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Research Article

The Effect of Anti-Inflammatory Drugs on MEFV, PSTPIP1, Siva, and ASC Gene Expression Levels

Yeliz Zulfiye Akkaya Ulum

Hacettepe University, Faculty of Medicine, Department of Medical Biology, Ankara, Türkiye

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Abstract

Aim: Familial Mediterranean Fever (FMF) is the one of the most common autoinflammatory diseases. FMF is characterized by fever attacks and inflammation and colchicine treatment reduces the frequency and severity of FMF attacks. The FMF gene, MEditerranean FeVer (*MEFV*), encodes a protein called Pyrin, which regulates inflammation through its interactions with several proteins. These proteins are; Apoptosis-associated speck like protein with a CARD (*ASC*), Proline serine threonine phosphatase interacting protein 1 (*PSTPIP1*), 14.3.3 proteins and *Siva* proteins. In this study, we aimed to study the effect of anti-inflammatory drugs with different mechanisms of action on *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression levels.

Material and Methods: We used differentiated monocytic cell line called THP-1 cells. Cells treated with colchicine, naproxen, prednol-L, acetylsalicylic acid, or azathioprine w and w/o lipopolysaccharide (LPS). After incubation, quantitative RT-PCR (qRT-PCR) was performed to measure *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression levels.

Results: *MEFV* gene expression level was down regulated in colchicine, naproxen, and azathioprine treated cells whereas *PSTPIP1* gene expression level was down regulated in naproxen and azathioprine treated cells with LPS. *Siva* gene expression level was up regulated in all treatments although *ASC* gene expression level was up regulated in only prednol-L treated cells with LPS.

Conclusion: These anti-inflammatory drugs are known to have different mechanisms of action however they are all used to treat pain or inflammation. Since Pyrin, *PSTPIP1*, *Siva*, and *ASC* have pro and anti-inflammatory roles, the results showing an alteration in gene expression levels with specific drugs may indicate the possible mechanisms of therapeutic action.

Keywords: Familial Mediterranean Fever, anti-inflammatory drugs, inflammation, inflammation-related genes

INTRODUCTION

FMF (MIM 249100) is one of the most common hereditary autoinflammatory disorders, which is caused by unprovoked inflammation and tissue destruction (1). FMF is caused by mutations in the *MEFV* (MEditerranean FeVer) (OMIM: 608107) (2) gene which encodes a protein called Pyrin (NP_000234.1). Pyrin is expressed predominantly in neutrophils, monocytes, and dendritic cells (3). It is claimed that through the interactions of several Pyrin interacting proteins, Pyrin may function in regulation of cell death, cytokine secretion, and cytoskeletal signaling (4).

In recent studies for elucidating the pathogenesis of FMF, Xu et al. (5) have shown that pyrin is a specific immune sensor for bacterial modifications of Rho GTPases and responds to Clostridium difficile, the cause of nosocomial diarrhea. In a study by Park et al. (6), it was shown that when pyrin mutated or in response to bacterial modification of RhoA GTPase, RhoA activated pyrin-binding and phosphorylating serine-threonine kinases PKN1 and PKN2. Phosphorylated pyrin has been found to bind 14-3-3 proteins that block pyrin infiltration. The FMF-associated mutant pyrin binding of 14-3-3 and PKN proteins was significantly reduced and IL-1β released from peripheral blood mononuclear cells of FMF patients by activation of PKN1 and PKN2. The same was true for hyperimunoglobulinemia D syndrome (HIDS) as a result of mevalonate kinase (MVK) mutations, and defects in prenylation in HIDS resulted in RhoA inactivation and consequently activation of pyrin infiltration (6). These data suggest that there is a common basic molecular link between two apparently different autoinflammatory disorders, while providing important findings for pyrin function.

CITATION

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Corresponding Author: Zulfiye Yeliz Akkaya Ulum, Hacettepe University, Faculty of Medicine, Department of Medical Biology, Ankara, Türkiye

E-mail: akkayayeliz@gmail.com

Several Pyrin-interacting proteins have been identified. One of the Pyrin-interacting proteins, *PSTPIP1* is a key protein associated with cytoskeleton (7). *PSTPIP1* has a C-terminal SH3 domain that is important for the binding of several PEST phosphatase substrates and also coiled-coiled domain of Pyrin protein (7). *PSTPIP1* is expressed especially in neutrophils and macrophages (8). *PSTPIP1* is involved in regulating inflammation through a complex of proteins termed the inflammasome and also has function in cell migration through actin-based processes. Mutations in the gene encoding *PSTPIP1* leads to the human autoinflammatory disease PAPA (pyogenic sterile arthritis, pyoderma gangrenosum, and acne) syndrome (9). The disease is characterized by recurrent, destructive inflammation of the skin and joints. Treatment can be achieved by using either infliximab or etanercept (10, 11).

Pyrin and *PSTPIP1* proteins are the components of an inflammasome complex that mediates the generation of activated Interleukin (IL)-Iβ (12). *PSTPIP1* mutant proteins bind more strongly to the protein Pyrin (7, 13) and leads to more IL-1β production and more inflammation (13). The dysregulated production of IL-1β contributes to many of the symptoms and the recurrent episodes of inflammation occurring in patients. In addition, it is demonstrated that Pyrin and *PSTPIP1* forms branched and reticulated fibrils in Pyrin transfected cells (14). Our group also showed that this reticulated fibril ratio was decreased by colchicine in Pyrin-*PSTPIP1* co-transfected COS-7 cells (15). Furthermore, *PSTPIP1* has shown to be a part in regulation of directed neutrophil migration and leukocyte uropod formation (16) and localized in actin polymerization side with Pyrin during cell migration (17). Pyrin also localized at the leading edge of the cell together with actin and *PSTPIP1*. Based on the studies that have been done so far, it is indicated that, Pyrin and *PSTPIP1* are important proteins for many cellular processes including inflammation and inflammatory cell migration. For understanding of the pathophysiology of FMF and PAPA syndrome, the function of the proteins should be carefully analyzed either alone or when they are together.

The second Pyrin-interacting protein, *ASC* protein plays a crucial role in regulating the immune system and controlling inflammation. The *ASC* gene is primarily expressed in immune cells. *ASC* protein is composed of 195 amino acids and contains Pyrin domain (PyD) at amino terminus and caspase recruitment domain at carboxyl terminus (CARD) (18). The *ASC* protein acts as a bridge between a receptor that senses the presence of the pathogen and an enzyme called caspase. When the receptor detects the pathogen, it signals *ASC* to assemble the inflammasome (3). This assembly triggers a c*ASC*ade of reactions that activate caspase, leading to the release of molecules called cytokines. The interaction between *ASC* and Pyrin occurs when certain triggers, such as pathogenic signals or cellular stress, activate Pyrin. The PYD domain of Pyrin interacts with the PYD domain of *ASC*, leading to the formation of a molecular complex called the *ASC* speck. The formation of the *ASC* speck is a critical step in the activation of the inflammasome pathway (18). The *ASC*-Pyrin interaction and subsequent formation of the *ASC* speck are particularly relevant in autoinflammatory disorders associated with Pyrin mutations.

Siva was identified as Pyrin-associated proteins, as a result of yeast two-hybrid screen experiments (19). The *Siva* gene contains 6490 nucleotides and has two different transcription products called *Siva*-1 and *Siva*-2 proteins (20). *Siva* protein can promote apoptosis through multiple mechanisms. In two different studies, it was observed that Pyrin has no role in *Siva*'s pro-apoptotic activity (21, 22). The *Siva* protein itself acts as a pro-apoptotic factor by promoting cell death through multiple mechanisms, including caspase activation, disruption of mitochondrial function, modulation of cell signaling pathways, and regulation of DNA repair.

In clinical practice, colchicine is highly effective in the treatment of FMF, preventing the development of recurrent attacks and amyloidosis (23). Its effect on the pyrin and its interacting proteins on cellular level and gene expression profile has already been characterized by our group (15). There are some other specific or nonspecific anti-inflammatory agents, but their effects on *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression levels have yet to be defined. In this study, we have compared the effects of methylprednisolone, acetylsalicylic acid (ASA), naproxen and azathioprine (AZA) on *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression levels. We used THP-1 cells, which naturally express Pyrin, *PSTPIP1*, *Siva*, and *ASC* for this purpose we treat cells with colchicine, naproxen, prednol-L, acetylsalicylic acid, and azathioprine containing mediums, individually. The results of this study provide an outline of the effect of selected drugs on *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression levels.

MATERIAL AND METHOD

Cell Culture and Drugs Treatment

THP-1 cells were obtained from ATCC (American Type Culture Collection) and grown in RPMI (Gibco by Invitrogen) supplemented with 10% FBS (vol/vol), 1% Penicillin/Streptomycin (vol/vol) and 1% glutamine (vol/vol). Cells were differentiated with PMA as previously described in Daigneault et al (24). THP-1 cells were treated 100 ng/ml, 5 uM, 50 nmol/L, 600 uM, and 10 uM of colchicine, naproxen, prednol-L, acetylsalicylic acid, and azathioprine containing medium respectively, for 24 h after starting with the 7th day of differentiation. THP-1 cells were also treated with 10 ng/mL LPS (Sigma–Aldrich, Steinheim, Germany) for 2 hours where indicated. All experiments were performed in triplicate.

RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was extracted by Qiagen (Valencia, CA) RNeasy Mini kit according to manufacturer's instructions. RNA yield and quality were assessed based on spectrophotometric measurements at wavelengths of 260 and 280 nm at NanoDrop ND 1000 (Thermo Scientific Inc., Waltham,

MA). The reverse transcription was carried out using Qiagen (Valencia, CA) QuantiTect Reverse Transcription kit according to manufacturer's recommendations with 400 ng total RNA.

qPCR was performed with SYBR Green JumpStart Taq Ready Mix kit (Sigma, St. Louis, MO) on Corbett Rotor Gene 6000 Light Cycler. The qPCR conditions were 2 min at 94°C, 5 s at 94°C, and 20 s at 59°C. The specific primers were designed for human ACTB (human beta-actin), *MEFV*, *PSTPIP1*, *Siva*, and *ASC*. The relative amount of mRNA, normalized to an internal control ACTB and relative to a calibrator (normal), was calculated by $2^{-\Delta\Delta CT}$. All samples were run in triplicate.

Statistical Analysis

Student's t test was used for comparison of the means among groups. All statistical analyses were performed using the software GraphPad Prism (version 9.0; GraphPad Software Inc., CA) for the Macintosh. P-values of <0.05 were considered statistically significant.

RESULTS

For determining the possible effect of colchicine, naproxen, prednol-L, acetylsalicylic acid, and azathioprine on *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression levels, qRT-PCR analysis was performed. All the experiments were also performed in the presence of 10 ng/mL LPS (bacterial lipopolysaccharide). As shown in Figure 1, LPS treatment induced all of the 4 genes expression levels. Although the expression levels of all the genes were increased, only *PSTPIP1* gene level increased significantly.

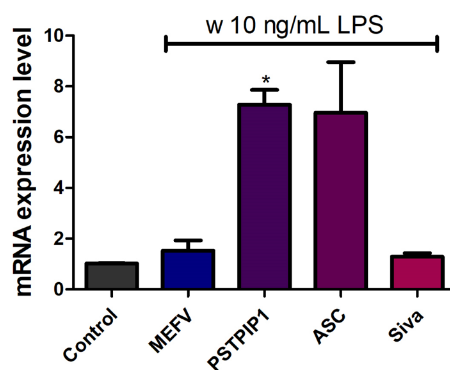

Figure 1. The expression levels *MEFV*, *PSTPIP1*, *Siva*, and *ASC* genes in the presence of LPS (10ng/ml). The qRT-PCR results for *MEFV*, *PSTPIP1*, *Siva*, and *ASC* genes in THP-1 cells that incubated with drugs for 24 h in the absence or presence of LPS (10ng/mL). Gene expressions were normalized to ACTB. Values represent means±SD of three separate experiments. *, P<0.05; **, P<0.01; ***, P<0.001

In colchicine treated cells, *MEFV* gene level was not changed although *PSTPIP1*, *ASC*, and *Siva* genes were down regulated in the absence of LPS (Figure 2). In the presence of LPS, a decrease in *MEFV* mRNA level was observed. Colchicine did not affect the *PSTPIP1* and *ASC* mRNA levels whereas interestingly it caused an increase in *Siva* gene expression level in response to LPS (Figure 2).

After naproxen treatment, in the absence of LPS, *MEFV* and *PSTPIP1* genes were down regulated, *ASC* gene expression level didn't change and *Siva* gene was up regulated. In the presence of LPS, *MEFV* and *PSTPIP1* mRNA levels were also decreased although *ASC* mRNA level was similar and *Siva* mRNA level was increased compared to control (Figure 2).

In prednol-L treated cells, *MEFV* and *ASC* mRNA expression levels were significantly inhibited in the LPS (-) cells. As shown in Figure 2, prednol-L treatment didn't affect *PSTPIP1* and *Siva* mRNA levels in the absence of LPS. In the presence of LPS, *MEFV* and *PSTPIP1* gene expression levels weren't changed whereas *ASC* and *Siva* gene expression levels were increased (Figure 2).

In the absence of LPS, in acetylsalicylic acid treated cells, *MEFV*, *ASC*, and *Siva* mRNA levels were down regulated although *PSTPIP1* gene expression level was shown dramatic increase. *MEFV*, *PSTPIP1*, and *ASC* mRNA levels weren't effected whereas *Siva* mRNA level was increased in the presence of LPS (Figure 2).

After azathioprine treatment, *MEFV* mRNA level was down regulated but the transcription level of *PSTPIP1*, *ASC*, and *Siva* genes weren't affected in LPS (-) cells. In the presence of LPS, *MEFV* and *PSTPIP1* genes were both down regulated whereas *ASC* mRNA level was similar and *Siva* mRNA level was increased compared to control (Figure 2).

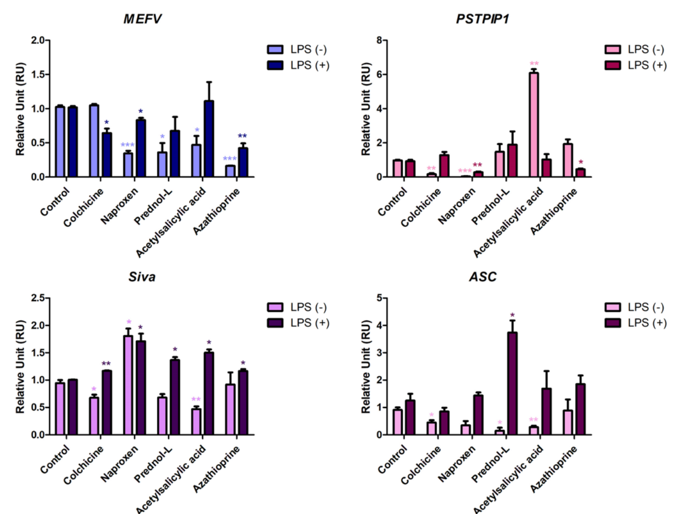

Figure 2. The expression analysis of *MEFV*, *PSTPIP1*, *Siva*, and *ASC* genes in colchicine, naproxen, prednol-L, acetylsalicylic acid, and azathioprine treated THP-1 cells. The qRT-PCR results for *MEFV*, *PSTPIP1*, *Siva*, and *ASC* genes in THP-1 cells that incubated with drugs for 24 h in the absence or presence of LPS (10ng/mL). Gene expressions were normalized to ACTB. Values represent means±SD of three separate experiments. *, P<0.05; **, P<0.01; ***, P<0.001

DISCUSSION

In this study, we have tested the effect of several anti-inflammatory drugs on *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression levels. In order to do that, we used a monocytic cell line (THP-1) and treat cells with colchicine, naproxen, prednol-L, acetylsalicylic acid, and azathioprine in the presence or absence of LPS. The results of LPS treated cells

are more valuable because they mimic the inflammation state which causes FMF disease pathogenesis.

Our data showed that in LPS treated cells, *MEFV* gene level was decreased significantly in colchicine, naproxen and AZA treated cells. The effect of colchicine is expected as it is the mainstay therapeutic option in preventing attacks of FMF via effecting the level of *MEFV* expression. The absence of colchicine effect on basal (non-LPS stimulated) conditions may be dose related as our previous work showed that dramatic decreases of *MEFV* was observed at or above 100 ng/ml colchicine concentrations (15). Of additional interest, naproxen and AZA were also found to regulate the *MEFV* gene level both in the presence and absence of inflammation. Naproxen is a member of nonsteroidal anti-inflammatory drugs (NSAIDs) and it may be used to alleviate symptoms during FMF attacks (25). Naproxen has also been used during the attacks of HIDS and several other auto-inflammatory disorders. Its effect on both basal and LPS-induced *MEFV* gene expression may have an additional role in its rather nonspecific anti-inflammatory effect. Azathioprine, which is a cytotoxic and immunosuppressive drug used in organ transplantation and several autoimmune diseases, had also decreased *MEFV* gene levels both in basal and LPS-stimulated conditions. AZA acts as a prodrug for mercaptopurine, which blocks DNA synthesis (26). Thus, it mostly affects proliferating cells, such as the T cells and B cells of the immune system (20). There is anecdotal evidence that AZA treatment had effectively controlled FMF attacks and ameliorated nephrotic range proteinuria in colchicine-resistant patients (27). The effect of AZA on *MEFV* gene mutation may complement its nonspecific anti-inflammatory effect in FMF patients.

In this study, *PSTPIP1* gene level was decreased significantly in colchicine and naproxen treated cells in basal conditions. In the presence of inflammation *PSTPIP1* gene level was significantly decreased only in naproxen and AZA treated cells. Colchicine treatment was not found to be associated with *PSTPIP1* gene level in the presence of inflammation. The therapeutic approaches are different for FMF disease and PAPA syndrome even though related genes have similar roles in inflammatory pathways. In PAPA syndrome, colchicine is less effective and there is need for additional treatments (28). Data in this study support that naproxen may act on *PSTPIP1* gene level as a therapeutic choice. AZA is also effective in various manifestations of the disease such as fever, peritonitis and pleuritis.

In the presence of LPS, expression levels both *Siva* and *ASC* genes were increased. Interestingly, pro-apoptotic protein *Siva* expression level was up-regulated significantly in all treatments. This result indicated that treatments of these drugs triggered the apoptosis pathway regardless of specify to a certain disease type. Apoptosis is one of the important types of the cell death and does not trigger any inflammatory reaction (29). Apoptotic cells can release anti-inflammatory signals which help to reduce the inflammation (30). All these features showed that the increase of *Siva* gene expression level may be related with the apoptosis pathway which is important for silencing the inflammation. *ASC* gene expression level was significantly up-regulated in only prednol-L treatment. This increase may be nonspecific as *ASC* is a component of inflammasome complex and act as pro-inflammatory protein so this anti-inflammatory, immune suppressing drug should have reduced the *ASC* gene expression level. But on the other hand, *ASC* can trigger apoptosis therefore this increase may be for reducing the inflammation. Dual role of *ASC* and *Siva* have also been monitored in cancer. *ASC* can either be increased in tumor cells and overexpressed in the myeloid compartment within the tumor microenvironment, or it can be downregulated in malignancies, primarily by aberrant methylation (31). Therapeutic methods that are already in use or being developed with the goal of increasing *ASC* expression or interfering with inflammasome components. *Siva*, a crucial regulator of apoptosis and metastasis, is abundantly expressed in a variety of malignant tumors, including ovarian cancer, osteosarcoma, non-small cell lung cancer, and gastric cancer, and promotes carcinogenesis (32). *Siva* may also be a promising target to address the main difficulties of therapeutic intervention in cancer patients, including cancer relapse and chemotherapy resistance in addition to treatment in inflammatory diseases.

It is well known that current treatments for many autoinflammatory disorders including FMF and PAPA are innovative biologic agents like recombinant IL-1 receptor antagonist (IL-1Ra), anti-IL-6 receptor antibodies or anti-TNF antibodies. These drugs have beneficial effects by ameliorating disease pathogenesis and progression via their systemic blocking of pro-inflammatory pathways, however they risk the patients by abrogating the host defenses against infectious pathogens. In these conditions, more precise understanding of pathogenic links between several genes (like *MEFV* and *PSTPIP1*) and their interacting proteins may guide us in finding more precise therapeutic targets. The findings of this study show versatility of various mechanisms and effects of anti-inflammatory agents. Further studies are obviously warranted for better identification of similarities and differences linking *MEFV* and *PSTPIP1* genes in FMF and PAPA.

This study has several strengths and limitations. Various anti-inflammatory drugs with different mechanisms of actions on *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression patterns were tested first time. This is critically important not only for elucidating the effects of these drugs but also deciphering the precise pathogenic links between *MEFV*, *PSTPIP1*, *Siva*, and *ASC* genes. Testing only one, fixed doses of the drugs was a limitation of this study.

CONCLUSION

To our knowledge, though, this is the first study that has investigated the effects of any of anti-inflammatory drugs on *MEFV*, *PSTPIP1*, *Siva*, and *ASC* gene expression patterns. This is critically important to understanding how these drugs have their effect on FMF and disease related

gene Pyrin and its interacting proteins *PSTPIP1*, *Siva*, and *ASC* by altering their expression levels. In addition, altered expression patterns are also essential for explaining the drugs on action of mechanism.

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Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *This article does not contain any studies with human or animal subjects.*

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