Zhou, D.; Li, W.; Zhao, X. (2023) ANTI-INFLAMMATORY EFFECTS OF ULINASTATIN IN LPS-INDUCED BV2 CELLS BY A20: IMPLICATIONS FOR SPORTS AND FITNESS PLAYERS' HEALTH. Revista Internacional de Medicina y Ciencias de la Actividad Física y el Deporte vol. 23 (92) pp. 124-137.
DOI: https://doi.org/10.15366/rimcafd2023.92.011

ORIGINAL

ANTI-INFLAMMATORY EFFECTS OF ULINASTATIN IN LPS-INDUCED BV2 CELLS BY A20: IMPLICATIONS FOR SPORTS AND FITNESS PLAYERS' HEALTH

Dan Zhou[1], Wen Li[1], Xi Zhao[2]*

[1]People's Hospital of Deyang City, department of anesthesiology, Deyang, Sichuan 618000, China

[2]People's Hospital of Deyang City, department of stomatology, Deyang, Sichuan 618000, China

*Corresponding author: Xi Zhao
E-mail: zhaoxi@zcmu.edu.cn

Recibido 22 de agosto de 2022 **Received** August 22, 2022
Aceptado 24 de julio de 2023 **Accepted** July 24, 2023

ABSTRACT

This study delves into the anti-inflammatory role of ulinastatin (UTI) in BV2 microglia cells stimulated with lipopolysaccharide (LPS), focusing on its relevance to sports and fitness players. A crucial aspect of athletic health is managing inflammation, which can impact performance and recovery. We constructed an inflammatory response model in BV2 microglia using LPS and divided the sample into four groups (n=12 each): a control group (C), an LPS-induced inflammation group (L), a UTI treatment group (U+L), and a group with A20 protein down-regulation (U+L+Si). The study evaluated IL-1β and TNF-α protein concentrations via ELISA, NF-κB/P65 and A20 protein expressions through Western blot, and microglial Iba-1 expression via immunofluorescence staining. Compared to the control, the L and U+L+Si groups showed significant increases in IL-1β, TNF-α, NF-κB P65 expression, and decreased A20 protein expression ($P<0.05$). The L and U+L+Si groups also exhibited higher levels of IL-1β, TNF-α, NF-κB P65, and Iba-1 compared to the U+L group ($P<0.05$), with reduced A20 expression. Interestingly, the U+L group displayed no significant differences in IL-1β, TNF-α, and NF-κB P65 compared to the control ($P>0.05$). The findings suggest that UTI significantly mitigates LPS-induced inflammation in BV2 microglia, primarily through upregulation of A20 protein. For athletes and fitness enthusiasts, these insights offer potential strategies for managing exercise-induced inflammation, enhancing recovery, and optimizing performance.

KEY WORDS: ulinastatin; lipopolysaccharide; BV2 microglia; A20 protein; Athletic Health and Recovery

1. INTRODUCTION

In recent years, the world of sports and fitness has witnessed an unprecedented surge in popularity, with an ever-growing number of individuals actively engaging in various physical activities to maintain and enhance their health. However, this surge has also brought to light the importance of addressing the associated health concerns, particularly those related to inflammation and immune response. For athletes and fitness enthusiasts alike, the ability to recover quickly from strenuous workouts or sports-related injuries is crucial for sustained performance and overall well-being. Inflammation, while a natural part of the body's defense mechanism, can become detrimental when it is chronic or excessive(Weir, 2017). The molecular underpinnings of inflammation have been extensively studied, and researchers have identified various pathways and molecules involved in regulating this complex physiological response. One such molecule of significance is A20, a multifunctional protein known for its anti-inflammatory properties. A20 has emerged as a key player in modulating the immune response and maintaining immune homeostasis, making it an attractive target for therapeutic intervention(ARUNACHALAM, VIJAYAKUMAR, & MAYDEN, 2017).

In this context, the present study delves into the intricate world of inflammation, focusing on the potential therapeutic application of Ulinastatin in mitigating inflammation in BV2 microglial cells induced by lipopolysaccharide (LPS). Ulinastatin, a serine protease inhibitor, has been used clinically to treat conditions characterized by excessive inflammation, such as sepsis and acute pancreatitis(Oh et al., 2015). Our investigation seeks to unravel the underlying mechanisms by which Ulinastatin may exert its anti-inflammatory effects in the context of A20 regulation, shedding light on its potential implications for the health and performance of sports and fitness players(Ullah et al., 2022).

The aim of this study is to elucidate how Ulinastatin may modulate the inflammatory response in BV2 cells exposed to LPS through the upregulation of A20. By examining the molecular pathways and interactions involved, we hope to provide valuable insights into the potential therapeutic utility of Ulinastatin for sports and fitness enthusiasts who often grapple with inflammation-related issues(Lee, Choi, Ju, & Yune, 2018). Understanding the mechanisms at play in this process could lead to the development of targeted interventions aimed at enhancing recovery, reducing the risk of injuries, and ultimately improving the overall health and performance of individuals engaged in physical activities(Tyrtyshnaia et al., 2020).

This investigation not only contributes to our understanding of the

intricate interplay between inflammation and immune regulation but also offers a promising avenue for the advancement of sports medicine and fitness-related healthcare (Xue et al., 2022). As we delve deeper into the anti-inflammatory effects of Ulinastatin and its association with A20, we embark on a journey that holds the potential to revolutionize the way we approach and manage inflammation in the world of sports and fitness.

2. METHODS

2.1 Main Materials

BV-2 cells, mouse TNF-α ELISA detection kit, mouse IL-1β ELISA detection kit, musTnfaip3 siRNA set [HonorGene (Changsha, China) Aibiwei Biotechnology Co., Ltd.], UTI [Tianpu (Guangdong, China)], DMEM medium, pancreatic Enzyme digestion solution, fetal bovine serum (GIBCO, USA), Iba-1 antibody (Thermo Fisher, China), rabbit A20 antibody, rabbit NF-κB P65 (CST, USA), rabbit goat anti-mouse antibody, goat anti-rabbit antibody, anti-β-actin (Proteintech, USA)

2.2 Cell culture

We placed BV-2 cells in DMEM medium that contained 10% FBS + 1% double antibody for culturing, 37°C, 5% CO2, in a saturated humidity incubator. When the cell density reached 80%, the cells were passaged. spare disk.

2.3 The musTnfaip3 siRNA kit was used for cell transfection.

From the previous qPCR detection results, the siRNA musTnfaip3-si1 has the highest interference efficiency on the target gene A20, which is 66.7%. Therefore, subsequent experiments were conducted with musTnfaip3-si1 interference experiments.

a. Take out the desired siRNA NC, musTnfaip3-si1 plasmid, and thaw on ice. b. Take 8 sterile centrifuge tubes, take two of them, add 95uL of serum-free DMEM medium to each tube, and then add 5uL of siRNA and 5uL of Lip2000 to the centrifuge tubes, respectively. Others were also added to the corresponding centrifuge tubes in this way. c. Gently mix, let stand at room temperature for 5 minutes, then mix the two tubes gently, a total of about 200uL, and let stand at room temperature for 20 minutes. d. Finally, evenly add the mixed solution to wells to be transfected and mix well. After 6 h of culturing in a 37 °C incubator, replace with fresh complete medium.

2.4 Experimental Grouping

Four groups of BV-2 cells received different culturing and treatment. The specific groups are as follows: Group C: Used siRNA NC (final concentration 150nM) to transfect BV-2 cells, and harvested cells 48h after transfection;

Group L: Used siRNA NC (final concentration 150nM) to transfect BV-2 cells, then added LPS (final concentration 10ug/ml) 24h after transfection, and cells were harvested after 24h treatment; Group U+L: BV-2 cells were first transfected with siRNA NC (final concentration 150nM), 23.5h after transfection, UTI (final concentration 1000U/ml) was added for 0.5h, and then LPS (final concentration 10ug) was added /ml) treatment, cells were harvested after 24h treatment; Group U+L+Si: BV-2 cells were first transfected with musTnfaip3-si1 (final concentration 150nM), 23.5h after transfection, UTI (final concentration 1000U/ml) was added for 0.5h, and then LPS (The final concentration was 10ug/ml), and the cells were harvested after 24h of treatment.

2.5 ELISA detection of IL-1β and TNF-α concentration

Cells received 10 min of centrifugation at 3000 rpm at 2-8°C. The supernatant was taken out for detection. 10 μl of sample were added to the well of the sample to be examined. 40 μl of diluent and 100 μl of enzyme labeling reagent were added to each well. A sealing film was used to seal the plate, followed by one hour of incubation at 37°C.

The following operations were then carried out: add each well with 50 μl of color developer A, followed by adding 50 μl of color developer B. Shake and mix gently, followed by developing color at 37°C for 15 minutes in the dark. Termination: Add each well with 50 μl of stop solution, and detect the absorbance (OD value) of each well in sequence at 450 nm wavelength. It was required to conduct measurement within fifteen minutes after the addition of the stop solution.

2.6 Western blotting

For detecting the relative expression exhibited by NF-κB/P65 and A20 after treatment, cells received 24 h of culturing, and the proteins of 4 groups of cells were extracted, detected by Western blotting, and analyzed by Quantity One Vesion 4.5.0 software to obtain the gray value of the resulting images.

2.7 Immunofluorescence detection of Iba-1

Take out the slides, wash 2~3 times with PBS, fix the slides with 4% paraformaldehyde for 30 minutes, rinse with PBS for 5 min x 3 times, add 0.3% Triton, 30 minutes at 37°C transparent. Rinse with PBS for 3 min x 3 times; block with 5% BSA at 37°C for 1 hour; rinse with PBS for 3 min x 3 times; incubate with primary antibody: add appropriately diluted primary antibody (Iba-1) overnight at 4°C.

Rinse with PBS for 5 min x 3 times; incubate with secondary antibody: add 50~100ul anti-mouse-IgG-labeled fluorescent antibody dropwise, incubate at 37°C for 1.5 hours, rinse with PBS for 5 min x 3 times; stain nuclei with DAPI

working solution at 37°C for 10 min, Rinse with PBS for 5 min x 3 times; cover with buffered glycerol. Protected from light, the images were taken by a fluorescence microscope computer, and the pictures were 200 × 400 times.

2.8 Statistical Analysis

Data processing and graphing were analyzed and produced using GraphPad Prism 8.3 statistical software. The measurement data with normal distribution were in the form of the mean ± standard deviation (x ± s), and one-way analysis of variance or two-sample t test served for the comparison. P<0.05 means the distinction is obvious.

3. RESULTS

3.1 ELISA measurement

According to the ELISA measurement of the concentrations of IL-1β and TNF-α protein in the four groups, group L presented remarkably higher concentrations relative to group C, and the distinction was clear (*P*=0.0008 and *P=0.0057*). Group U+L presented remarkably lower concentrations than group L after statin, and the distinction was obvious (*P*=0.0023 and *P=0.0036*). After the inhibition of A20 expression in group U+L+Si, the protein concentrations of IL-1β and TNF-α presented an obvious increase relative to group U+L, and the distinction was obvious (*P*=0.0003 and *P=0.0003*).

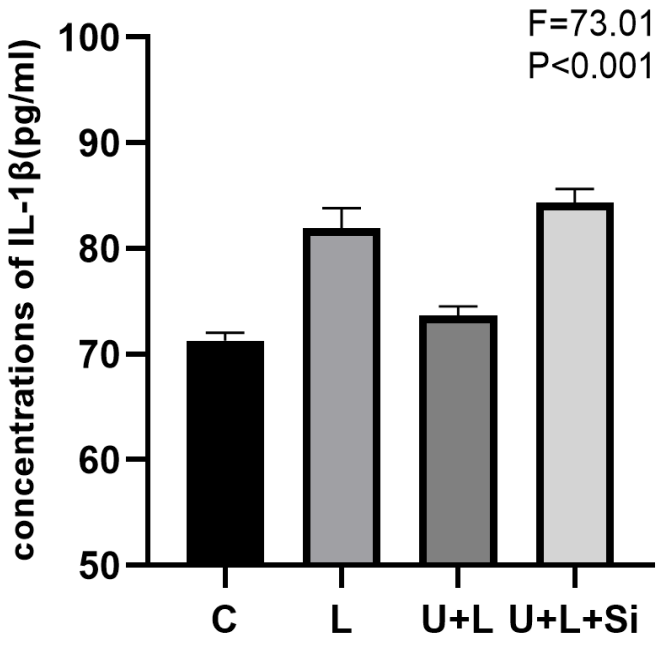

Figure 1: Comparison of IL-1β concentrations in BV2 cells in four groups (n=3). After the expression of A20 in group U+L+Si was inhibited, the protein concentrations of IL-1β was obviously increased in comparison with that in group U+L, and the distinction was obvious (P=0.0003).

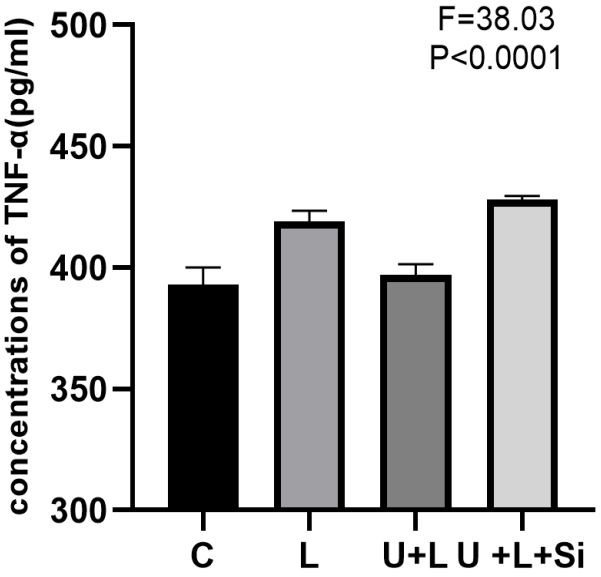

Figure 2: Comparison of TNF-α concentrations in BV2 cells in four groups (n=3). After the expression of A20 in group U+L+Si was inhibited, the protein concentration was obviously increased in comparison with that in group U+L, and the distinction was obvious (P=0.0003)

3.2 Western blot

Western blot assisted in detecting the protein expressions of NF-κB p65 and A20 in the cells of the 4 groups, finding that in comparison with group C, group L presented up-regulated NF-κB p65 and down-regulated A20, and the distinction was obvious (*P*=0.0004, *P*=0.0011); in comparison with group L, group U+L presented up-regulated A20 and down-regulated NF-κB p65, and the distinction was obvious (*P*=0.0005, *P*=0.0068).

In comparison with group U+L,the expression of NF-κB p65 was obviously up-regulated, while the expression of A20 protein was down-regulated in group U+L+Si and the distinctions were obvious (*P*=0.0026,*P*<0.0001). See Figure 3, Figure 4, Figure 5.

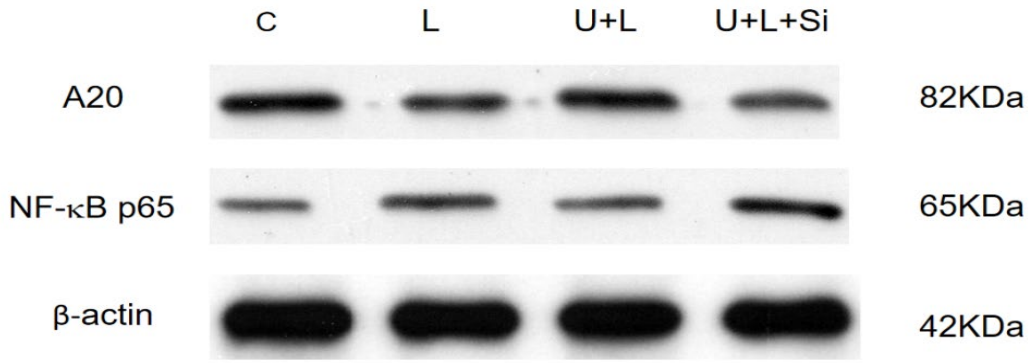

Figure 3: Western blot experiment electrophoresis of A20 and NF-κB p65 expression in four groups of BV2 cells.

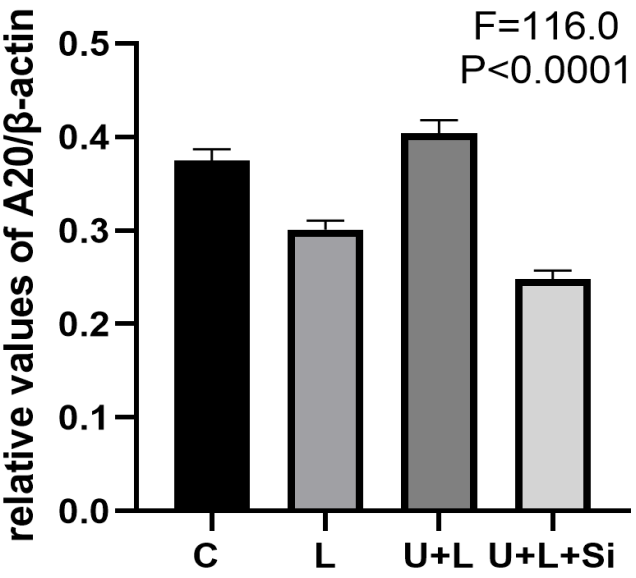

Figure 4: Comparison of the relative values of A20 gray levels in four groups of cells (n=3).In comparison with group U+L, the A20 protein expression presented an obvious down-regulation in group U+L+Si(P<0.0001).

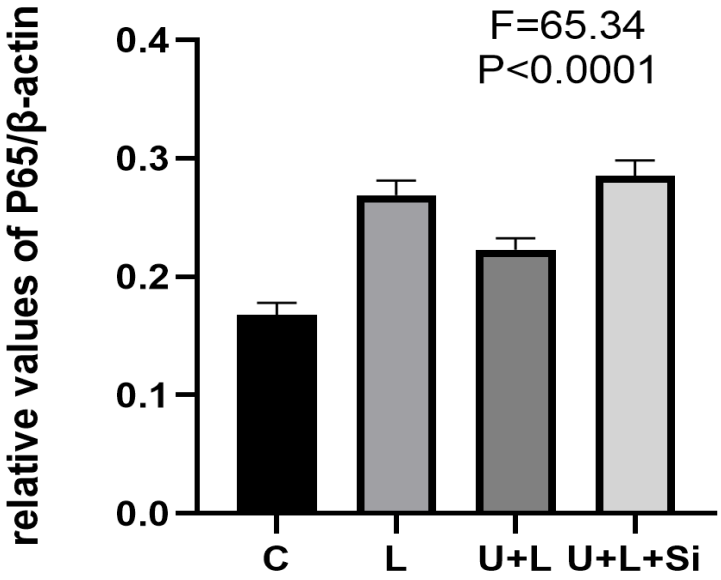

Figure 5: Comparison of the relative values of P65 gray levels in four groups of cells (n=3). In comparison with group U+L, the NF-κB p65 expression presented an obvious up-regulation in group U+L+Si (P=0.0026).

3.3 Immunofluorescence staining

Immunofluorescence staining was performed with anti-Iba-1 (red) antibody, and DAPI (blue) counterstained the nuclei. The expression of Iba-1 in four groups of cells was observed by fluorescence microscope. The results showed that in comparison with group U+L, the expression of Iba-1 in group L was upward adjustment (*P*=0.0026), while that was no different in group U+L+Si (*P*=0.0026). See Figures 6 and 7.

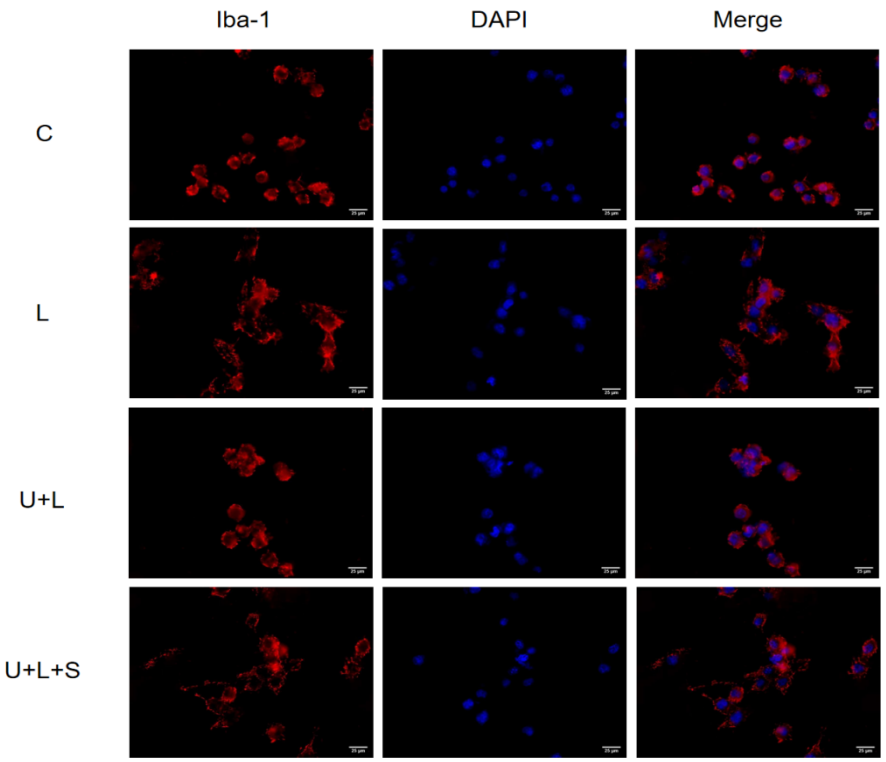

Figure 6: Immunofluorescence image of microglia (immunofluorescence staining × 400)

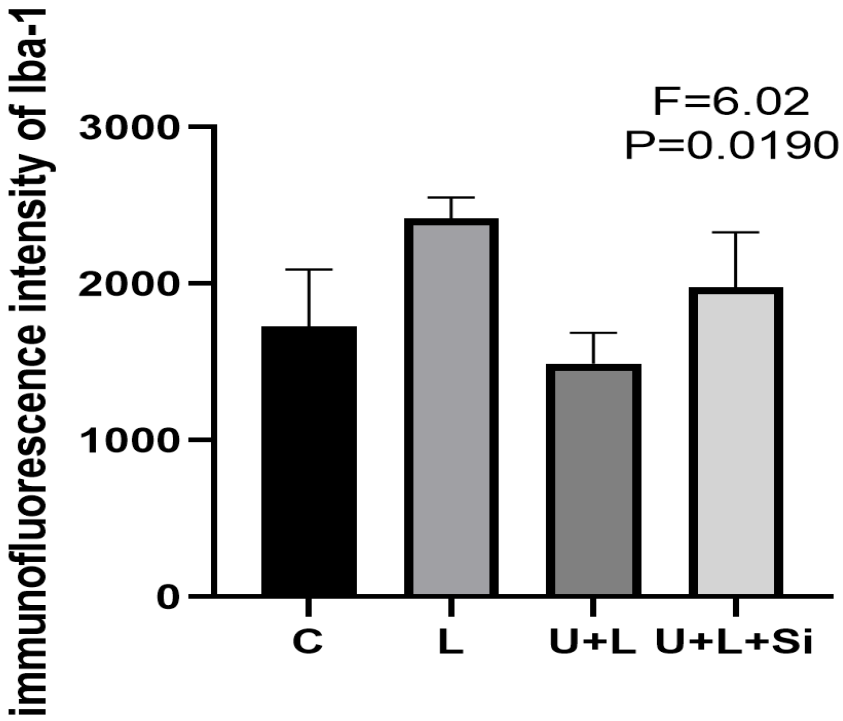

Figure 7: Comparison of Iba-1 immunofluorescence intensity in four groups of cells (n=3). The results showed that in comparison with group U+L, the expression of Iba-1 in group L was up-regulated (P=0.0026), while that was no different in group U+L+Si (P=0.0026) .

4. DISCUSSION

Neuropathic pain (NP) act as a chronic pain resulted from infection-

induced impairment or disfunction of the central or (and) peripheral neurological system, inflammation, trauma and other factors, which is lack of specific treatment (Perga et al., 2021). Epidemiological studies have shown that NP has a prevalence of between 7% and 10% in the population, and unlike acute pain, NP is resistant to nonsteroidal anti-inflammatory drugs and opioids. Due to the difficulty of diagnosis, the poor response of existing painkillers, and poor understanding of the pathogenesis of NP, 40 to 50% of NP patients are not well treated. In recent years, more and more researchers have devoted themselves to the study of the mechanism and treatment of NP.

Recently, more and more studies have revealed the crucial impact of glial cells on NP occurrence and maintenance. Glial cells mainly have 3 types in the central nervous system: microglia, astrocytes and oligodendrocytes. Microglia and astrocytes are mainly studied in pain. After nerve injury or inflammation, astrocytes activate later than microglia, but function is maintained for a longer time. Therefore, microglia activation may impact NP development, and astrocyte activation is related to the maintenance. Under normal circumstances, glial cells are quiescent. After nerve injury, the injured peripheral nerve endings will release cytokines to activate microglia and astrocytes and release a large number of pain-causing factors: Neuropeptides (SP, CGRP), amino acids (glutamate), chemokines, neurotrophic factors (NGF, BDNF), and cytokines (TNF-α, IL-1β, IL-6), etc., thereby more strongly enhancing the central sensitization. At present, the commonly used drugs for treating NP are anticonvulsants and Tricyclic antidepressants. TCAs, selective serotonin and norepinephrine reuptake inhibitors (SSNRIs) and opioids, etc. Nevertheless, there are still some problems such as poor efficacy, many adverse reactions and great individual differences.

More and more evidence demonstrate that immune responses remarkably impact development of neuralgia, especially through neuroinflammation in the central and peripheral nervous systems caused by immune cell activity, which mediates chronic NP development and maintenance 214. Neuroinflammation related to chronic NP features immune cell infiltration in the dorsal root ganglion (DRG) and microglia activation in the spinal cord and brain. Through studies on various animal models of chronic neuralgia, it has been found that CD11b is highly expressed in the spinal cord under pathological conditions, which indicates that microglia in the spinal cord are activated. This is because after nerve injury, microglia form a dense cell population around the ventral horn of the spinal cord, similar to that in the peripheral nervous system. Macrophages encircle injured sensory neurons in the form of larger cell bodies, increased cell branches and antennae, and secrete a variety of cytokines and pain-causing substances to regulate the conduction of pain signals in neurons.

In order to increase the means of clinical treatment of NP, we started from

the idea of inhibiting inflammatory response, thereby inhibiting or treating NP (Ouyang et al., 2016). Ustatin is an endogenous protease inhibitor with anti-inflammatory and cell membrane stabilising effects and is widely used in clinical practice (Wong et al., 2022). Based on previous research results, it has been demonstrated that activation and proliferation of microglia occur earlier than astrocytes after nerve injury(Jensen & Finnerup, 2014), so activation of microglia in the initial stage of neuropathic pain trigger the activation and proliferation of astrocytes(Shi et al., 2017), and the activation of astrocytes by bioactive factors released by microglia has also been confirmed in vitro(Jain, 2008). So the activation pathway of microglia may become a target to prevent the occurrence of neuropathic pain(Chang, Mondshine, Hill, Fleury, & Kleiber, 2022). NF-κB is the main transcription factor of bioactive molecules during the inflammatory response (Röhl, Lucius, & Sievers, 2007). It is one of the key transcription factors related to inflammation discovered so far(Kotliarova & Sidorova, 2021). It induces the expression of various cytokines and chemokines in the inflammatory response of neuropathic pain (Shen et al., 2022). It essentially regulates the expression of many key enzymes, and participates in the pathophysiological process of neuropathic pain (Owoyele et al., 2022). Therefore, how to inhibit the NF-κB activation becomes the key to preventing the occurrence of inflammatory response and treating neuropathic pain. A20, (tumor necrosis factor alpha-inducible protein 3 (TNFAIP3)), is an editing enzyme that ubiquitinates or deubiquitinates its target proteins (Sun, Li, Zhou, Wang, & Liu, 2022). Studies have shown that A20 is activated downstream of NF-κB(Boni, Gross, Gunn, & Levine, 2021; Borgonetti, Meacci, Pierucci, Romanelli, & Galeotti, 2022), and interfering with the ubiquitination of multiple substrates can become a key negative regulator of inflammation by terminating the activation of NF-κB. At the same time, studies have shown that A20 can also inhibit the development of malignant tumors by inhibiting the inflammatory response (Goebeler et al., 2001), and inhibit the attack of allergic asthma and reduce the inflammatory response of ulcerative colitis, etc. Recent studies have confirmed that there is also a regulatory relationship between A20 and microglia in the nervous system, which is relevant to different neurological diseases, like multiple sclerosis. Partial or complete germinal deficiency of A20 results in spontaneous neuroinflammation in puppies with marked reactive microgliosis and astrogliosis.

Therefore, we selected the experimental research subjects, and through the experimental design and analysis of experimental results, we found that when LPS-treated microglia, the expression of A20 decreased, while the concentrations of TNF-α and IL-1β elevated, LPS resulting in up-regulation of the microglial activation marker Iba-1 indicates an increased inflammatory response, whereas when A20 expression is increased, TNF-α and IL-1β protein concentrations decrease and the inflammatory response decreases. In Western blot and immunofluorescence staining, we also confirmed that NF-κB P65 and Ibal decreased as A20 expression increased. Thereby, the increase of A20 expression can inhibit the activation of NF-κB pathway, restricting

inflammatory factor release, preventing the activation of microglia, astrocytes and the destruction of neuronal cells. Through experimental comparison, we found that UTI can increase the protein expression of A20, thereby reducing the protein content of NF-κB P65 in cells. The inhibitory effect on the release of inflammatory factors weakened, and the expression of P65 increased accordingly. ELISA measured the concentrations of TNF-α and IL-1β, which also confirmed that the effect of UTI on inflammatory factor release was inhibited in cells knocked down by A20 obviously. It indicated that UTI restricted the inflammatory factor release by up-regulating the expression of A20 in microglia.

The pathogenesis of NP is complex, with central and peripheral mechanisms forming a huge and complex molecular network. Research on the existing drugs for NP is helpful to select the appropriate treatment plan. Antiepileptic drugs and tricyclic antidepressants are classic drugs for NP, but they have some serious adverse reactions, which need to be monitored and dose adjusted. At present, the existing analgesics have poor efficacy on NP and large adverse reactions. In the absence of effective alternatives, opioid and gabapentin abuse has become a serious problem. Therefore, new painkillers with good efficacy and few side effects shall be developed quickly. There is still a long way to go from a target to a new drug.

Summary and Implications

The study exploring the anti-inflammatory effects of Ulinastatin in LPS-induced BV2 cells by A20 regulation has provided valuable insights into the potential implications for the health and performance of sports and fitness players. The intricate interplay between inflammation and immune response, as well as the role of A20 as a modulator of these processes, has been illuminated through our research.

Throughout the investigation, it became evident that Ulinastatin, a serine protease inhibitor with established clinical applications in treating inflammatory conditions, holds promise as a therapeutic agent for mitigating inflammation in sports and fitness-related contexts. The upregulation of A20 in BV2 microglial cells exposed to LPS, mediated by Ulinastatin, presents a compelling mechanism through which this anti-inflammatory effect may be achieved. This finding suggests that Ulinastatin could potentially be harnessed to enhance recovery, reduce the risk of injuries, and improve overall health and performance for athletes and fitness enthusiasts.

Moreover, the implications of this study extend beyond the laboratory and into the realm of sports medicine and healthcare. By understanding the molecular pathways involved in Ulinastatin's action and its relationship with A20, we pave the way for targeted interventions that could revolutionize the

management of inflammation in the sports and fitness community. These interventions could lead to personalized approaches for athletes and fitness players, tailored to their unique needs and challenges, ultimately optimizing their training regimens and promoting long-term well-being.

In anti-inflammatory effects of Ulinastatin, as mediated by A20 regulation in LPS-induced BV2 cells, offer a promising avenue for further research and application in the field of sports and fitness. As we continue to unravel the complexities of inflammation and immune response, we hold the potential to enhance the quality of life and performance of individuals engaged in physical activities, ushering in a new era of sports and fitness healthcare. This study underscores the importance of scientific inquiry in addressing the evolving needs of athletes and fitness enthusiasts and highlights the exciting prospects that lie ahead in the pursuit of optimal health and performance.

REFERENCES

ARUNACHALAM, M., VIJAYAKUMAR, C., & MAYDEN, R. L. (2017). Two new species of Psilorhynchus (Teleostei: Psilorhynchidae) from Bihar and Uttar Pradesh, India. *FishTaxa, 2*(4), 210-225.

Boni, A. A., Gross, D. M., Gunn, M. A., & Levine, D. S. (2021). Recognizing and Celebrating Innovation and Innovators in Biopharma Table of Contents for Special Edition of the Journal of Commercial Biotechnology. *Journal of Commercial Biotechnology, 26*(2).

Borgonetti, V., Meacci, E., Pierucci, F., Romanelli, M. N., & Galeotti, N. (2022). Dual HDAC/BRD4 Inhibitors Relieves Neuropathic Pain by Attenuating Inflammatory Response in Microglia After Spared Nerve Injury. *Neurotherapeutics, 19*(5), 1634-1648.

Chang, B. L., Mondshine, J., Hill, A., Fleury, C. M., & Kleiber, G. M. (2022). Prevalence of Comorbid Psychiatric Conditions and Chronic Pain in Patients Seeking Peripheral Nerve Surgery. *Plastic and Reconstructive Surgery Global Open, 10*(7).

Goebeler, M., Gillitzer, R., Kilian, K., Utzel, K., Bröcker, E.-B., Rapp, U. R., & Ludwig, S. (2001). Multiple signaling pathways regulate NF-κB–dependent transcription of the monocyte chemoattractant protein-1 gene in primary endothelial cells. *Blood, The Journal of the American Society of Hematology, 97*(1), 46-55.

Jain, K. K. (2008). Current challenges and future prospects in management of neuropathic pain. *Expert review of Neurotherapeutics, 8*(11), 1743-1756.

Jensen, T. S., & Finnerup, N. B. (2014). Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. *The Lancet Neurology, 13*(9), 924-935.

Kotliarova, A., & Sidorova, Y. A. (2021). Glial cell line-derived neurotrophic factor family ligands, players at the interface of neuroinflammation and neuroprotection: Focus onto the glia. *Frontiers in Cellular Neuroscience,*

15, 679034.

Lee, J. Y., Choi, H. Y., Ju, B.-G., & Yune, T. Y. (2018). Estrogen alleviates neuropathic pain induced after spinal cord injury by inhibiting microglia and astrocyte activation. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1864*(7), 2472-2480.

Oh, S. H., So, H. J., Lee, H. Y., Lim, K. J., Yoon, M. H., & Jung, K. T. (2015). Urinary trypsin inhibitor attenuates the development of neuropathic pain following spinal nerve ligation. *Neuroscience Letters, 590*, 150-155.

Ouyang, H., Nie, B., Wang, P., Li, Q., Huang, W., Xin, W., . . . Liu, X. (2016). Ulinastatin attenuates neuropathic pain induced by L5-VRT via the calcineurin/IL-10 pathway. *Molecular pain, 12*, 1744806916646785.

Owoyele, B. V., Bakare, A. O., Olaseinde, O. F., Ochu, M. J., Yusuff, A. M., Ekebafe, F., . . . Roi, T. (2022). Synergistic interaction between acetaminophen and L-carnosine improved neuropathic pain via NF-κB pathway and antioxidant properties in chronic constriction injury model. *The Korean Journal of Pain, 35*(3), 271-279.

Perga, S., Montarolo, F., Martire, S., Bonaldo, B., Bono, G., Bertolo, J., . . . Bertolotto, A. (2021). Overexpression of the ubiquitin-editing enzyme A20 in the brain lesions of Multiple Sclerosis patients: moving from systemic to central nervous system inflammation. *Brain Pathology, 31*(2), 283-296.

Röhl, C., Lucius, R., & Sievers, J. (2007). The effect of activated microglia on astrogliosis parameters in astrocyte cultures. *Brain research, 1129*, 43-52.

Shen, C.-L., Wang, R., Yakhnitsa, V., Santos, J. M., Watson, C., Kiritoshi, T., . . . Neugebauer, V. (2022). Gingerol-Enriched Ginger Supplementation Mitigates Neuropathic Pain via Mitigating Intestinal Permeability and Neuroinflammation: Gut-Brain Connection. *Frontiers in Pharmacology*, 2546.

Shi, Y., Qin, W., Nie, F., Wen, H., Lu, K., & Cui, J. (2017). Ulinastatin attenuates neuropathic pain via the ATP/P2Y2 receptor pathway in rat models. *Gene, 627*, 263-270.

Sun, X., Li, X., Zhou, Y., Wang, Y., & Liu, X. (2022). Exogenous TIPE2 Inhibit TAK1 to Improve Inflammation and Neuropathic Pain Induced by Sciatic Nerve Injury Through Inactivating NF-κB and JNK. *Neurochemical Research, 47*(10), 3167-3177.

Tyrtyshnaia, A. A., Egorova, E. L., Starinets, A. A., Ponomarenko, A. I., Ermolenko, E. V., & Manzhulo, I. V. (2020). N-Docosahexaenoylethanolamine attenuates neuroinflammation and improves hippocampal neurogenesis in rats with sciatic nerve chronic constriction injury. *Marine drugs, 18*(10), 516.

Ullah, R., Ali, G., Rasheed, A., Subhan, F., Khan, A., Halim, S. A., & Al-Harrasi, A. (2022). The 7-Hydroxyflavone attenuates chemotherapy-induced neuropathic pain by targeting inflammatory pathway. *International*

Immunopharmacology, 107, 108674.

Weir, T. (2017). Clear aligners in orthodontic treatment. *Australian dental journal, 62*, 58-62.

Wong, C.-E., Hu, C.-Y., Lee, P.-H., Huang, C.-C., Huang, H.-W., Huang, C.-Y., . . . Lee, J.-S. (2022). Sciatic nerve stimulation alleviates acute neuropathic pain via modulation of neuroinflammation and descending pain inhibition in a rodent model. *Journal of Neuroinflammation, 19*(1), 1-14.

Xue, S., Cao, Z.-x., Wang, J.-n., Zhao, Q.-x., Han, J., Yang, W.-j., & Sun, T. (2022). Receptor-Interacting Protein Kinase 3 Inhibition Relieves Mechanical Allodynia and Suppresses NLRP3 Inflammasome and NF-κB in a Rat Model of Spinal Cord Injury. *Frontiers in Molecular Neuroscience, 15*.