Experimed 2023; 13(3): 213-217

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1364227

Indomethacin Affects the Inflammatory Response via Interaction with the RhoA-Actin Cytoskeleton in THP-1 Cells

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Cite this article as: Haciosmanoglu Aldogan E, Dal Yontem F, Bulut S, Yapislar H, Guncer B, Bektas M. Indomethacin affects the inflammatory response via interaction with the rhoA-actin cytoskeleton in THP-1 cells. Experimed. Experimed 2023; 13(3): 213-217.

ABSTRACT

Objective: Inflammation is a complex reaction present in numerous disorders. Indomethacin, a compound possessing an indoline core, is a Nonsteroidal Anti-Inflammatory Drug (NSAID) that is commonly prescribed for inflammation and pain. The actin network, plays a major role in cellular activities and it's regulated by by Rho GTPases has important implications for cellular dynamics and orientation. In this research, we explore the effects of indomethacin on the inflammatory response as mediated via RhoA and pyrin inflammatory complexes using an inflammatory disease model with relation actin cytoskeleton.

Materials and Methods: This study used Western blotting to examine the impact of indomethacin on the assembly processes related to the pyrin inflammasome complex and the RhoA signaling pathway in Lipopolysaccharide-stimulated THP-1 cells. Actin-indomethacin interaction was analyzed by Differential Scanning Fluorimetry (DSF).

Results: We found that while the expression levels of pyrin decreased, phosphorylated-RhoA increased but overall RhoA levels did not change. The equilibrium dissociation constant (K_D) for the G-actin-indomethacin complex was calculated to be 9.591± 1.608 ng/mL (R^2= 0.8582) using ΔT_m measurements of indomethacin by DSF.

Conclusion: Moreover, the effects of indomethacin on inflammation pathways may provide insight into the molecular mechanisms of pyrin inflammasome formation in various autoimmune diseases.

Keywords: Actin, differential scanning fluorimetry, GTPases, indomethacin, RhoA, Rho pyrin

INTRODUCTION

Autoinflammatory disorders arise from chronic inflammatory episodes caused by malfunctions in the body's innate immune defense mechanisms (1). For example, Familial Mediterranean Fever (FMF) stands out as a prevalent autoinflammatory condition and is characterized by pyrin synthesis caused due by Mediterranean Fever (*MEFV*) gene mutations (2). Pyrin plays a central role in the pyrin inflammasome, and mutations or reactions to bacterial-induced changes in RhoA GTPase can result in the activation of caspase-1, which subsequently leads to the secretion of Interleukin-1β (IL-1β)

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Submitted: 25.09.2023 **Revision Requested:** 08.11.2023 **Last Revision Received:** 20.11.2023 **Accepted:** 27.11.2023

(3, 4). Interestingly, pyrin has also been found to associate with cellular skeletal components including actin and related binding proteins (4). The actin structure plays a major role in a variety of cellular activities, ranging from movement and cell division to vesicle transport and phagocytosis (5). Many Actin Binding Proteins (ABPs), which arrange actin polymerization, filament nucleation, and depolymerization, are downstream targets of Rho GTPases (6). A recent study suggested that the activation of RhoA GTPase can result in the inhibition of pyrin due to the phosphorylation activities of Protein Kinases N1 and N2 (PKN1 and PKN2) (3). Indomethacin, a widely recognized Non-steroidal Anti-Inflammatory Drug (NSAID) that is a member of the indole compound family, addresses pain-inducing inflammatory conditions by interrupting prostaglandin production (7). Recent evidence also indicates that indomethacin suppresses the inflammation process by reducing the levels of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) (8). Thus, this study aimed to investigate the role of indomethacin in the phosphorylation of pyrin and RhoA, the mechanism responsible for this role, and the relationship between indomethacin and the actin cytoskeleton *in vitro*.

MATERIALS AND METHODS

Differential Scanning Fluorimetry (DSF)

DSF experiments were performed using a real time polymerase chain reaction (qPCR) system (BioRad CFX 96, California, USA). Reaction mixtures (25 µL) comprised 2 µM rabbit skeletal muscle actin (1 mg/mL) procured from Cytoskeleton, Inc. (AKL99, Denver, CO, USA), suspended in a buffer (pH 7.4) containing 20 mM HEPES, 150 mM NaCl, 1mM DTT, and 5% sucrose. These reaction mixtures were combined with 50X SYPRO Orange (diluted from a 5000X stock at Thermo Fisher Scientific, Waltham, MA, USA) and incubated in the presence of indomethacin ($C_{19}H_{16}ClNO_4$- Sigma Aldrich, St. Louis, MO) at concentrations ranging from 1-100 ng/mL. After incubation, these mixtures were placed in a PCR plate and subjected to gradual heating from 25°C to 90°C (using ascending increments of 0.5°C every 15 seconds) in the thermocycler (9). Three replicates per compound and six internal controls (which contained only free protein in 10% DMSO) were included in 96-well plates. The melting point (T_m) was assessed by plotting the derivative of initial fluorescence against temperature, then determining the midpoint temperature during the transition via the Boltzmann equation (10).

Cell Cultivation Process

THP-1 human monocytic cells (TIB-202, ATCC, Manassas, Virginia, USA) were grown in RPMI-1640 medium (Gibco, MD, USA) enriched with 10% FBS and 1% penicillin/streptomycin. Cells were housed in a 5% CO_2 humidified environment. Following standard trypsinization methods, cells were detached and subsequently passaged two to three times. For our experimental setup, cells were seeded into 6-well plates (at 1 x 10^5 cells/ per well) then incubated with a 20 µg/mL lipopolysaccharide (LPS) treatment for 1h followed by treatment with increasing concentrations of indomethacin for 24h.

SDS-PAGE and Western Blotting Procedure

Cells were lysed using RIPA buffer and a protease/phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO) using a half-hour ice incubation. Next, lysates were centrifuged at 10,000 g for 15 minutes at 4°C, after which supernatants were collected. SDS-PAGE was performed to separate proteins (40 µg), which were then transferred onto PVDF membranes (Bio-Rad, Marnes-la-Coquette, France) (11). These membranes were then incubated with primary antibodies targeting RhoA, phosphorylated-RhoA (Ser188), pyrin, and β-actin (Santa Cruz Biotechnology, Texas, USA). Subsequently, membranes were incubated with secondary peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA) to reveal specific protein bands via chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Protein quantification was then performed using densitometry analysis as implemented by NIH ImageJ software (NIH, Bethesda, MD, USA). For all blotting experiments, β-actin was used as a reference standard (12).

Statistical Analyses

Data analysis was performed with using CFX Manager and GraphPad software and protein–ligand dissociation constants (K_D) were calculated. Graphs were then presented as normalized plots and were generated using Microsoft Excel. $p<0.05$ was accepted as the significance limit.

Table 1. T_m and the difference (ΔT_m) in T_m in the absence and presence of indomethacin.

Concentration of indomethacin (ng/mL)	T_m (°C)	T_m Slope (ΔT_m, °C)
Control	57.65	5.4
1	58.42	5.7
5	59.54	6.1
10	59.96	6.3
50	60.08	6.7
100	61.22	7.1

RESULTS

Effect of Indomethacin on Actin Thermodynamic Stability

DSF measurements were taken to evaluate how indomethacin binding affects the thermodynamic stability of G-actin and to quantify the binding affinity of these proteins. The thermal unfolding characteristics of G-actin, whether alone or in the presence of indomethacin, were tracked by observing changes in the fluorescence intensity of SYPRO Orange. This dye exhibits an enhanced fluorescence signal as it binds to hydrophobic regions that become exposed during protein unfolding. For example, when a ligand binds to a protein, there is a shift in ΔT_m. These data suggest that the newly observed peak corresponds to the actin–indomethacin complex. The observed T_m was 57.65 °C for G-actin alone but 61.06 °C when G-actin was incubated with 100 ng/mL indomethacin (Figure 1, Table1). This obviously differs from the ΔT_m of untreated actin. The elevated T_m value of the actin monomers bound to indomethacin suggests that the conformational alteration induced by indomethacin on monomeric actin is associated with an enhancement in the thermodynamic stability of the actin monomers. Using ΔT_m measurements for indomethacin, the equilibrium dissociation constant (K_D) for the G-actin–indomethacin complex was determined to be 9.591± 1.608 (R^2= 0.8582) (10). These results therefore show that indomethacin binding to G-actin can affect pyrin inflammasome formation and the actin cytoskeleton.

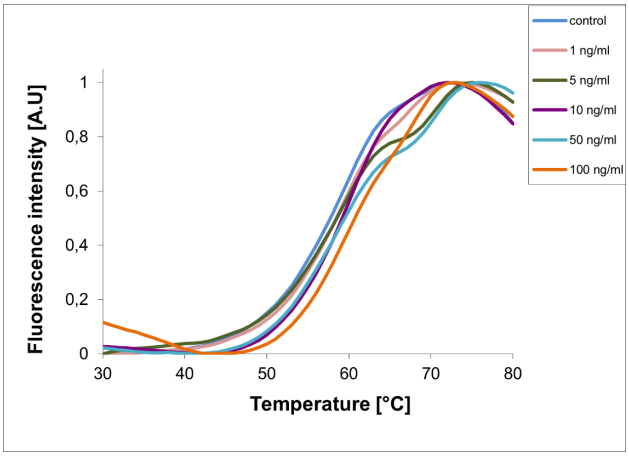

Figure 1. *In vitro* characterization of indomethacin binding to G-actin. DSF of G-actin alone and G-actin incubated with indomethacin at different concentrations. G-actin alone is shown in blue, 1 ng/mL in pink, 5 ng/mL in green, 10 ng/mL in purple, 50 ng/mL in turquoise and 100 ng/ mL in orange.

The Expression Levels of RhoA, Phosphorylated-RhoA, and Pyrin Protein Following Indomethacin Treatment

Western blotting was performed to determine the effects of indomethacin on the expression of RhoA, phosphorylated-RhoA, and pyrin in LPS-induced THP-1 cells (Figure 2A). Pyrin expression levels n significantly decreased after indomethacin

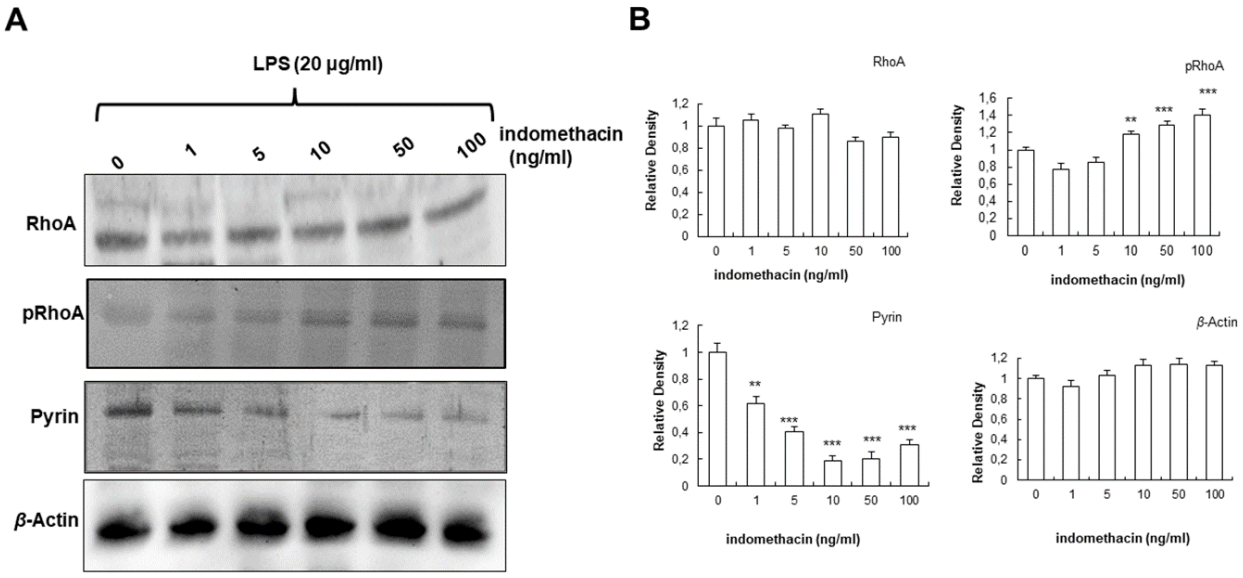

Figure 2. Effects of indomethacin on RhoA, phospho-RhoA, and pyrin expression in LPS-induced inflammation. (A) Western blots showing proteins extracted from THP-1 cells pretreated with 20 µg/mL LPS for 1 h and exposed to 1, 5, 10, 50, or 100 ng/mL indomethacin for 24 h. **(B)** Protein expression levels of RhoA, phospho-RhoA, and pyrin as determined via analysis with ImageJ. Values were expressed as mean ± SEM of three experiments. ** p < 0.01, ***p < 0.001 compared to cells treated only with LPS.

treatment relative to the control group ($p < 0.001$) (Figure 2B). Moreover, the RhoA expression level remained unchanged post-treatment, but the level of phosphorylated-RhoA increased relative to the control ($p < 0.005$) (Figure 2B). Taken together, these data suggest that indomethacin reduces inflammation by indirectly decreasing the pyrin component of the pyrin inflammasome, potentially facilitated by induced phosphorylation of the RhoA protein. Furthermore, changes to the phosphorylation of RhoA and pyrin may be result of indomethacin-G-actin.

DISCUSSION

The actin cytoskeleton, which is traditionally associated with cellular structure and motility, has also been found to be a key player in the regulation of the inflammasome. Actin dynamics in cells depend on changes between its monomeric (G-actin) and filamentous (F-actin) forms (13). Actin dynamics modulate the mobility of inflammasome components within the cell, thereby affecting their ability to form functional complexes. Studies have indicated that actin polymerization is crucial for both the formation and activation of the NLRP3 inflammasome (14). Furthermore, the RhoA-actin cytoskeletal system is known to play a major role in facilitating various cellular functions, including modulating cell morphology, mobility, and adhesion. RhoA, which belongs to the Rho GTPase family, serves as a crucial molecular switch governing the dynamics of actin. Activation of RhoA triggers actin polymerization, the development of stress fibers, and the formation of focal adhesions. These processes are indispensable for cell mobility and mechanosensory responses (15). Indomethacin, an NSAID, is primarily known for its role in inhibiting prostaglandin synthesis. However, recent studies have revealed that indomethacin can also modulate inflammasome activation. This effect is attributed to its ability to inhibit caspase 1, a key enzyme involved in inflammasome assembly, via mechanisms independent of cyclooxygenase (COX) inhibition (7).

In the present study, we demonstrated the effect of indomethacin on the pyrin inflammasome and explored how indomethacin affects the relationship between actin cytoskeleton and RhoA. DSF analysis revealed that indomethacin binds to G-actin and induces the thermal stability of monomeric actin. In addition, according to the shifting T_m values recorded here, the binding constant of indomethacin to-actin was 9.591 ± 1.608 ng/mL. Furthermore, Western blotting indicated a reduction in the expression of pyrin, one of the main components of the pyrin inflammasome, following indomethacin treatment. Together, these data suggest that the indomethacin-monomeric actin complex may be responsible for the observed decrease in the inflammatory response attributed to the pyrin inflammasome complex. For example, a previous study found that indomethacin restores actin cytoskeleton dynamics disrupted by amyloid β in differentiated human neuroblastoma cells (16). In our study, an increase in the level of phosphorylated-RhoA was observed, despite the fact that the total RhoA level remained unchanged. Phosphorylated-RhoA is known to be one of the key regulators of the actin cytoskeleton (17). Apart from binding to G-actin, indomethacin can influence Rho-kinase activity or indirectly lead to RhoA phosphorylation. In conclusion, the effect of indomethacin on the actin–pyrin inflammasome and the RhoA pathway represents an important area of research that has implications for our understanding of inflammation regulation. However, further investigation should be undertaken to understand the molecular mechanisms involved in this pathway as well as its potential significance for health and disease.

Ethics Committee Approval: Since this study is a cell culture study, no ethics committee approval is required.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.H.A., M.B., B.V.; Data Acquisition- E.H.A., F.D.Y., S.B.; Data Analysis/Interpretation- E.H.A., F.D.Y., H.Y., S.B.; Drafting Manuscript- E.H.A., F.D.Y., H.Y.; Critical Revision of Manuscript- F.D.Y., H.Y., M.B., B.V.; Final Approval and Accountability- E.H.A.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: This study was funded by Scientific Research Projects Coordination Unit of Bezmialem Vakif University (project number: 2.2019/17).

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