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The anti-inflammatory activity of Glycyrrhizin in LPS-stimulated Raw264.7 macrophage cell line

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Abstract: The main sweet-tasting bioactive component of licorice (the root of Glycyrrhiza glabra) is glycyrrhizin (GL), which has poor oral absorption. Gut microbes convert GL to glycyrrhetinic acid (GA), which has a variety of pharmacological effects. The aim of this study was to investigate the potential anti-inflammatory effects of glycyrrhizic acid (GA), which is found in licorice roots and rhizomes, on macrophages that were stimulated by lipopolysaccharide (LPS). The results indicated that GA reduced the levels of two specific proteins (STAT3 and NF-κB) in LPS-induced macrophages, and suppressed the expression of pro-inflammatory genes, including TNF-α and INF-γ. These findings suggest that GA has anti-inflammatory properties and may be beneficial in treating inflammatory diseases by inhibiting the activity of STAT3 and NF-κB.

keywords: Glycyrrhizin, NF-κB, anti-inflammatory activity; lipopolysaccharide

1. Introduction

The body's initial defense against harmful stimuli like infections and poisons is inflammation, which is a biological response. Macrophages, which are cells that help protect against infections, play an important role in this response by detecting lipopolysaccharides (LPS) found on the outer membrane of bacterial toxins. This triggers a chain of events that mobilizes the body's defense against the infection [1]. Macrophages, when activated by pathogens, trigger the activation of nuclear factor-κB (NF-κB) which leads to the production of various inflammatory mediators such as ROS (reactive oxygen species), prostaglandins (PG), cytokines, (TNF-α), nitric oxide, (NO) and other eicosanoid mediators. These mediators are responsible for promoting inflammation and causing cellular injury.[2]. STAT3 activation is involved in several infectious agents that are known to cause inflammation-induced cancer and these agents likely rely on STAT3 for their carcinogenic potential [3].

New bioactive natural chemicals with anti-inflammatory properties are currently being discovered and developed. by a rising public preference for plant food components over traditional medication therapies in the prevention and treatment of chronic inflammatory diseases. Scientific studies have shown that a variety of plant extracts and isolated chemicals have anti-inflammatory effects by modifying the levels of inflammation-related gene expression.[4] The licorice root and rhizome contain the pentacyclic triterpenoid glycyrrhizinic acid (glycyrrhizin, GN) (*Glycyrrhiza glabra*) [5]. It has been shown to have a variety of pharmacological actions, including anticancer, antioxidant, antiviral, anti-allergic, anti-inflammatory, and antibacterial properties. It is made up of a diglucuronic acid connected to a triterpenoid aglycone glycyrrhetinic acid (GA) [6]. Triterpenoids are used as anti-inflammatory, analgesic, antipyretic, hepatoprotective, cardiotonic, sedative, and tonic medications in many Asian countries [7]. The objective of this research was to examine the effectiveness of various pentacyclic triterpenoids, including glycyrrhizin, in reducing inflammation.

2. The materials and methods used in the study

2.1. Chemicals and reagents of cell culture

RPMI 1640 medium (Cegrogen Biotech GmbH, Stadtallendorf, Germany, E0500-380), Lipopolysaccharide (LPS; Sigma, Cat no. 297-473-0), FBS, and Penicillin-Streptomycin, ELISA kits were also used for detecting TNF-α and IFN-γ. The primary rabbit monoclonal antibodies against STAT3 and β-actin were obtained from Cell Signaling Technology, while the NF-κB antibody was purchased from Biospes. Dilution ratios were 1:1000 and 1:800 for STAT3/β-actin and NF-κB, respectively.

2.2. Cell culture

The RAW264.7 macrophages cell line (ATCC, Cat. No. TIB-71) was obtained from the American Tissue Culture Collection. The cells were cultured in RPMI-1640 medium with

15% FBS and 1% penicillin-streptomycin at 37°C and 5% CO2 until they reached full confluence. The cells were then washed and collected using a scraper.

2.3. Enzyme linked immunosorbent assay.

Raw264.7 cells were added to a 96-well plate at a concentration of 1×10^5 cells/mL. The next day, the cells were exposed to glycyrrhizin at a concentration of 50 µM for 3 hours, followed by treatment with LPS at a concentration of 5 µg/mL for 24 hours, or they were treated with LPS alone for 24 hours. After the incubation period, the culture medium was removed and spun at 3000 rpm for 20 minutes. The supernatant was collected, and the levels of TNF-α and IFN-γ were measured in the sample to evaluate their expression.

2.4. Western blot assay

Raw264.7 cells were exposed to glycyrrhizin for 24 hours, followed by treatment with LPS at a concentration of 5 µg/mL for 24 hours, or they were treated with LPS alone for 24 hours. After the 24-hour incubation with glycyrrhizin, the cells were lysed using a 1x RIPA buffer that contained 1x protease and phosphatase inhibitor. The supernatant was collected, and the protein concentration was determined using the Pierce™ BCA Protein Assay Kit. A mixture of different proteins was separated using SDS-PAGE (15%) at a voltage of 120 V for a duration of 60 minutes. The protein (20 µg/µL) was transferred to a nitrocellulose membrane for 70 min at 90 V. Primary antibody against STAT3 (CST, #9132, 1:1000 dilution) and NF-κB (BBP1066, 1:800 dilution) were added to the membrane after blocking then incubated overnight. The secondary antibody anti-rabbit HRP-conjugated was applied to the membrane and was detected by WesternBright™ ECL. The signal was recorded by a ChemiDoc BioRad documentation system.

3. Results and Discussion

3.1. The impact of glycyrrhizin on the expression of pro-inflammatory cytokines induced by LPS

The study investigated the impact of glycyrrhizin on the production of pro-inflammatory cytokines, specifically TNF-α and INF-γ, induced by LPS in RAW 264.7 cells. ELISA was used to analyze cytokine expressions. The results demonstrated that the concentrations of TNF-α and INF-γ were significantly lower in the cells that were pre-treated with glycyrrhizin and then treated with LPS, as compared to the cells that were only treated with LPS. (Figure 1).

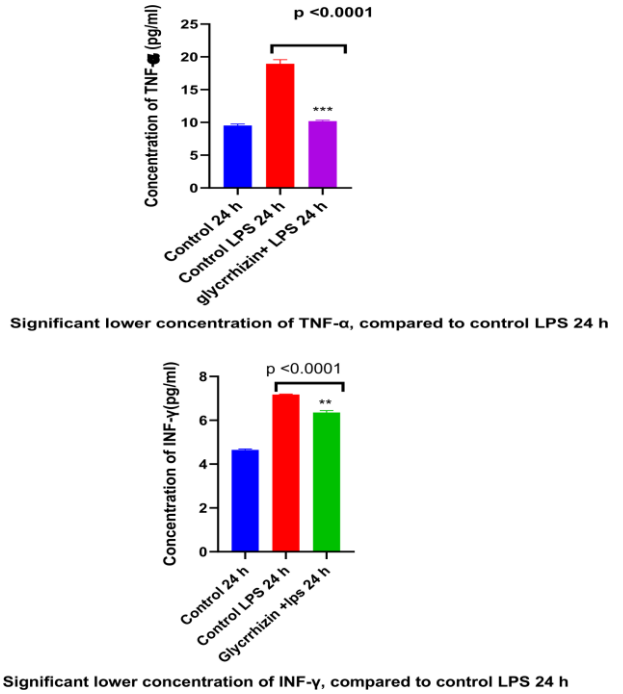

Figure 1: The study investigated how glycyrrhizin affects the concentration of TNF-α and INF-γ in cell cultures. The data, which represents the mean ± standard deviation (n = 3), shows that there were significant differences between the control LPS group and the glycyrrhizin-treated groups. Specifically, the

results indicate that glycyrrhizin had a statistically significant effect in reducing the production of pro-inflammatory cytokines, with p-values of less than 0.001 and 0.0001.

3.2. The impact of glycyrrhizin on the expression of STAT3 and NF-κB protein in response to LPS stimulation.

The study aimed to understand how glycyrrhizin's anti-inflammatory actions are related to the NF-κB and STAT3 pathways by analyzing the expression of related proteins. The findings indicate that in macrophage cells treated with glycyrrhizin, the levels of NF-κB and STAT3 proteins were reduced compared to the LPS control group. This suggests that the anti-inflammatory effects of glycyrrhizin may be related to its regulation of the NF-κB and STAT3 pathways (Figure 2).

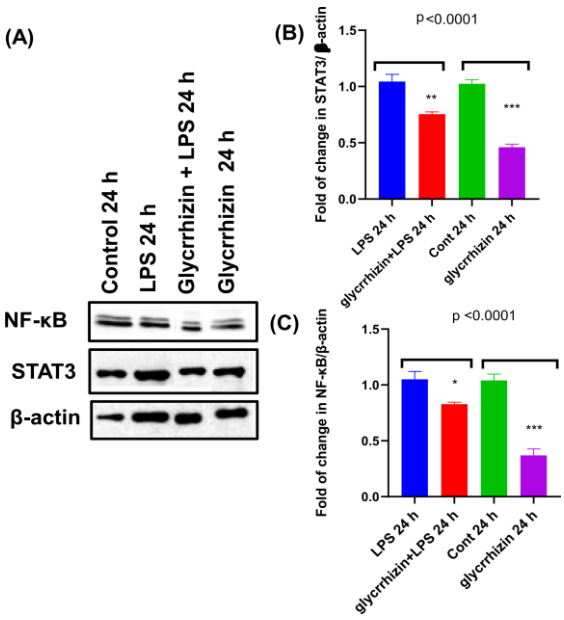

Figure 2: The expression of proteins related to the STAT3 (B) and NF-κB (C) pathways in RAW 264.7 cells treated with LPS were suppressed by glycyrrhizin. The results are shown as the average value with standard deviation (n=3); statistical analysis revealed significant differences (*P < 0.05, **P < 0.001, ***P < 0.0001) between the glycyrrhizin treated group and the control group treated with LPS.

4. Discussion

According to epidemiological studies, the occurrence of inflammatory illnesses linked to various diseases is inversely correlated with consuming a diet high in plant nutraceuticals. [8]. GA is considered to be the main physiologically active component, along with the triterpene saponins, flavonoids, isoflavonoids, and chalcones that have been found from licorice. [9]. Previously, we stated that GA exhibits anti-inflammatory properties. In the current investigation, we show that GA decreases STAT3 and NF-κB protein levels as well as TNF-α and INF-γ production at nontoxic dosages (50 μM).

Inflammatory cytokines, nitrogen reactive species, and large amounts of free radicals are typically created in conjunction with inflammatory disorders. The overproduction of pro-inflammatory cytokines by macrophages, such as TNF-α and INF-γ, which depends on the coexistence of TNF-α responsive NF-κB signal transducers and activators of transcription protein binding sites inside the gene promoter, may disrupt cellular macromolecules. [10, 11] It is likely that GA interferes with TNF-α and INF-γ transcription and causes levels of these cytokines to drop after NF-κB activation because GA inhibits LPS-induced TNF-α and INF-γ synthesis.

Important intracellular signaling pathways like NF-κB and STAT3 are activated by LPS, and as a result, many inflammatory mediators and cytokines, including COX-2 and iNOS, are produced [12, 13]. A widely present transcription factor called nuclear factor-kappa B controls large number of other genes involved in cell survival, proliferation, and differentiation. Numerous triggers can cause NF-κB to become active. The transcription of numerous genes, including TNF-α, as well as enzymes like COX-2 and iNOS, is then activated by the unbound form of NF-κB, which translocate to the nucleus [14-16].

In conclusion, our research has demonstrated that GA has protective effects by preventing the generation of TNF-α and INF-γ and reducing STAT3 and NF-κB protein levels. Thus, this substance might be helpful in the treatment of inflammatory diseases.

5. References

1. Bhattacharyya, J., S. Biswas, and A.G. (2004)Datta, Mode of action of endotoxin: role of free radicals and antioxidants. Current medicinal chemistry,. **11(3):** p. 359-368.

2. Yen, G.-C., et al., (2008) Protective effect of pine (Pinus morrisonicola Hay.) needle on LDL oxidation and its anti-inflammatory action by modulation of iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages. Food and Chemical Toxicology,. **46(1)**: p. 175-185.
3. Yu, H., D. Pardoll, and R. Jove, (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. Nature reviews cancer,. **9(11)**: p. 798-809.
4. Darshan, S. and R. Doreswamy, (2004) Patented antiinflammatory plant drug development from traditional medicine. Phytotherapy Research: An *International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*,. **18(5)**: p. 343-357.
5. Graebin, C.S., (2018) The pharmacological activities of glycyrrhizinic acid ("glycyrrhizin") and glycyrrhetinic acid. Sweeteners,: p. 245.
6. Wei, B., et al., (2018). Characteristics and molecular determinants of a highly selective and efficient glycyrrhizin-hydrolyzing β-glucuronidase from Staphylococcus pasteuri 3I10. Applied microbiology and biotechnology, **102(21)**: p. 9193-9205.
7. Ovesna, Z., et al., Pentacyclic triterpenoic acids: new chemoprotective compounds Minireview. Neoplasma, 2004. **51(5)**: p. 327-333.
8. Mueller, M., S. Hobiger, and A. Jungbauer, (2010) Anti-inflammatory activity of extracts from fruits, herbs and spices. Food chemistry,. **122(4)**: p. 987-996.
9. Asl, M.N. and H. Hosseinzadeh, (2008) Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds. Phytotherapy Research: An International *Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*,. **22(6)**: p. 709-724.
10. An, S., et al. (2002), Inhibition of TNF-α, IL-1β, and IL-6 productions and NF-κB activation in lipopolysaccharide-activated RAW 264.7 macrophages by catalposide, an iridoid glycoside isolated from Catalpa ovata G. Don (Bignoniaceae). International Immunopharmacology,. **2(8)**: p. 1173-1181.
11. Lee, K.H., A.J. Kim, and E.M. Choi, (2009) Antioxidant and antiinflammatory activity of pine pollen extract in vitro. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives,. **23(1)**: p. 41-48.
12. Guha, M. and N. Mackman, (2001). LPS induction of gene expression in human monocytes. Cellular signalling, **13(2)**: p. 85-94.
13. Ahuja, A., et al., (2020) STAT3 differentially regulates TLR4-mediated inflammatory responses in early or late phases. *International Journal of Molecular Sciences*,. **21(20):** p. 7675.
14. Giuliani, C., I. Bucci, and G. Napolitano, (2018)The role of the transcription factor nuclear factor-kappa B in thyroid autoimmunity and cancer. Frontiers in endocrinology,. **9:** p. 471.
15. Shih, R.-H., C.-Y. Wang, and C.-M. Yang, (2015) NF-kappaB signaling pathways in neurological inflammation: a mini review. Frontiers in molecular neuroscience,. 8: p. 77.
16. Ahn, K.S. and B.B. Aggarwal, (2005)Transcription factor NF-κB: a sensor for smoke and stress signals. Annals of the new York Academy of Sciences,. **1056(1):** p. 218-233.