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Original Contribution

Rhein exerts pro- and anti-inflammatory actions by targeting IKKβ inhibition in LPS-activated macrophages

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ABSTRACT

Because steroids and cyclooxygenase inhibitors may cause serious side effects, the IκB kinase (IKK) β/nuclear factor-κB (NF-κB) system has become an intriguing candidate anti-inflammatory target. Rhein, the active metabolite of diacerein, possesses anti-inflammatory ability with a gastrointestinal protective effect. However, in a preliminary study, we accidentally found that rhein showed both anti- and proinflammatory activities in lipopolysaccharide (LPS)-activated macrophages. Thus, in this study, we explored the underlying molecular mechanisms of the dual effects of rhein. In LPS-activated macrophages, rhein inhibits NF-κB activation and sequentially suppresses its downstream inducible nitric oxide synthase, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) transcription and supernatant nitric oxide and IL-6 levels by inhibiting IKKβ ($IC_{50} \approx 11.79$ μM). But in the meantime, rhein enhances the activity of caspase-1 by inhibiting intracellular (in situ) IKKβ, in turn increasing the IL-1β and high-mobility-group box 1 release, which can be amplified by rhein's reductive effect on intracellular superoxide anion. Unexpectedly, it is because of IKKβ inhibition that rhein significantly enhances TNF-α secretion and phagocytosis in macrophages with or without LPS. These results indicate that rhein exerts anti- and proinflammatory activities by targeting IKKβ inhibition, providing a molecular mechanism for the unanticipated role of rhein in macrophages. Furthermore, our study also highlights the potential complications of IKKβ inhibitor (e.g., rhein, diacerein, etc.) application in inflammation disorders, for the overall effects of IKKβ inhibition in various organ systems and disease processes are not easily predictable under all circumstances.

Nuclear factor-κB (NF-κB) is a classical and key dimeric transcription factor in inflammatory processes [1]. At baseline, NF-κB is kept inactive through the action of IκB. When bacterial components such as lipopolysaccharide (LPS) engage Toll-like receptor 4 on macrophage membranes, the signals transduced converge on the IκB kinase (IKK) complex, consisting of two catalytic subunits, IKKα and IKKβ, and the regulatory subunit NEMO [2]. IKKβ, but not IKKα [3], phosphorylates IκBα, leading to its ubiquitination and eventual degradation, thus freeing p50/p65, primarily bound to IκBα proteins under basal conditions, to enter the nucleus and activate transcription of genes such as inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), pro-tumor necrosis factor-α (pro-TNF-α), pro-interleukin-1β (pro-IL-1β), etc.

Unlike nitric oxide (NO) and IL-6, pro-TNF-α and pro-IL-1β are inactive intracellular precursors that are released only after their maturation by their respective converting enzymes, TACE [4] and caspase-1 [5]. Different from IL-1β, high-mobility-group box 1 (HMGB1), a highly conserved nuclear protein lacking a classic secretion signal, does not undergo caspase-1-mediated processing, but its extracellular release from activated immune cells relies on activation of caspase-1 [6]. Currently, HMGB1 is regarded as a prototypical endogenous danger signal, or alarmin, acting as a proinflammatory factor in many disorders including endotoxemia and sepsis [7].

Rhubarb (*Rheum palmatum* L. or *R. tanguticum* Maxim), a traditional Chinese herbal medicine with antibacterial [8], antipyretic [9], and purgative activities [10], has been widely used to treat local and systemic infectious inflammation for about 2000 years in China [11]. Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), a major bioactive compound enriched in rhubarb [12], could effectively inhibit NO production with little effect on prostaglandin E_2

Abbreviations: AP-1, activator protein-1; HMGB1, high-mobility-group box 1; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; IL-6, interleukin-6; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; $O_2^{\bullet-}$, superoxide anion; OA, osteoarthritis; PGE_2, prostaglandin E_2; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; YC, YVAD-CHO

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(PGE_2) production in LPS-activated RAW 264.7 macrophages [13]. Its prodrug diacerein, which is completely metabolized by humans into rhein [14], is currently used for the treatment of osteoarthritis (OA) [15]. Rhein and diacerein, in contrast to most of the nonsteroidal anti-inflammatory drugs, are potent inhibitors of supernatant NO induced by IL-1β in chondrocytes and cartilage, without reducing PGE_2 production [16]. This unique feature seems to be the reason for the excellent gastric safety profile of diacerein during OA treatment [17]. Surprisingly, in our preliminary study, we found a seemingly contradictory and unbelievable phenomenon that rhein could suppress supernatant NO and IL-6, but meanwhile could increase IL-1β, TNF-α, and HMGB1 release in LPS-activated primary and immortal macrophages. Thus, this paper aims to explore the underlying molecular mechanisms of these unanticipated effects of rhein.

Materials and methods

Materials and reagents

Rhein was purchased from the National Institutes for Food and Drug Control (Beijing, China) and dissolved in dimethyl sulfoxide (DMSO) at the concentration of 4 mg/ml. We also tested rhein from Sigma and found that it exhibited equivalent potency at inhibiting NF-κB signaling. Mouse TNF-α and IL-6 ELISA kits were obtained from Biolegend Co. (San Diego, CA, USA). Mouse IL-1β and HMGB1 ELISA kits were obtained from ExCell Bio Co. (Shanghai, China) and R&D Systems (Minneapolis, MN, USA), respectively. IKKβ shRNA plasmid and its negative control plasmid, antibodies for IKKβ (sc-34673), iNOS, IκBα, and p65 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (Lancaster, PA, USA). Caspase-1 colorimetric assay kit, superoxide dismutase (SOD), and the plasmids NFκB-TA-luc, AP1-TA-luc, and their control GL6-TA were from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Antibodies for mitogen-activated protein kinase and phospho-IκBα (Ser32) and biotinylated IκBα (Ser32) peptide were from Cell Signaling Technology (Danvers, MA, USA). Recombinant IKKβ and YVAD-CHO (YC; a caspase-1 inhibitor) were from Millipore (Billerica, MA, USA) and Enzo Life Sciences (Farmingdale, NY, USA), respectively. Streptavidin, fluorescein isothiocyanate (FITC), lucigenin, dichlorofluorescin diacetate (DCFH-DA), thioglycollate medium, and puromycin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Yeast (*Saccharomyces cerevisiae*) was from Angel Yeast Co., Ltd (Yichang, Hubei, China). The luciferase assay system and Lipofectamine 2000 reagent were obtained from Promega Co. (Madison, WI, USA) and Invitrogen Life Technology (Carlsbad, CA, USA), respectively. All other reagents were of analytical grade.

Cell isolation, culture, and treatment

The RAW 264.7 cell line was purchased from the American Type Culture Collection. Mouse peritoneal macrophages were isolated from male Balb/c mice 3 days after intraperitoneal injection of 1 ml of 3.8% thioglycollate medium as previously described [18]. These two cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 1.0% penicillin–streptomycin solution in a humidified incubator with 5.0% CO_2 at 37 °C. For the rhein treatments, rhein was first added to the culture medium and then mixed thoroughly (the final concentrations of DMSO were $\leq 0.25\%$). The cell culture medium was replaced with the medium already mixed with rhein. All the experiments related to animals were carried out according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Animals Ethics Committee of the Institute of Medicinal Plant Development of the Chinese Academy of Medical Sciences.

Measurement of proinflammatory mediators in supernatant

The level of NO production was monitored by measuring the nitrite level in the supernatant of macrophages using the Griess method as we previously described [19]. For the measurements of TNF-α, IL-6, HMGB1 and IL-1β, macrophages were pretreated with rhein for 2 h and then stimulated with LPS (10 ng/ml) for 24 h. TNF-α, IL-6, HMGB1 and IL-1β in the cell supernatants were assayed using ELISA kits according to the manufacturer's instructions. The concentrations were calculated from the standard curves.

Superoxide anion ($O_2^{\cdot -}$) scavenging assays

Measurements of $O_2^{\cdot -}$ scavenging activity of rhein were performed as we previously described [20].

Phagocytosis assay

Conjugation of FITC to yeast and phagocytosis assays using FITC-conjugated yeast as targets were performed following previously described methods [21,22]. The inactivated yeast was washed once in 0.1 M CBS (carbonate bicarbonate-buffered saline, pH 9.0) and the pellets were resuspended in CBS at 4×10^8 cells/ml. An equal volume of DMSO containing 100 μg/ml FITC was added to the yeast suspension and then stirred at 25 °C for 30 min. An equal volume of phosphate-buffered saline (PBS) was added to the suspensions and the yeast was washed by centrifugation. After unconjugated FITC was removed, the final FITC-conjugated yeast was resuspended in medium at 4×10^7 cells/ml. Assay was initiated by supplied cells with this yeast suspension yielding a 40:1 ratio of yeast to cells. 2 h after incubation, extracellular fluorescence was quenched by adding 200 μl of trypan blue (250 μg/ml, pH 4.4). After 1 min, the dye was removed. The fluorescence intensity was determined at λ_{ex} 485 nm and λ_{em} 538 nm.

Western blot analysis

Proteins in the nucleus or cytoplasm were extracted and separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 4 h with 5.0% nonfat dry milk and then incubated with each primary antibody at 4 °C overnight. After being washed, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The blots were visualized using enhanced chemiluminescence, and data were analyzed using the Gel Doc EQ System (Bio-Rad).

Plasmids, transfection, luciferase reporter assay, and RNA interference

For the luciferase reporter assay, RAW 264.7 cells were transfected with pNFκB-TA-luc, pAP1-TA-luc, or their control plasmid pGL6-TA by using Lipofectamine 2000 according to the manufacturer's protocol. 24 h after transfection, RAW 264.7 cells were preincubated with various concentrations of rhein for 2 h and then exposed to LPS for 6 h. The cells were lysed and luciferase activity was measured using the luciferase assay system.

For the IKKβ knockdown experiments, RAW 264.7 cells were transfected with IKKβ shRNA plasmid and its negative control plasmid using Lipofectamine 2000. Stable IKKβ (−) cells and IKKβ