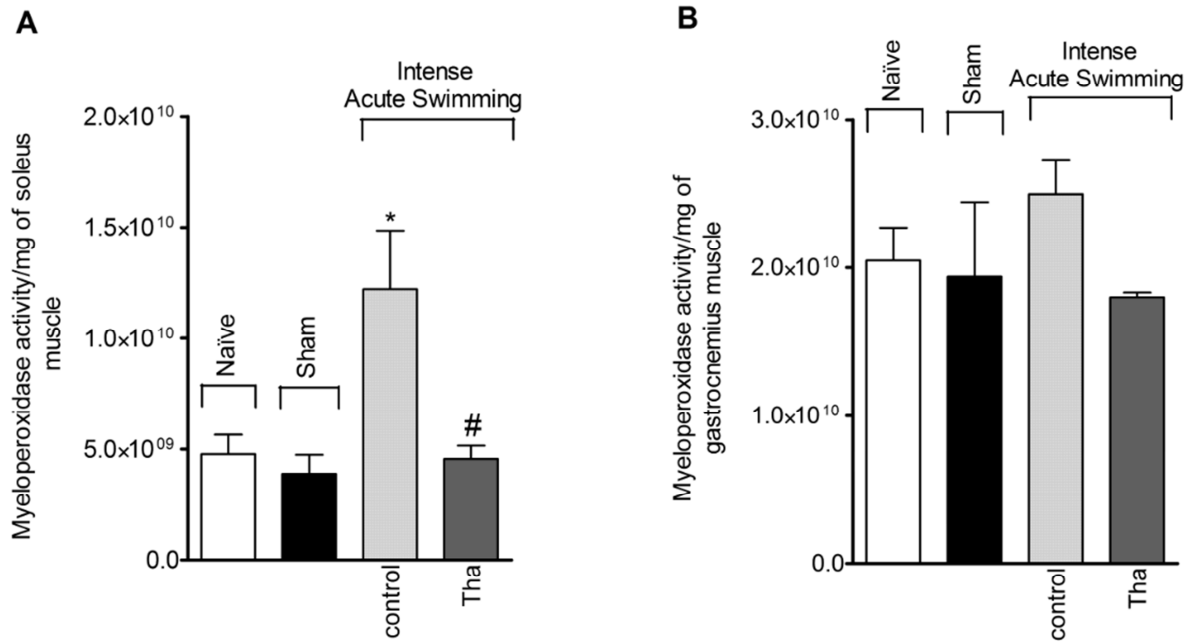
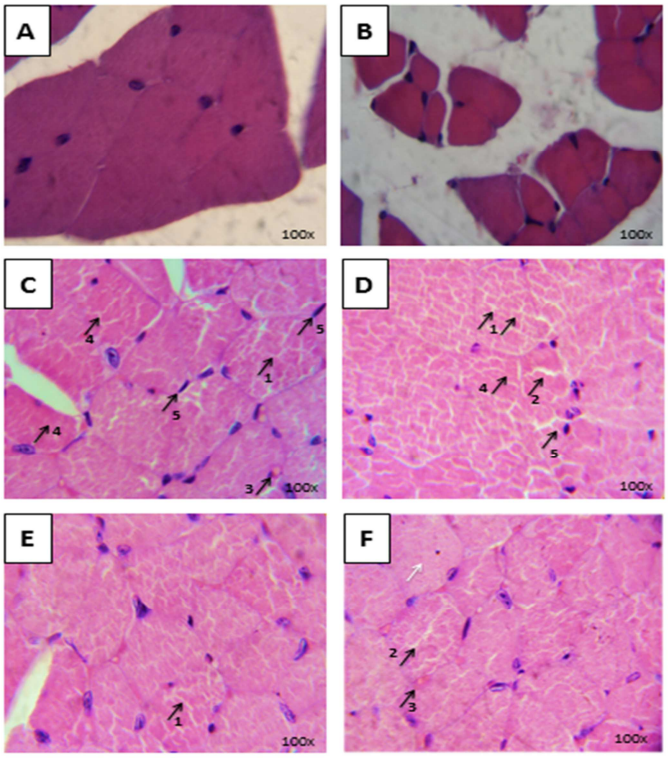


Fig. 5. Thalidomide inhibited intense acute swimming-induced increase of myeloperoxidase (MPO) activity in the soleus muscle but not in the gastrocnemius muscle. Mice were treated with thalidomide (45 mg/kg i.p., diluted in DMSO/saline) 30 minutes before, and 12 h after acute swimming session. The samples of the soleus (Panel A) and gastrocnemius (Panel B) muscles were collected 24 h after the intense acute swimming session and the MPO activity was determinate by an enzymatic assay. Results are presented as myeloperoxidase activity per milligram of the soleus and gastrocnemius muscles (*n* = 6 mice per group per experiment, representative of two separated experiments). *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).



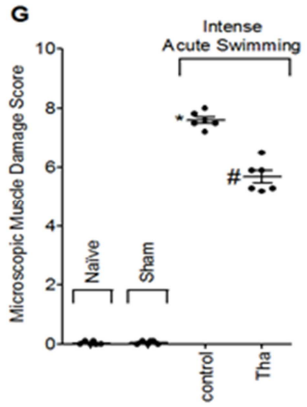


Fig. 6. Thalidomide inhibited intense acute swimming-induced cellular infiltration to the soleus muscle. Mice were treated with thalidomide (45 mg/kg i.p., diluted in DMSO/saline) 30 minutes before and 12 hours after acute swimming session. Histological analysis of the soleus muscle from naïve (Panel A), sham (Panel B), intense acute swimming (Panels C and D) and thalidomide treated (Panels E and F) groups were carried 24 hours after the exercise session. Panel G shows a final microscopic muscle damage score graphic. The samples were stained with hematoxylin and eosin. Original magnification 100X, *n* = 6 mice per group per

experiment, representative of two separated experiments. Black arrows indicate: (1) loss of myofibrillar architecture, (2) splitting areas, (3) vacuoles formation, (4) sarcolemmal disruption / necrotic sites and (5) cellular infiltration. White arrow indicates an intact myocyte. *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with non-treated group (One-way ANOVA followed by Tukey's t test).

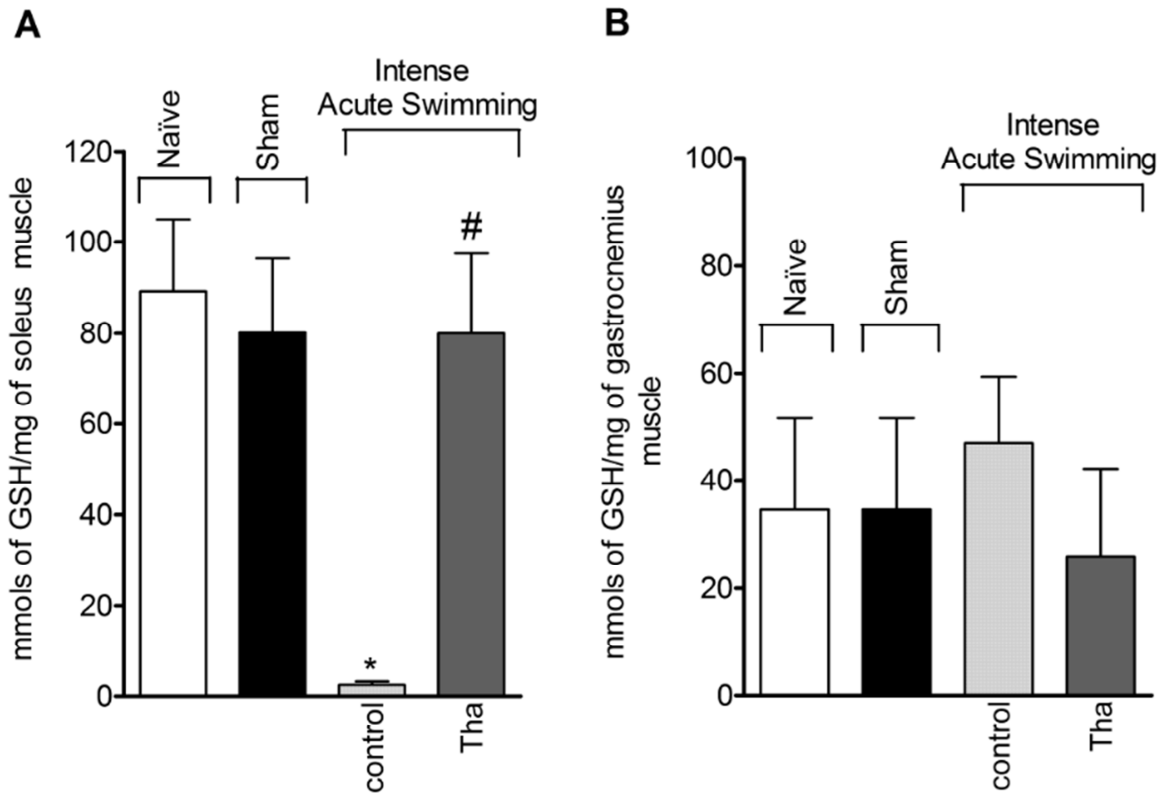


Fig. 7. Thalidomide inhibited intense acute swimming-induced reduced glutathione (GSH) depletion in the soleus muscle, but not in the gastrocnemius muscle. Mice were treated with thalidomide (45 mg/kg i.p., diluted in DMSO/saline) 30 minutes before swimming session. Samples of the soleus (Panel A) and gastrocnemius (Panel B) muscles were collected 4 h after the beginning of the intense acute swimming session and endogenous GSH levels were evaluated by kinetic-colorimeric assay. Results are presented as mmols per milligram of the soleus and gastrocnemius muscles (*n* = 6 mice per group per experiment, representative of two separated experiments). *P<0.05 compared to the naïve and sham groups, #P<0.05 compared with control non-treated group (One-way ANOVA followed by Tukey's t test).