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Auranofin Downregulates Nuclear Factor-κB Activation via Nrf2-Independent Mechanism

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Transcription factors Nrf2 and NF-κB are important regulators of the innate immune response, and their cross-talks in inflammation have been reported. Previously, we demonstrated that gold(I)-compound auranofin, an inhibitor of NF-κB signal, induced Nrf2 activation in human synovial cells and monocytic cells. To investigate whether the Nrf2 activation is involved in the mechanism of the auranofin-attenuated NF-κB signaling, we examined the effects of Nrf2 knockdown on NF-κB activation using rheumatic synovial cells. When the cells were transfected with a specific siRNA for Nrf2, the gene expression was perfectly blocked. However, the Nrf2 knockdown did not cancel the suppressive effect of auranofin on TNF-α-induced IκB-α degradation. Treatment with a specific siRNA for HO-1, which is a target of Nrf2 and plays a role in anti-inflammation, also did not affect the blocking activity of auranofin on IκB-α degradation. In addition, auranofin-inhibited ICAM-1 expression was not restored by Nrf2 knockdown. These findings indicate that the activated Nrf2 and HO-1 are not associated with the suppressive action of auranofin on the pro-inflammatory cytokines-stimulated NF-κB activation. This suggests that Nrf2/HO-1 and NF-κB signals, which are regulated by auranofin, participate in the anti-inflammatory action of auranofin via independent pathways in rheumatic synovial cells.

Key words : Auranofin, nuclear factor-erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), nuclear factor-κB (NF-κB), intercellular adhesion molecule-1 (ICAM-1)

Introduction

Auranofin (2, 3, 4, 6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-S-[triethylphosphine] gold) is a thiol-reactive gold(I) compound that has been used for the treatment of rheumatoid arthritis for a long time [1]. The drug possesses the inhibitory activity for nuclear factor κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), and toll-like receptor 4 [6,12,26]. Recently, we reported a novel anticancer activity of auranofin by demonstrating that auranofin induced apoptosis and promoted all-*trans* retinoic acid-mediated differentiation of acute promyelocytic leukemia cells [11,20,21]. Therefore, the gold(I) compounds including auranofin have been studied for therapeutic application to inflammation and cancer [3,4,19,22,24].

In the mechanisms on anti-inflammatory and anti-rheumatic actions of auranofin, the inhibitory effect on NF-κB signal pathway has been well known. The thiol-reactive auranofin modifies Cys-179 in beta subunit of inhibitory κB kinase (IKK), which results in the blockade of inhibitory κB-α (IκB-α) phosphorylation and degradation [6,7]. Consequently, auranofin attenuates the transcriptional activity of NF-κB and downregulates the expression of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).

Heme oxygenase-1 (HO-1), which suppresses an inflammatory response, is also associated with the pharmacological property of auranofin in rheumatoid arthritis [14,16]. In the previous study, we found that auranofin induced HO-1 via the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) in human synovial cells and THP-1 monocytic cells. Rac1/NADPH oxidase/inducible nitric oxide synthase signaling and MAPK activation were involved in the mechanism of auranofin-stimulated Nrf2 activation [13].

The Nrf2 is a redox-sensitive transcription factor. In the cytoplasm of unstimulated cells, Nrf2 binds to the Kelch-like ECH-associated protein 1 (Keap1) with ubiquitin ligase activity, and is rapidly degraded through 26S proteasome system. In the cells stimulated by oxidative stress, Nrf2 dissociates from Nrf2-Keap1 complex. The released Nrf2 translocates to the nucleus, where it binds specifically to the anti-

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oxidant response element (ARE) and induces phase II detoxifying and antioxidant molecules, including HO-1 [10,15].

Recent evidences indicated that Nrf2 was involved in downregulation of NF-κB signaling [5,17,23]. After traumatic brain injury, NF-κB activity and intercellular adhesion molecule-1 (ICAM-1) expression were upregulated in Nrf2 (−/−) mice compared with Nrf2 (+/+) wild-type mice [9]. In addition, various Nrf2 activators, such as curcumin and sulforaphane, strongly inhibited lipopolysaccharide (LPS)-induced NF-κB activation [8,18]. These findings suggest that Nrf2 acts as a critical regulator of the innate immune response via inhibition of NF-κB signal pathway, although it is still unclear whether Nrf2 directly inhibits NF-κB activation.

Auranofin functions as an inhibitor of NF-κB signaling as well as a potent activator of Nrf2 [7,13]. To date, however, it is unknown whether the Nrf2 activation is involved in the mechanism of the auranofin-regulated NF-κB signaling. To solve the problem, in the present study, we examined the effect of knockdown of Nrf2 on the NF-κB activation in the auranofin-treated synovial cells originated from the patient of rheumatoid arthritis.

Materials and Methods

Cell culture and treatment

Human rheumatic synovial cells transformed by SV40 T antigen were obtained from Dr. Wan-Uk Kim (The Catholic University of Korea, Seoul, Korea) and THP-1 human monocytic cell line was purchased from the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI 1640 medium (Gibco Life Technology, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Gibco). For induction of Nrf2 and HO-1, the cells were plated at a density of 5×10^5/ml. After incubation for 16 hr, the cells were treated with 0.3 and 0.5 μM of auranofin (Alexis, Lausen, Switzerland) for the indicated periods in serum-free medium. To examine the inhibitory effect of auranofin on NF-κB activation, cells were plated at a density of 5×10^5/ml and cultured overnight. At the next day, the cells were preincubated for 3 hr with 0.3 or 0.5 μM of auranofin and then stimulated with TNF-α (10 ng/ml; R & D Systems Inc., Minneapolis, MN) or IL-1β (5 ng/ml; R & D Systems Inc.) for the indicated times in serum-free medium. The IκB-α degradation and ICAM-1 expression were analyzed by Western blot.

Nrf2 and HO-1 knockdown

Specific small interference RNAs (siRNAs) for Nrf2 and HO-1 were purchased from Dharmacon (Lafayette, CO). The siRNAs were introduced into human synovial cells using transfection reagent DharmaFECT® (Dharmacon) by incubating the cells with 50 nM of the siRNA oligonucleotides for 24 hr in serum- and antibiotics-free media, according to the manufacturer's protocol.

The siRNA-transfected cells were preincubated without or with auranofin (0.3-0.5 μM) for 3 hr, and then stimulated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) for the indicated times. In the cells treated with the cytokines, IκB-α and ICAM-1 were detected by Western blot analysis.

Western blot analysis

The cells were lysed in lysis buffer (25 mm Tris-HCl (pH 7.2), 0.1% sodium dodecyl sulphate, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mm NaCl, 1 mm ethylene diaminetetraacetic acid, 1 mm sodium orthovanadate, aprotinin (10 μg/ml), leupeptin (5 μg/ml), and 1 mm phenylmethylsulphonyl fluoride). After determining the protein concentration of each lysate, equal amounts of the proteins were loaded on a 12% SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose membrane and analyzed using following specific antibodies and an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech, Piscataway, NJ). The antibodies against human Nrf2, HO-1, IκB-α, ICAM-1, and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Results and Discussion

Induction of Nrf2 and HO-1 by auranofin

To examine the Nrf2 induction by auranofin, Western blot was performed using auranofin-treated cells. Fig. 1 showed that 0.3-0.5 μM of auranofin increased Nrf2 level. The expression of HO-1, which is an important Nrf2 target protein and participates in anti-inflammatory response, was also enhanced by auranofin treatment (Fig. 1A and B). These results were consistent with the findings demonstrated in our previous study [13].

The inhibitory effect of auranofin on ICAM-1 expression stimulated by proinflammatory cytokines

Auranofin exerts the pharmacological activity on anti-inflammation by inhibiting NF-κB activation [19,25]. We con-

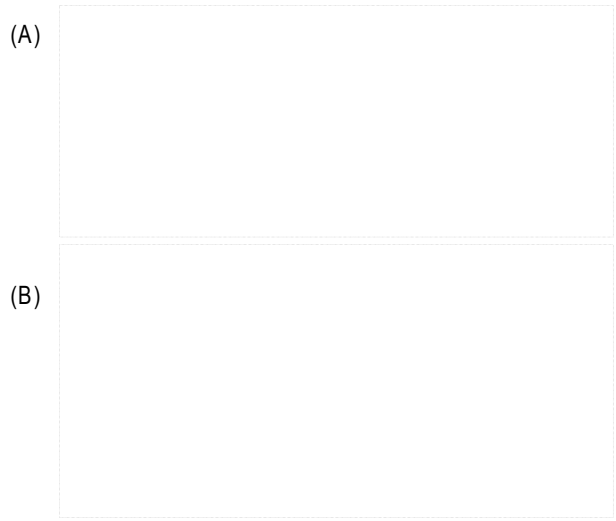

Fig. 1. Induction of Nrf2 and HO-1 expressions by auranofin. Cells were treated with 0.5 μM of auranofin for the indicated times (A), or treated for 6 hr with 0.3- 0.5 μM of auranofin (B). Cellular proteins were analyzed by Westen blot using antibodies against Nrf2 and HO-1. α-tubulin was used as an internal marker to ensure equal protein loading. The results shown here are representative of two independent experiments.

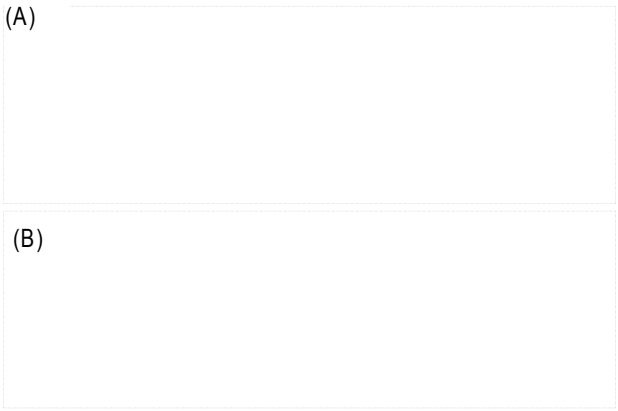

Fig. 2. Down-regulation of ICAM-1 expression by auranofin in human rheumatic synovial cells. (A) and (B) Cells were plated at a density of 5×10^5/ml. After culture for 16 hr, the cells were treated with 0.3-0.5 μM of auranofin for 3 hr in serum-free medium. The auranofin-pretreated cells were stimulated with 10 ng/ml of TNF-α (A) or 5 ng/ml of IL-1β (B) for the indicated times. The change of ICAM-1 expression was analyzed by Western blot. The results shown here are representative of two separate experiments. Figure (A) shows the results of duplicate experiments.

firmed the effect of this compound on expression of ICAM-1 which is a pivotal target gene of transcription factor NF-κB. As shown in Fig. 2A and B, the preincubation of synovial cells with auranofin effectively inhibited the ICAM-1 expression induced by TNF-α and IL-1β.

Nrf2 and HO-1 are not associated with the inhibitory activity of auranofin on NF-κB activation in synovial cells

Accumulated evidences have indicated that Nrf2 plays an important role in regulation of NF-κB activation [5,9,17,23]. Therefore, we examined whether Nrf2 activation is involved in the mechanism of the auranofin-regulated NF-κB signaling. When synoviocytes were transfected with control siRNA and treated with 0.5 μM auranofin, the protein levels of Nrf2 and HO-1 were significantly increased and IκB-α degradation mediated by TNF-α was completely blocked (Fig. 3A and B). These results were similar to that of figure 1 and 2. To test the effects of Nrf2 and HO-1, the gene expressions were knockdown by specific siRNAs. After preincubation of the knockdown cells with 0.5 μM auranofin for 3 h, the cells were stimulated by TNF-α treatment. The specific siRNAs perfectly blocked the expression of Nrf2 and HO-1, however, the knockdown of Nrf2 and HO-1 did not cancel the suppressive effect of auranofin on TNF-α-induced IκB-α degradation (Fig. 3A and B). In addition, the ICAM-1 expression diminished by auranofin treatment was not restored by Nrf2 knockdown (Fig. 4A and B). These findings indicate that the activated Nrf2 and HO-1 are not associated with the inhibitory effect of auranofin on the proinflammatory cytokines-stimulated NF-κB activation. It suggests that Nrf2/HO-1 and NF-κB signals, which are regulated by auranofin, participate in anti-inflammatory action of auranofin via independent pathways in rheumatic synovial cells.

Although the cross-talks between Nrf2 and NF-κB signal pathway in the inflammatory response have been suggested, the inhibitory effects of Nrf2 and HO-1 on LPS or TNF-α -induced NF-κB activation are still controversial. Several researchers demonstrated that Nrf2-deficient mice showed more NF-κB activation compared to wild-type mice, indicating that Nrf2 plays an important role in the regulation of NF-κB activity [9,23]. However, others reported that Nrf2 did not inhibit NF-κB activation in endothelial cells [2]. In our synovial cell system, the treatment of Nrf2 and HO-1 siRNAs did not affect on IκB-α degradation and ICAM expression which were stimulated by TNF-α. This result suggests that Nrf2 and HO-1, which are increased by auranofin,

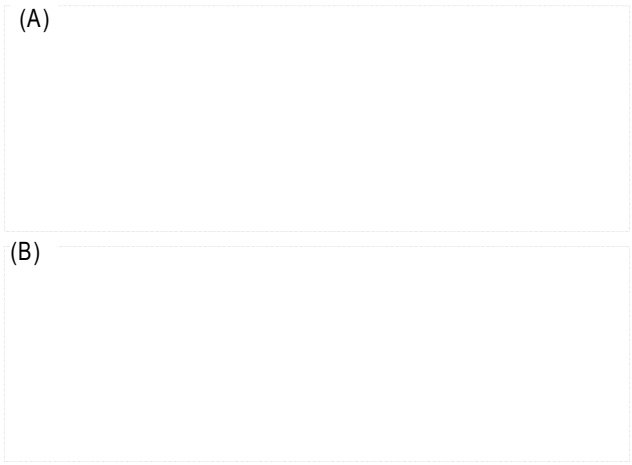

Fig. 3. The effect of knockdown of Nrf2 and HO-1 on IκB-α degradation. (A) and (B) Human synovial cells were transfected with control siRNA or specific siRNAs for Nrf2 (A) and HO-1 (B) as described in Materials and Methods. The cells were stimulated with TNF-α (10 ng/ml) for 15 min, after preincubation for 3 hr without or with 0.5 μM of auranofin. IκB-α degradation was examined by Western blot analysis. The experiments were performed three times and the results were similar.

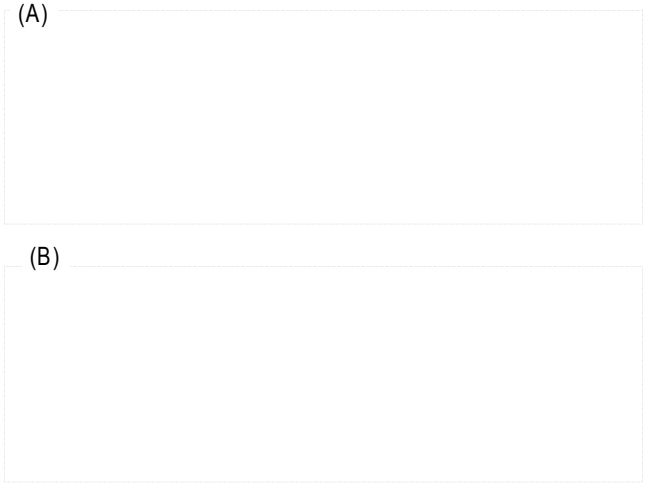

Fig. 4. The ICAM-1 expression down-regulated by auranofin is not restored by Nrf2 knockdown. (A) and (B) The cells transfected with control siRNA or Nrf2 siRNA were preincubated with auranofin for 3 hr and then stimulated with 10 ng/ml of TNF-α (A) or 5 ng/ml of IL-1β (B) for 6 hr. In the cells treated with cytokines, ICAM-1 was detected by Western blot analysis. N and C mean the samples from cells transfected with Nrf2 siRNA and control siRNA, respectively. The results are representative of three independent experiments.

are not involved in the mechanism underlying the auranofin-induced attenuation of NF-κB activation. Further studies are required to elucidate this discrepancy.

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초록 : 오라노핀에 의한 nuclear factor κB 활성저해는 Nrf2 활성화와 무관한 기전에 의함

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내재면역반응의 중요한 조절자인 Nrf2와 NF-κB는 염증시에 교차 작용을 통하여 서로의 전사활성을 조절할 수 있다고 보고된 바 있으나 상반된 결과도 제시되고 있어서 아직까지 확실하게 규명되어 있지 않다. 저자들은 선행연구에서 NF-κB 저해제인 금(I)-화합물 오라노핀이 인간 관절활막세포와 단핵구성 세포에서 Nrf2를 활성화시킴을 확인한 바 있기 때문에, 본 연구에서는 Nrf2를 knockdown 시킨 류마티스성 활막세포를 사용하여 오라노핀에 의해 저해되는 NF-κB 신호전달 과정에 Nrf2가 관여하는지를 조사하였다. 세포를 Nrf2 siRNA로 transfection 시켰을 때 Nrf2 발현은 대부분 차단됨을 확인하였다. 하지만 Nrf2 knockdown은 TNF-α에 의해 유도되는 IκB-α 분해를 막는 오라노핀의 작용에는 영향을 주지 않았다. Nrf2 target 단백질로서 항염 작용에 관여하는 HO-1을 knockdown 시켰을 경우에도 IκB-α 분해를 저해하는 오라노핀의 작용에 영향을 미치지 않았다. 또한, Nrf2 knockdown은 오라노핀에 의해 저해된 ICAM-1 발현을 다시 복원시키지 못했다. 이러한 결과들은 염증성 싸이토킨에 의해 유도되는 NF-κB 활성화를 오라노핀이 저해하는 기전에 Nrf2 및 HO-1이 관련되어 있지 않음을 시사한다. 따라서 류마티스성 관절활막세포에서 오라노핀의 항염작용 기전으로 알려진 Nrf2/HO-1 활성유도와 NF-κB 활성저해는 교차작용 없이 각각 독립적인 기전을 통해 나타나는 것으로 생각된다.