Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Glucocorticoids promote joint microenvironment alteration of GABBR1 expression associated with mitigating rheumatoid arthritis

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Article Info

Article history:

Received: January 15, 2024
Accepted: April 13, 2024
Published: June 30, 2024

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Abstract

GABBR1 receptors have been implicated in the progression of rheumatoid arthritis (RA), and p38 MAP kinase (MAPK) was shown to be downregulated by GABA and result in unchecked production of pro-inflammatory cytokine. GABBR1 is a member of GABA receptors, and it is known to be upregulated and plays a vital role in RA. Glucocorticoids are efficient therapeutics in rheumatoid arthritis (RA) and are known to regulate GABA actions; therefore, we intended to investigate the potential of glucocorticoids in RA concerning the potential pathway GABBR1/MAPK. Joint specimens were obtained from collagen-induced arthritis mouse model. A double-blind semi-quantitative analysis of vascularity, cell infiltration, as well as lining thickness by help of a 4-point scale setting was used to assess joint inflammation. Expression of GABBR1 and p38 was evaluated immunohistochemically. In vitro peripheral blood (PB), synovial fluid (SF), and mononuclear cells (MCs) were acquired from RA mice. Western blotting was used for detecting expression of GABBR1 and p38 proteins. The presence of high levels of GABBR1 and p38 was prevalent in RA joints relative to healthy joints and related to the inflammation level. Glucocorticoid treatment alters GABBR1 along with p38 protein expression in joints while reducing joint inflammation. Ex vivo and in vitro assays revealed glucocorticoids have a direct impact on p38, such as the decreased GABBR1 expression level after dexamethasone incubation with SFMC. GABBR1 together with p38 expression in RA joints depends on local inflammation and can be targeted by glucocorticoids.

Keywords: Rheumatoid arthritis, Glucocorticoids, GABBR1, P38, Joint microenvironment.

1. Introduction

Rheumatoid arthritis (RA) belongs to a symmetrical, chronic, and autoimmune inflammatory disorder that primarily influences the small joints and ultimately the skin, heart, eyes, lungs, and kidneys. The joints' bone and cartilage often deteriorate, and the tendons and ligaments are weakened [1]. This level of joint damage causes deformability and bone defacement, usually excruciating for patients. The common RA symptoms include fatigue, weight loss, fever, and stiffness of the affected joints; the affected joints tend to be swollen, tender, as well as warm, with rheumatoid nodules below the skin. The onset of RA generally occurs between 35 and 60 years. RA can also occur in children under the age of 16, called juvenile RA (JRA), which is analogous to RA but with absent rheumatoid factor [2-5]. The prevalence of RA in the West is considered to be 2% [2, 5], as well as 1% globally [6].

In the recent era of targeted therapy, the first-line management of RA has been primarily dependent on nonsteroidal anti-inflammatory drugs (NSAIDs) as well as corticosteroids [7]. The overall goal of major therapy is to alleviate pain as well as lessen inflammation-NSAIDs are fast-acting drugs in RA. Therapeutic approaches and management of comorbidities in RA mice have spotlighted the future position glucocorticoids (GCs) may have in RA. So far, glucocorticoids are more potent anti-inflammatory drugs than NSAIDs, but they possess significant adverse effects. Although GCs therapy was a significant therapeutic for RA during the twentieth century, the current emphasis is on the proper application of GCs to avoid its adverse side effects. For this reason, recent investigations reported that a low dose of GCs has a significant therapeutic effect on RA and during flares or exacerbations of RA [8-10]. Glucocorticoids act by decreasing the actions of eosino-

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.6.12

phils and preventing the release of phospholipids, thereby reducing inflammation. Their side effects include weight gain, bone-thinning, immunosuppression, and diabetes. It is crucial not to abruptly discontinue GCs as this can result in hypothalamic-pituitary-adrenal axis (HPA) suppression or RA flares [11].

The search for a novel target for the treatment of RA has been surging in the past decade. Impaired GABA function may enhance intemperate inflammation. This dysfunction may happen at a particular GABA receptor. $GABA_B$ receptors release prolonged repressive signals, which are more likely implicated in chronic inflammatory responses. GABBR1 and GABBR2 encode the subunits of the heterodimer $GABA_B$. GABBR1 is encoded in the Major Histocompatibility Complex (MHC) extended class I region (6p21.3) [12], a location of the MHC linked to MS, Alzheimer's disease, epilepsy, narcolepsy, as well as RA. While MHC class II, specifically HLA-DRB1 alleles, strongly contribute to RA susceptibility. The experimental characterization of GABBR1 polymorphisms in RA mice has not been made yet, but silico analyses imply that some may influence protein structure [13] or alternative splicing [14]. The inhibition of p38 MAP kinase (MAPK) was reported to reduce joint inflammation in the rat model of RA by an unclear mechanism [15]. It suppressed the pro-inflammatory mediators IL-1, IL-6, and MMP3 in the periphery [15]. GABBR1 and MAPK14 are likely to be regulated together since they are encoded on 6p21.3, typical for functionally linked genes within the MHC [16, 17]. Since GABA suppresses inflammation and requires a receptor to transmit signals, GABBR1 polymorphism may impair downstream inhibition of p38 MAPK, resulting in the production of pro-inflammatory agents containing IL-1, IL-6, as well as MMP3 agents implicated in RA [18]. Also, an inefficient GABA signaling system leads to uncontrolled production of pro-inflammatory cytokine via the p38 MAPK pathway [18].

Since glucocorticoids are known to regulate glutamate and GABA [19], we investigated the therapeutic role of glucocorticoids (dexamethasone, DMX) in RA concerning the potential GABBR1/p38 MAPK pathway.

2. Materials and methods

2.1. RA mouse model

The 8-week-old C57BL/6 mice obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. were used to construct the mouse model of RA. Animal experiments using rodents were performed under the Guideline of Institutional Animal Care and Use Committee of the Affiliated Hospital of Nanjing University of Chinese Medicine. Type II collagen (4 mg/ml; #C8000; Solarbio, Beijing, China) from bovine nasal septum is dissolved in 50 mM acetic acid and emulsified with the same volume of Freund's complete adjuvant (#P2036; Beyotime). Mice were stimulated with RA by intradermal injection at the bottom of the tail of 100 μL (4 mg/ml) emulsion solution at day 0 and a booster intraperitoneal injection of 100 μL emulsion solution at day 21. Mice in RA group were intraperitoneally injected with 1 mg/kg dexamethasone or Methotrexate daily. After sacrificing the mice, ankle joints were isolated from each mouse. Peripheral blood mononuclear cells (PBMCs) were isolated from mice eyeball blood with mouse lymphocyte isolation solution (TBD, Tianjin, China).

2.2. Joint biopsies processing

Joint specimens were instantly frozen in isopentane and cooled with dry ice. Sequential cryostat sections (7 μm) were immobilized with 4% (vol/vol) paraformaldehyde for 20 minutes and stored at -80°C.

2.3. Cell culture

Synovial fluid mononuclear cells (SFMCs), as well as paired peripheral blood mononuclear cells (PBMCs) from RA mice (n = 6), were cultivated in RPMI 1640 containing heat-inactivated fetal bovine serum (FBS, 20%), L-glutamine (2 mM), Penicillin-Streptomycin Solution (50 μg/ml) (#PB180120; Procell Life Science&Technology Co., Ltd., Wuhan, China) at 37°C and 5% CO_2 in a humidified atmosphere. Cells were cultured/grown until they reached a confluence of 1×10^6 per ml, followed by cultivating with dexamethasone (1 and 100 μM) for twenty-four hours. Next, cells could be resuspended in PBS and then planted on Falcon® glass slides (Corning). Adhered cells were immobilized for twenty minutes with 4% (vol/vol) paraformaldehyde (#P0099, Beyotime, Shanghai, China).

2.4. Immunohistochemical and histological analysis

The protein levels of GABBR1 and p38 were detected with a mouse IgM monoclonal antibody (GABBR1 and p38 MAPK). To detect GABBR1 expression, we used a rabbit polyclonal antibody (#ab238130, Abcam, Shanghai, China) to identify its expression. An appropriate negative control was used with each employed antibody.

The two experts used a 4-point scale to evaluate the presence of GABBR1 and p38 proteins in joint tissues on a double-blind scale (0 points without staining; Low staining, 1; Moderate staining, 2; High staining, 3). Parallelly, the histologic scoring of the inflammation degree was analyzed via a 4-point scale in a double-blind semi-quantitative analysis of the infiltration level, lining thickness, and vascularity in serial H&E staining. In all examinations, the final scores represent the mean of the 2 observations by the experts. The presence of GABBR1 and p38 proteins in PBMCs as well as SFMCs was evaluated by manual counting of positive cells and exhibited to be the percentage of positive cells/total number of cells.

2.5. Western blotting

Cells were lysed with RIPA lysis buffer which included 1 μL protease inhibitor along with 1 μL phenylmethanesulfonyl fluoride. Following denaturation, protein samples were isolated with SDS-PAGE, followed by shifting to PVDF membranes. Next, the membranes were cultivated with primary antibodies which contained anti-GABR1, anti-p38, and anti-GAPDH (Abcam) and subsequent secondary antibody HRP-labeled IgG (Abcam). The enhanced chemiluminescence kit (Cell Signaling Technology, USA) was adopted for measurement.

2.6. Statistical analysis

Mann-Whitney test was used for analyzing independent samples. No Bonferroni correction was implemented as all comparisons were planned. The Fisher Exact test was employed to analyze the difference between proportions. *In vitro* results were analyzed via one-way analysis of variance. $P<0.05$ meant statistical significance.

3. Results

3.1. High expression of GABBR1 and p38 is likely correlated with RA progression

Previously, it was hypothesized that GABA and p38 MAPK are correlated in RA pathogenesis [18]. Therefore, we investigated this hypothesis in RA mice samples. Immunohistochemical analysis of the joint tissue samples unveiled that GABBR1, as well as p38 expression, presented higher in RA joint relative to the healthy joint (Figure 1).

3.2. The expression of GABBR1 and p38 is correlated with the degree of inflammation

As expected, GABBR1 and p38 were related to the degree of cell infiltration, lining thickness, as well as vascularity (Table 1).

3.3. Intraarticular GC, but not MTX, declines the expression level of GABBR1 and p38 in joint tissues

The noted correlation of inflammation and GABBR1 and p38 reminded us to probe the impact of anti-rheumatic treatment on joint GABBR1 and p38 expression. We found that the local administration of GC significantly lessened GABBR1 expression from an average score of 2.17 to an average score of 1.00. p38 expression was also declined after treatment from an average score of 1.67 to an average score of 0.67 (Figure 2). The GC impact on GABBR1 along with p38 expression was paralleled by a reduction in cell infiltration, and in the lining thickness but not vascularity. All patients' joint inflammation samples during arthroscopy were analyzed microscopically and showed that all patients displayed good clinical responses as concluded from the retrospective scoring of microscope images. In contrast, methotrexate treatment did not affect joint inflammation or local expression of GABBR1 along with p38 (Figure 3).

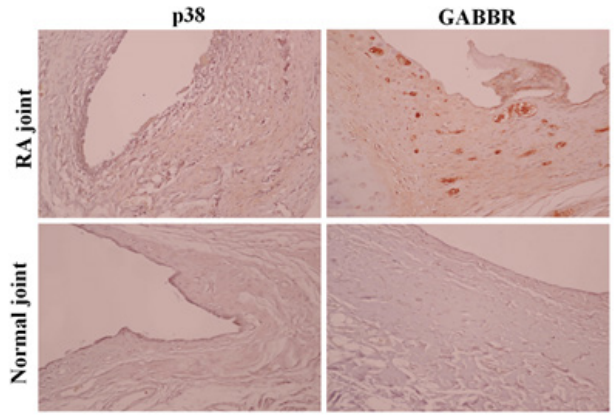

Fig. 1. Representative microscopic images show higher expression of p38 and GABBR1 in RA (upper panel) relative to healthy (lower panel) joint tissues. Frozen joint specimens showed diaminobenzidine (brown) immunoperoxidase staining (hematoxylin counterstained) for p38 as well as GABBR1.

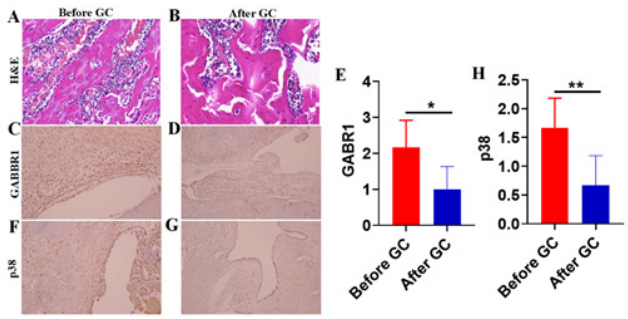

Fig. 2. Intraarticular glucocorticoid (GC) treatment lessens joint expression of GABBR1 along with p38. Hematoxylin-eosin (HE) staining illustrated the histologic pattern of frozen joint specimens of an RA mouse before (A) as well as after (B) intraarticular GC treatment. (C) Brown diaminobenzidine immunoperoxidase staining showed a reduced GABBR1 expression before GC treatment and (D) GABBR1 after GC treatment. (E) Semi-quantitative analysis for GABBR1 proteins. (F) Brown diaminobenzidine immunoperoxidase staining showed a reduced p38 expression before GC treatment and (G) p38 after GC treatment. (H) Semi-quantitative analysis for p38 proteins. *P<0.05, **P<0.01.

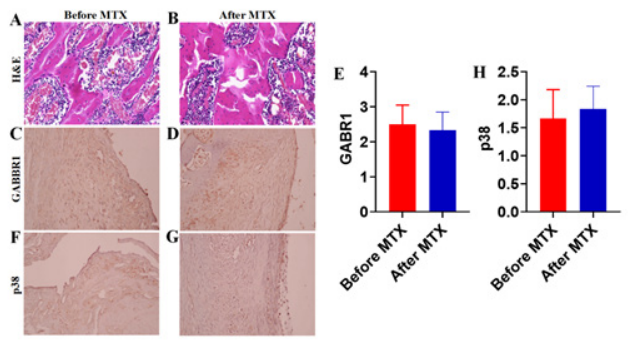

Fig. 3. Methotrexate (MTX) treatment does not affect the joint expression of GABBR1 and p38 proteins. Hematoxylin-eosin (HE) staining illustrated the histologic pattern of frozen joint biopsy sections of an RA mouse before (A) as well as after (B) intraarticular MTX treatment. (C) Brown diaminobenzidine immunoperoxidase staining showed no change on GABBR1 expression before MTX treatment and (D) GABBR1 after MTX treatment. (E) Semi-quantitative analysis for GABBR1 proteins. (F) Brown diaminobenzidine immunoperoxidase staining showed a reduced p38 expression before MTX treatment and (G) p38 after MTX treatment. (H) Semi-quantitative analysis for p38 proteins.

3.4. *In vitro* GCs have directly affected the cellular expression of p38 through GABBR1-dependent mechanism

Away from the anti-inflammatory effects of GCs, they may also directly target GABA receptors and their downstream signaling. To assess this, the impacts of DXM

Table 1. Relation between p38 and GABBR1 expression and joint inflammation.

	Mean lining thickness	Infiltration	Vascularity
Total p38	0.4 (< 0.05)	0.5 (< 0.001)	0.6 (< 0.001)
Intracellular p38	0.4 (< 0.05)	0.4 (0.001)	0.6 (< 0.01)
GABBR1	0.5 (< 0.05)	0.6 (< 0.001)	0.4 (< 0.05)

Value was represented by the Spearman rank correlation coefficient (P value).

on GABBR1 along with p38 protein expression were examined in PBMC as well as SFMC paired samples of RA mice. It was discovered that DXM treatment suppressed GABBR1 and p38 expression in SFMC (Figure 4) rather than PBMC (data not shown). Besides, methotrexate did not affect the protein expressions of GABBR1 and p38 when tested in PBMC (Figure 5).

4. Discussion

RA belongs to a prevalent systemic inflammatory disorder that gives rise to chronic joint inflammation, abrasion, as well as joint damage in some patients. The etiology of RA remains obscure but environmental factors might trigger its onset under the background of genetic predisposition [20]. Within that intricate system, we proposed a novel mechanism in which gamma-aminobutyric acid receptor (GABA), especially GABBAR1, downregulated p38 MAPK activity, led to the suppression of cell infiltration, vascularity, and lining thickness in joints affected by RA.

Both MAPK14 and GABBR1 are encoded on 6p21.3, and their expression might be modulated at the same time, as this usually occurs on function-linked genes near or within the MHC [16, 17]. Due that GABA is engaged in the downregulation of inflammation through its receptors, a polymorphism GABBR1 might repress downstream suppression of p38 MAPK [18]. Inhibitory signaling such as GABA promotes the downregulation of p38 MAPK and limits pro-inflammatory cytokines production [21]. This could stop RA from worsening due to excessive pro-inflammatory cytokines. Any alterations that influence this negative modulation, containing haplotype or SNP allele in GABBR1, might promote the uncontrolled production of pro-inflammatory cytokines of p38 MAPK and worsen RA. Therefore, the focus on GABBR1 is feasible to elucidate the underlying mechanism of RA.

Glucocorticoids (GCs) have been shown to exhibit anti-inflammatory characteristics and have been shown to alleviate RA significantly. Local GC treatment was reported to decrease joint citrullination in rheumatoid arthritis [22]. However, the fundamental role of GCs is to inhibit the production of pro-inflammatory cytokines [23], yet, novel GCs ought to function through distinct pathways. Therefore, we utilized dexamethasone in our investigations to investigate the potential novel mechanism of GCs in treating RA.

Our investigations have found that GABBR1 and p38 are prevalent in RA tissue samples. Interestingly, our dexamethasone treatment suppressed the expression levels of GABBR1 and p38. These findings indicate possible crosstalk between GABBAR1 and p38; nevertheless, the exact mode of action remains unknown.

Inhibited p38 MAPK can restrict joint inflammation [24,25], while GABA is able to decline p38 MAPK signaling [21]. Nevertheless, the essentiality of GABBR1 in this process seems to act in a distinct mechanism.

5. Conclusion

We hypothesize that GABBR1 might impact RA progression through the p38 MAPK pathway and that GC treatment suppresses GABBR1 expression and subsequently inhibits p38. One difficulty in relating the direct GABBR1 inhibition to the downregulation of p38 and expertise is required to assess his dilemma. While numerous researchers have focused on immunological or neurological factors of RA, few laboratories have been engaged to reveal novel mechanisms of rheumatic disorders. Our findings shed light on a novel mechanism that will help better understand the GC mode of action in RA and the possible mechanism of GABA receptors in regulating inflammatory responses. Furthermore, the present study requires additional work on GABBR1 receptor signaling on RA development and to establish detailed settings to elucidate whether GABBR1 directly affects p38 after the treatment with GC or via alternative mechanisms.

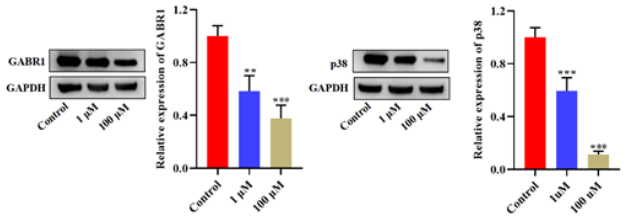

Fig. 4. Dexamethasone (DXM) lessens GABBR1 and p38 protein expression in SFMCs through a dose-dependent way. Enhanced chemiluminescence kit detects the expression levels of GABBR1 and p38 proteins in SFMCs after treatment of 1 μM DXM or 100 μM DXM. **P<0.01, ***P<0.001.

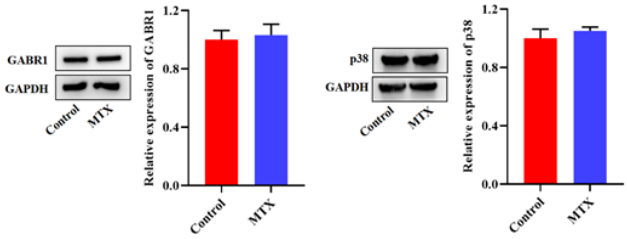

Fig. 5. Methotrexate (MTX) did not affect the expression of GABBR1 and p38 in SFMCs. Enhanced chemiluminescence kit detects the expression levels of GABBR1 and p38 proteins in SFMCs after treatment of MTX. The graphs are quantitative results of the grey density of left protein, which are normalized to GAPDH.

Acknowledgements

This work was supported by 521 Hospital of Norinco Group Xi'an Traumatology.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

SS and WY conducted the experiments and wrote the paper; FY, TF, YT, CL, WX and HR analyzed and organized the data; XM conceived, designed the study and revised the manuscript.

Funding

This work was supported by the Jiangsu Province Traditio-

nal Chinese Medicine Science and Technology Development Plan Project (No. MS2023028); the Jiangsu Provincial Hospital of Traditional Chinese Medicine Innovation Development Fund Special Project (No. Y2018CX16); The Third Batch of Academic Experience Inheritance Project for Veteran Traditional Chinese Medicine Experts in Jiangsu Province (No. [2019] 8).

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