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Full Paper

Auranofin, a Gold(I)-Containing Antirheumatic Compound, Activates Keap1/Nrf2 Signaling via Rac1/iNOS Signal and Mitogen-Activated Protein Kinase Activation

Nam-Hoon Kim[1], Mi-Kyung Oh[1], Hyo Jung Park[1], and In-Sook Kim[1,*]

[1]*Department of Natural Sciences, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea*

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Abstract. Auranofin (2,3,4,6-tetra-*O*-acetyl-1-thio-*β*-D-glucopyranosato-*S*-[triethylphosphine] gold) is a gold(I)-containing antirheumatic drug that possesses anti-inflammatory properties. The pharmacological activity of this drug is associated with its ability to induce heme oxygenase-1 (HO-1). However, the mechanism underlying auranofin-mediated HO-1 induction remains unclear. We investigated the action of auranofin on activation of nuclear factor erythroid 2–related factor 2 (Nrf2), an activator of HO-1. Auranofin elevated cellular levels of Nrf2 by increasing protein stability but not transcriptional activation. Coimmunoprecipitation and Western blot analysis indicated that auranofin inhibited Nrf2 degradation by inducing the dissociation of the Nrf2 / Kelch-like ECH-associated protein 1 (Keap1) complex, which resulted in nuclear accumulation of Nrf2. In addition, auranofin treatment activated cellular Rac1 and induced inducible nitric oxide synthase (iNOS) expression. An inhibitor of Rac1 (NSC23766) blocked the iNOS induction as well as Nrf2 activation and HO-1 expression. N^G-nitro-L-arginine methyl ester and aminoguanidine, inhibitors of iNOS, diminished the auranofin-induced Nrf2 activation and HO-1 expression. Phosphorylation of mitogen-activated protein kinases (MAPKs) was increased by auranofin treatment, and inhibitors of MAPKs partially diminished the Nrf2 activation. A chromatin immunoprecipitation assay showed that the Nrf2 activated by auranofin was involved in transactivation of the HO-1 gene. These findings indicate that auranofin leads to HO-1 upregulation by activating Keap1/Nrf2 signaling via Rac1/iNOS induction and MAPK activation.

Keywords: auranofin, nuclear factor erythroid 2–related factor 2 (Nrf2), Kelch-like ECH-associated protein 1 (Keap1), heme oxygenase-1 (HO-1), Rac1

Introduction

Gold(I) compounds have been used for the treatment of rheumatoid arthritis for a long time (1). Auranofin (2,3,4,6-tetra-*O*-acetyl-1-thio-*β*-D-glucopyranosato-*S*-[triethylphosphine] gold) is a sulfur-containing gold(I)-based antirheumatic drug (1, 2). Auranofin is a potent inducer of heme oxygenase-1 (HO-1) (3 – 5) and can also inhibit the activation of signal transducer and activator of transcription 3 (STAT3), nuclear factor *κ*B (NF-*κ*B), and homodimerization of toll-like receptor 4, all of which can be stimulated by interleukin 6 or lipopolysaccharide (LPS) (6 – 9). In addition, the anticancer activity of auranofin has been reported in various human cancer cells (10, 11). Accordingly, the structurally related gold(I)-containing compounds have been developed for application to the treatment of inflammation and cancer (12, 13).

Accumulated evidence indicates that nuclear factor erythroid 2–related factor 2 (Nrf2) and HO-1 play important roles in the attenuation of inflammation and immune reactions by regulating the expression of mediators such as tumor necrosis factor-*α* (TNF-*α*), interleukin-6, inducible nitric oxide synthase (iNOS), and cyclooxygenase 2, which can all be induced by LPS (3, 14, 15). Nrf2 and HO-1 have therefore been highlighted as pharmacological targets for anti-inflammatory drugs (16, 17). A recent report showed that auranofin inhibited the secretion of

*Corresponding author. ikim@catholic.ac.kr
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LPS-induced interleukin-6, interleukin-8, and TNF-α, and the suppressive effect was canceled by specific small interference RNA (siRNA) for HO-1 in synovial cells. These results indicated that the pharmacological property of auranofin in rheumatoid arthritis is partly associated with its ability to induce HO-1 (3). However, the mechanism underlying auranofin-mediated HO-1 induction remains unclear.

HO-1 catalyzes heme degradation and produces biliverdin, carbon monoxide, and Fe^{2+}. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. The anti-inflammatory effects of HO-1 are due to the biological activities of the byproducts generated during heme degradation (3, 18, 19). Nrf2, AP-1, and NF-κB are important transcription factors for HO-1 expression. A number of pharmaceutical compounds such as sulfur-containing compounds and Michael reaction acceptors containing an α,β-unsaturated carbonyl moiety can induce HO-1 expression, and the induction is dependent on Nrf2 activation (20).

Nrf2 is a redox-sensitive transcription factor of the basic leucine zipper family (21). In the cytoplasm, Nrf2 binds to the Kelch-like ECH-associated protein 1 (Keap1), which is anchored to the actin cytoskeleton (22). Keap1 promotes the ubiquitination of Nrf2 by cullin3-dependent ubiquitin ligase, and ubiquitinated Nrf2 is rapidly degraded via the 26S proteasome system (21). Nrf2 has a short lifetime in unstimulated cells (23), but stimulation by oxidative stress or electrophilic chemicals induces a conformational change of Keap1 and leads to the dissociation of Nrf2 from Keap1. The released Nrf2 translocates to the nucleus, where it forms Nrf2 / small Maf heterodimer and binds specifically to the antioxidant response element (ARE) in its target genes, including the HO-1 gene (24).

In the present study, we investigated the effect of auranofin on the Keap1/Nrf2 signaling pathway that leads to induction of HO-1 in THP-1 human monocytic cells and human synoviocytic cells.

Materials and Methods

Cell culture and treatment

THP-1 human monocytic cell line and MDA-MB 231 human breast cancer cell line were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 medium (Gibco Life Technology, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco). SV40 T antigen–transfected human synoviocytic cell line was obtained from Dr. Wan-Uk Kim (The Catholic University of Korea, Seoul, Korea) and maintained in the same medium. The cells were plated at a density of 1×10^6/ml. After 16 h, the cells were treated with auranofin (Alexis, Lausen, Switzerland) for the indicated periods in serum-free medium. To investigate the effect of various inhibitors, before auranofin treatment, the cells were preincubated for 30 min with 200 μM NSC23766 (Calbiochem, San Diego, CA, USA), 5 μM diphenyleneiodonium (DPI; Sigma Chemical, St. Louis, MO, USA), 40 mM N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), 20 mM aminoguanidine (AG, Sigma), 40 μM PD98059 (Sigma), 30 μM SB203580 (Sigma), 15 μM SP600125 (Sigma), or 5 μM MG132 (Sigma).

Western blot analysis

Western blotting was performed as previously described (10) using antibodies against Nrf2, HO-1, Keap1, iNOS, α-tubulin, and extracellular signal-regulated kinase (ERK), which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), as well as antibodies to phospo-ERK, phospo-p38, and phospo-jun N-terminal kinase (JNK), which were obtained from Cell Signaling Technology (Beverly, MA, USA).

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNA STAT-60 solution (TEL-TEST; Friendswood, TX, USA). A 2-μg sample of total RNA was reverse-transcribed using reverse transcriptase (Promega Corporation, Madison, WI, USA) for 1 h at 42°C. The cDNA was amplified by PCR using specific primers for Nrf2 (forward, 5′-TTGCCTGT AAGTCCTGGTC-3′ and reverse, 5′-ACTGCTCTTTG GACATCATTTCG-3′), HO-1 (forward, 5′-ACATCTA TGTGGCCCTGGAG-3′ and reverse, 5′-TGTTGGGG AAGGTGAAGAAG-3′) and GAPDH (forward, 5′-AC CACAGTCCATGCCATCAC-3′ and reverse, 5′-TC CACCACCCTGTTGCTGTA-3′). The PCR reaction was performed for 25 cycles under the following conditions: denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide.

Plasmid construction

Nrf2 full-length cDNA–inserted plasmid (pOTB7) was purchased from NIH mammalian gene collection (Open Biosystems, Huntsville, AL, USA), and the *Nrf2* gene was subcloned into a mammalian expression vector, pCR3.1 (Invitrogen, Carlsbad, CA, USA) at *BamHI* and *XhoI* sites. The DNA sequence was confirmed by commercial services (Cosmo Genetech, Seoul, Korea).

Immunoprecipitation

After preclearing with goat normal IgG (Sigma),

samples were incubated with anti-Keap1 antibody with shaking at 4°C overnight and followed by further incubation with agarose-conjugated protein G (Invitrogen) for 1 h at 4°C. The immune complexes were precipitated by centrifugation and washed with cold phosphate-buffered saline three times. The coimmunoprecipitated Nrf2 was detected by Western blotting.

Determination of Rac1 activation

The auranofin-treated cells were suspended in immunoprecipitation lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride] and then sonicated on ice. The active Rac1 was precipitated from total cell lysate using glutathione-agarose beads conjugated with a fusion protein, glutathione *S*-transferase (GST) fused to the p21-binding domain of human PAK-1, which specifically binds GTP-Rac1 (Upstate Biotechnology Industries, Lake Placid, NY, USA). The precipitated Rac1 was detected by Western blotting using anti-Rac1 antibody (BD Biosciences, San Jose, CA, USA).

Subcellular fractionation

To isolate cytoplasmic and nuclear fractions, cells were suspended in cold buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethylsulphonyl fluoride, 5 μg/ml leupeptin, and 10 μg/ml aprotinin] and allowed to swell on ice for 15 min. The cells were lysed by adding Nonidet P-40 (final 0.6%) and centrifuged at 14,000 × *g* for 1 min. The supernatant was saved as the cytoplasmic fraction; and the residual nuclei were resuspended in cold buffer B [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM ethylenediaminetetraacetic acid, 25% glycerol, 1 mM phenylmethylsulphonyl fluoride, 5 μg/ml leupeptin, and 10 μg/ml aprotinin) and then vigorously agitated. After centrifugation at 14,000 × *g* for 20 min, the supernatant was taken and used as a nuclear fraction.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously (25). Briefly, the cells were cross-linked with 1% formaldehyde for 10 min at 37°C and lysed. The lysates were sonicated to obtain fragmented DNA of 100 – 200 bp and immunoprecipitated with antibodies against Nrf2 or human IgG (negative control). The DNA was extracted from the immunoprecipitated protein–DNA complex, and the ARE region of HO-1 promoter was amplified by PCR with the specific primer (forward, 5′-GCTGCCCA AACCACTTCTGT-3′ and reverse, 5′-GCCCTTTCAC CTCCCACCTA-3′). The PCR products were analyzed on 1% agarose gels.

Statistical analyses

Data were expressed as means ± S.D. Statistical analyses were performed using the paired *t*-test for two data comparison and one-way analysis of variance (ANOVA) with Dunnett's test for multiple data comparison (version 11.5; SPSS Inc., Chicago, IL, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

The effect of auranofin on Nrf2 expression

Nrf2 protein levels were increased by auranofin treatment in a time- and dose-dependent manner in THP-1 cells (Fig. 1: A and B) and synoviocytes (Fig. 1: D and E); however, Nrf2 mRNA levels were not changed in either cell type (Fig. 1: C and F). Consistent with the Nrf2 increase, HO-1 expression was upregulated at both the transcript and protein levels (Fig. 1). These findings indicate that auranofin not only regulated the expression of Nrf2 post-translationally, but also induced the expression of the HO-1 gene in monocytes and synoviocytes.

To examine whether the upregulation of Nrf2 was caused by an increase in protein stability, we performed a cycloheximide (CHX) blocking assay. To inhibit Nrf2 degradation and allow its accumulation to levels that were detectable in control cells, the cells were preconditioned by treatment with a 26S proteasome inhibitor (MG132) for 1 h, followed by extensive washing. Subsequently, *de novo* synthesis of Nrf2 was blocked by pretreatment with CHX for 5 min and then followed by incubation with auranofin for the indicated times. In untreated control cells, a large proportion of Nrf2 was degraded within 1 h and the protein disappeared completely after 2 h of incubation. In contrast, Nrf2 was detectable in auranofin-treated cells for a longer period [i.e., up to 3 h after incubation (Fig. 1G)]. These results suggest that auranofin increased the stability of the Nrf2 protein.

In our Western blot system, the Nrf2 was detected as nearby double bands. However, some immunoblots showed it as a single band, which was thought to result from the two bands overlapping. Other researchers' results also showed the band pattern of Nrf2 protein as a single band (26) or double bands (27), although the reason was unclear.

Stimulatory effect of auranofin on Nrf2 release from the Keap1/Nrf2 complex

To investigate the mechanism underlying the stabilization of the Nrf2 protein, we examined whether auranofin promoted the release of Nrf2 from Keap1. Coimmunoprecipitation analysis of Nrf2 and Keap1 demonstrated that Nrf2 was not bound to Keap1 in the cytoplasm of

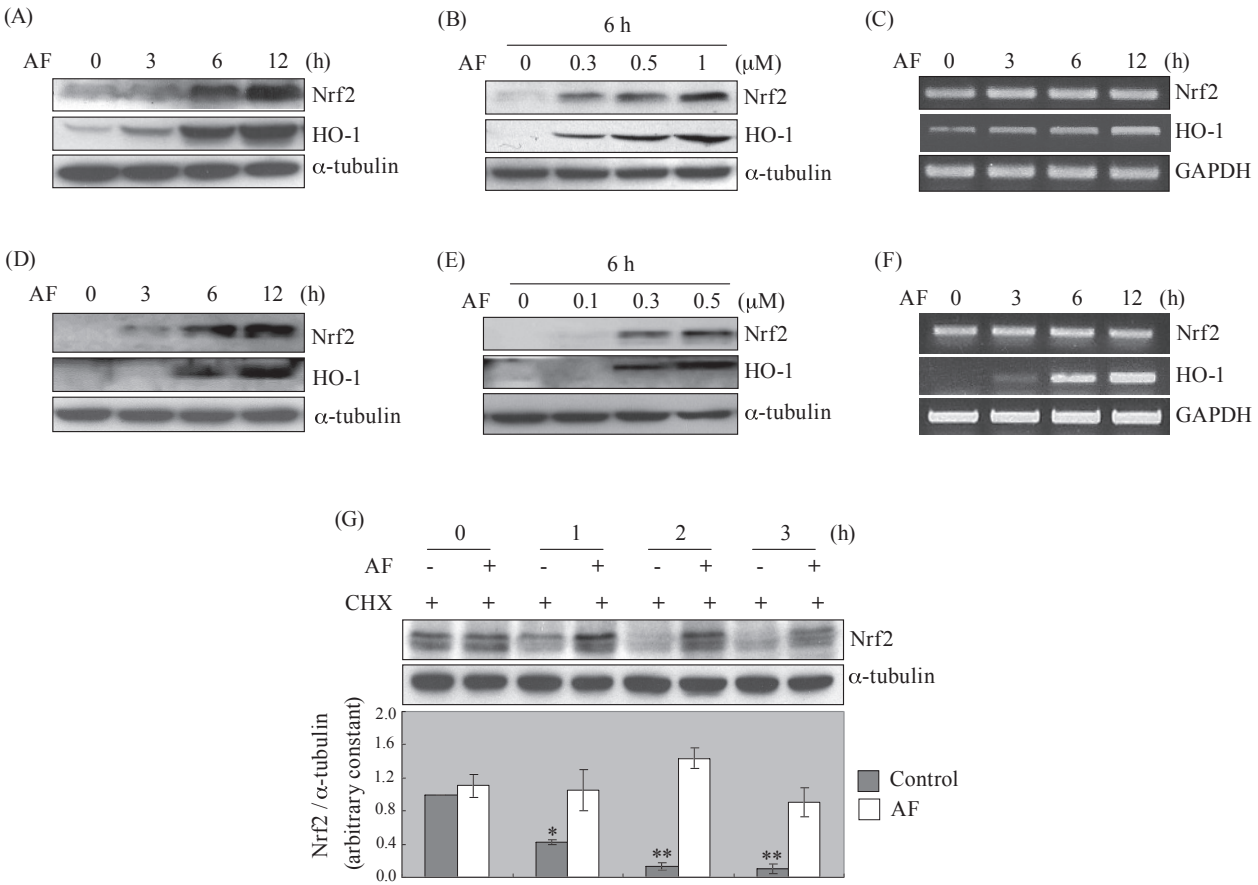

Fig. 1. Enhancement of Nrf2 levels via protein stabilization. Panels A – C are the results for THP-1 monocytic cells and panels D – F are the results for synoviocytes. All experiments were performed 2 or 3 times with similar results and a representative experiment is shown. A and D) Cells were treated with 1 μM auranofin (A) or 0.5 μM auranofin (D) for the indicated times. B and E) Cells were treated for 6 h with auranofin at 0.1 – 1 μM. Cellular proteins were extracted and analysed by Western blot using antibodies against Nrf2 and HO-1. α-Tubulin was used as an internal marker to ensure equal protein loading. C and F) After treatment with auranofin, Nrf2 and HO-1 mRNA levels were determined by RT-PCR. AF indicates auranofin. G) Upper panel: THP-1 cells were preconditioned by treatment with 5 μM MG132 for 1 h, which was followed by extensive washing. Subsequently, cells were pretreated with 10 μg/ml CHX for 5 min and then further incubated in the presence or absence of 1 μM auranofin for 1, 2, and 3 h. After the incubation, Nrf2 levels were analyzed by Western blotting. A representative result of three independent experiments is shown. Lower panel: each Nrf2 level was normalized to the corresponding α-tubulin level and the quantitative results of Nrf2/α-tubulin are each expressed as the mean ± S.D. of data from three separate experiments. *$P < 0.05$, **$P < 0.01$ vs. auranofin-untreated control at 0 time (ANOVA with Dunnett's test). There were no significant differences in the auranofin-treated group.

auranofin-stimulated cells (Fig. 2), indicating that auranofin induced the release of Nrf2 from the Nrf2/Keap1 complex. Taken together, the data shown in Figs. 1 and 2 suggest that auranofin induces the dissociation of Nrf2 from Keap1, leading to a longer Nrf2 protein half-life by protecting it from the ubiquitin–proteasome system.

Involvement of Rac1 / NADPH oxidase / iNOS signaling in the auranofin-mediated Nrf2 activation

Reactive oxygen species (ROS) and nitrogen species are mediators of Nrf2 activation (28, 29). We found previously that auranofin induced ROS production in leukemia cells (10). We could also detect ROS production at early times in auranofin-treated THP-1 cells (data not shown). In addition, it was reported that hydrogen peroxide activated cellular Rac, which is a member of the Rho family of small GTPases, and that prostaglandin J_2–activated Rac1 was closely correlated with Nrf2-dependent HO-1 expression (30 – 32). We wondered if the activation of Rac1 is involved in the mechanism of auranofin-stimulated Nrf2/HO-1 activation. To test this hypothesis, we tried to detect GTP-Rac1, the active form of Rac1, in auranofin-treated cells. As shown in Fig. 3, A and B, Rac1 activation was promoted by auranofin treat-

ment at 30 min in both THP-1 monocytes and synoviocytes. Experiments using inhibitors of Rac1 activation (NSC) and NADPH oxidase (DPI) revealed that the Rac1 / NADPH oxidase system is involved in auranofin-mediated Nrf2 activation (Fig. 3: C and D).

Cells treated with auranofin exhibited iNOS induction (Fig. 4: A and B) and pre-incubation with a Rac1 inhibitor for 30 min before auranofin treatment diminished the expression of iNOS, Nrf2, and HO-1 (Fig. 4C). In addition, the NOS inhibitors L-NAME and AG reduced the

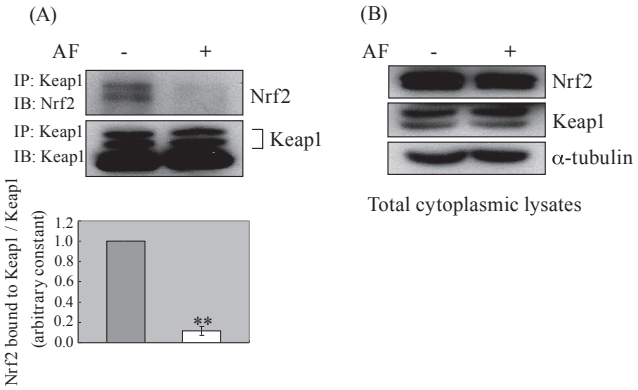

Fig. 2. Auranofin-mediated release of Nrf2 from the Nrf2/Keap1 complex. MDA-MB231 cells were transfected with the human *Nrf2* gene for 36 h. Transfected cells were pre-incubated with 5 μM MG132 for 30 min and then treated with 1 μM auranofin for 3 h. Cells were lysed and fractionated into cytoplasmic and nuclear fractions. A) Keap1 in the cytoplasmic fraction was immunoprecipitated with an anti-Keap1 antibody and the immunoprecipitated proteins were analyzed by Western blotting using antibodies to Nrf2 and Keap1. A representative result of three independent experiments is shown. The quantitative results of Nrf2 bound to Keap1/Keap1 are expressed as means ± S.D. $^{**}P < 0.01$ vs. auranofin-untreated condition (n = 3, paired *t*-test). B) The total amount of Nrf2 and Keap1 in the cytoplasmic fraction was also determined by Western blotting using samples collected before immunoprecipitation.

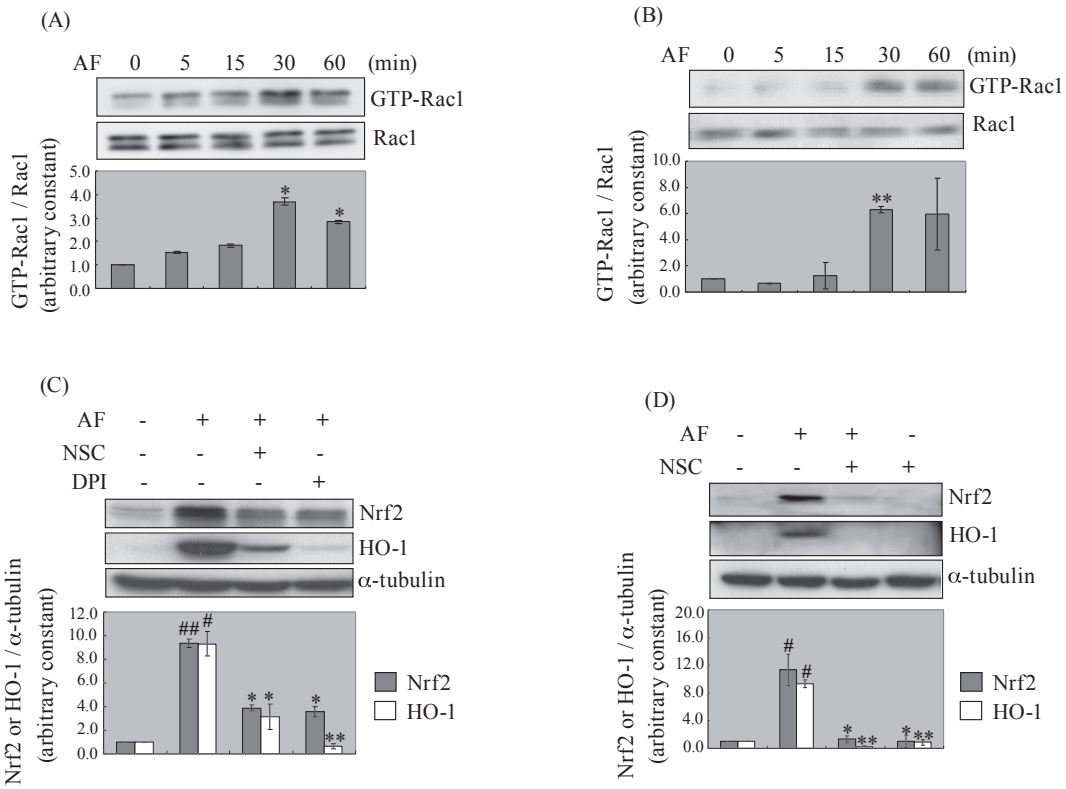

Fig. 3. Auranofin-mediated Rac1 activation and upregulation of Nrf2 and HO-1. A and B) THP-1 cells and synoviocytes were incubated for the indicated times in the presence of auranofin at 1 and 0.5 μM, respectively. Active Rac1 in the cell lysates was detected as described in Materials and Methods. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 time (n = 3, ANOVA with Dunnett's test). C and D) After pre-incubation with NSC23766 (200 μM) or DPI (5 μM) for 30 min, THP-1 cells (C) and synoviocytes (D) were treated with auranofin for 6 h. The effect of the inhibitors was analyzed by Western blotting. The quantitative results represent the means ± S.D. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. untreated control, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. treatment with auranofin without inhibitors (n = 3, ANOVA with Dunnett's test).

levels of Nrf2 and HO-1 significantly and also blocked the nuclear translocation of Nrf2, which was elevated by auranofin (Fig. 4: D and E). These results indicate that auranofin activates Nrf2 via Rac1/iNOS signaling and leads to the upregulation of HO-1.

Transactivation of the HO-1 gene by auranofin-mediated Nrf2 activation

We performed a ChIP assay to determine whether the HO-1 gene was a target of auranofin-activated Nrf2. Figure 5 shows that binding of Nrf2 to the HO-1 promoter was induced by auranofin and that this binding was attenuated by treatment with inhibitors of Rac1, NADPH oxidase, and iNOS. This suggests that auranofin upregulates HO-1 expression via Nrf2 activation, which is mediated by the Rac1 / NADPH oxidase / iNOS signaling pathway.

Association of mitogen-activated protein kinases (MAPKs) with auranofin-mediated Nrf2 activation

Because we found previously that auranofin increased p38 phosphorylation, we examined whether MAPKs

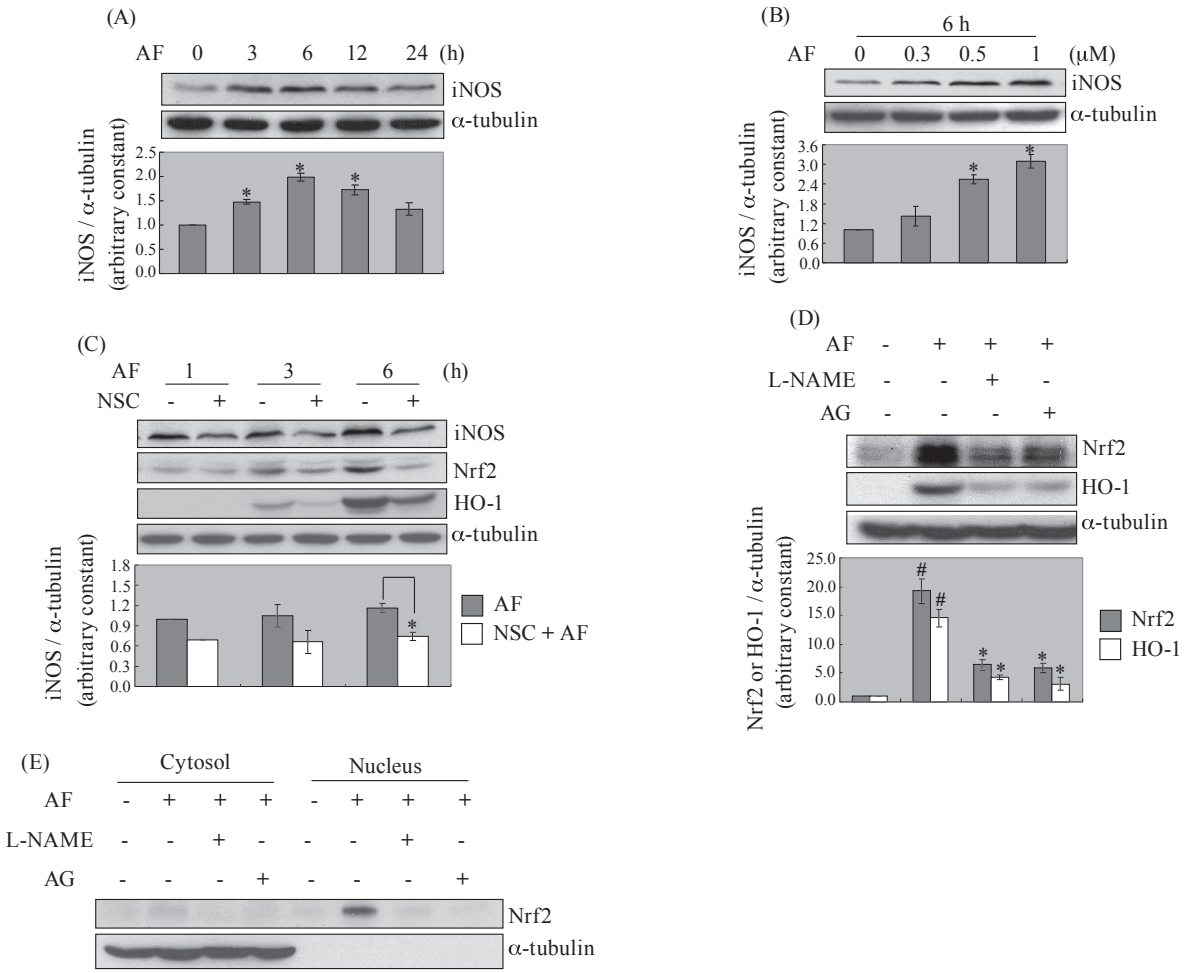

Fig. 4. Involvement of iNOS signaling in the auranofin-mediated upregulation of Nrf2 and HO-1. A and B) Monocytic cells were treated with 1 μM auranofin in a time-dependent manner or in an auranofin-dose–dependent manner for 6 h. The quantitative results represent the means ± S.D. $^{*}P < 0.05$ vs. auranofin-untreated condition (n = 3, ANOVA with Dunnett's test). C and D) Cells were pretreated with inhibitors [i.e., NSC23766 (200 μM), L-NAME (40 mM), and AG (20 mM)], for 30 min before addition of 1 μM auranofin. Western blotting was performed for the detection of iNOS, Nrf2, and HO-1. The quantitative results represent the means ± S.D. C) $^{*}P < 0.05$ vs. NSC-untreated cells at each time (n = 3, paired *t*-test). D) $^{\#}P < 0.05$ vs. untreated control, $^{*}P < 0.05$ vs. cells treated with auranofin without inhibitors (n = 3, ANOVA with Dunnett's test). E) After the cells were pretreated with NOS inhibitors (L-NAME and AG), they were incubated with auranofin. Fractionation into cytoplasmic and nuclear fractions was performed as described in Materials and Methods. The nuclear translocation of Nrf2 was identified by Western blot analysis of these fractions. α-Tubulin was used as an internal marker for the cytoplasmic fraction. The experiments were performed twice and the results were similar.

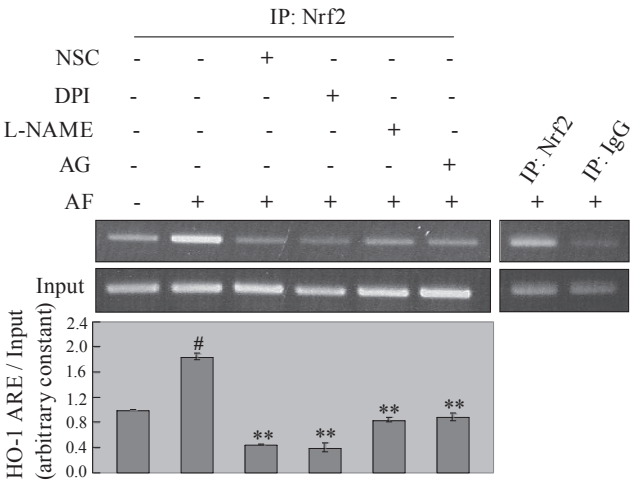

Fig. 5. Binding of auranofin-activated Nrf2 to the ARE of the *HO-1* promoter. THP-1 cells were pre-incubated with or without the inhibitors, NSC23766 (200 μM), DPI (5 μM), L-NAME (40 mM), and AG (20 mM) for 30 min, and were then treated with 1 μM auranofin for 3 h. Nrf2-linked chromatin was immunoprecipitated with an anti-Nrf2 antibody or with a normal rabbit IgG (negative control) and the specific *HO-1*–promoter region containing the ARE site was then amplified using PCR. The experiments were performed three times and a representative result is shown. The quantitative results are shown as the means ± S.D. $^{\#}P < 0.05$ vs. untreated control, $^{**}P < 0.01$ vs. cells treated with auranofin alone (n = 3, ANOVA with Dunnett's test).

signals were involved in Nrf2 activation and HO-1 induction. As shown in Fig. 6A, auranofin increased the phosphorylation of all three MAPKs (ERK, p38, and JNK), although the patterns of the increase in phosphorylation with time were not the same. Inhibitors of the MAPKs partially blocked the auranofin-mediated activation of Nrf2 and HO-1 (Fig. 6B), which suggests that MAPKs signaling was partly associated with Nrf2 activation. Pre-incubation with NSC or DPI before auranofin treatment inhibited ERK phosphorylation exclusively and did not affect phosphorylation of p38 and JNK (Fig. 6: C and D). These findings suggest that the activation of ERK is Rac1 / NADPH oxidase–dependent and that p38 and JNK are independently associated with auranofin-mediated Nrf2 activation. However, iNOS induction was not required for ERK activation (Fig. 6E).

Discussion

Elucidation of the action mechanisms for the pharmacological activity of specific compounds may provide new strategies for the development of target drugs. Kataoka et al. reported previously that auranofin activated the DNA binding of the Nrf2 / small Maf heterodimer and upregulated the expression of antioxidative

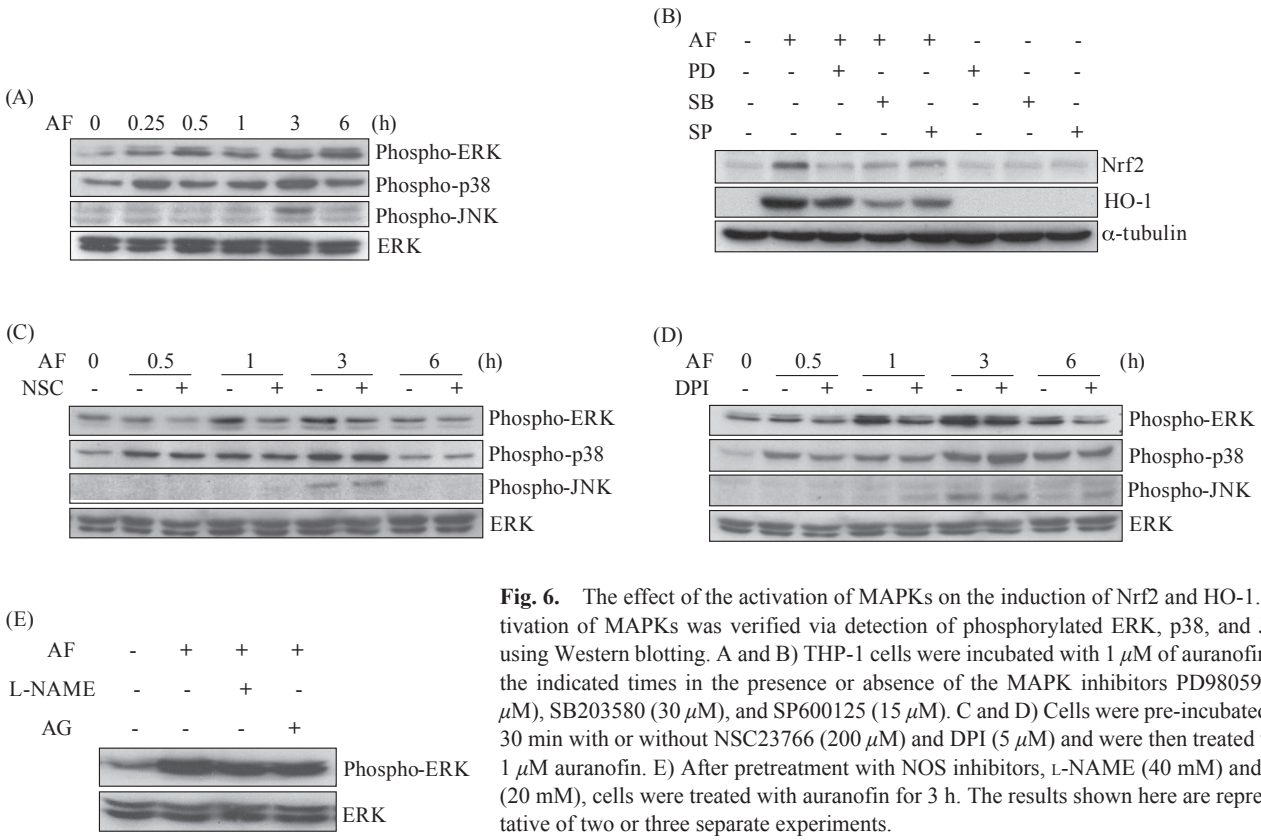

Fig. 6. The effect of the activation of MAPKs on the induction of Nrf2 and HO-1. Activation of MAPKs was verified via detection of phosphorylated ERK, p38, and JNK using Western blotting. A and B) THP-1 cells were incubated with 1 μM of auranofin for the indicated times in the presence or absence of the MAPK inhibitors PD98059 (40 μM), SB203580 (30 μM), and SP600125 (15 μM). C and D) Cells were pre-incubated for 30 min with or without NSC23766 (200 μM) and DPI (5 μM) and were then treated with 1 μM auranofin. E) After pretreatment with NOS inhibitors, L-NAME (40 mM) and AG (20 mM), cells were treated with auranofin for 3 h. The results shown here are representative of two or three separate experiments.

stress genes, including HO-1 (4). However, until now, the molecular mechanisms underlying the cytoplasmic events in auranofin-promoted Nrf2 activation were unknown. In this paper we elucidated the upstream signaling pathway for the auranofin-induced Nrf2/HO-1 activation in THP-1 monocytic cells and a human synoviocytic cell line. Our data indicate that auranofin activates the Rac1 / NADPH oxidase system and subsequently induces iNOS, which leads to the dissociation of Keap1/Nrf2 and the nuclear accumulation of Nrf2. iNOS-independent activation of MAPKs is also involved in the mechanism of auranofin-mediated Nrf2/HO-1 activation (Fig. 7).

We demonstrated, for the first time, that auranofin activates cellular Rac1 in monocytic cells and synoviocytes, although the underlying mechanism is unknown. We previously found that auranofin generated ROS, and Ho et al. reported that hydrogen peroxide activated Rac (30). Therefore, it is possible that ROS participates in auranofin-induced Rac1 activation. In THP-1 cells, auranofin produced cellular ROS within 15 min and pretreatment with *N*-acetyl-L-cysteine, a ROS scavenger, completely blocked the ROS production as well as Nrf2/HO-1 activation (data not shown). We are currently studying the ROS-dependency of Rac1/Nrf2/HO-1 activation induced by auranofin.

It has been reported that various compounds that react with cysteine thiol groups of specific proteins increase HO-1 expression by Nrf2 activation via diverse upstream signal pathways. For example, curcumin, caffeic acid phenethyl ester, and sulforaphane are known to modify specific cysteine residues in Keap1 and lead to HO-1 induction (20, 33). Auranofin is also a thiol-reactive compound. Jeon et al. showed that auranofin could modify Cys-179 of inhibitory κB kinase beta subunit (8). We show here that the NOS inhibitors L-NAME and AG block auranofin-mediated Nrf2 activation (Fig. 4: D and E), suggesting that auranofin indirectly changes the Keap1 structure (i.e., it modified Keap1 via iNOS signaling). Recent reports that NO provokes (*S*)-nitrosation of cysteine residues in Keap1 and induces nuclear accumulation of Nrf2 (34, 35), in combination with our data, suggest the possibility that auranofin triggers Keap1 modification through (*S*)-nitrosation and results in the nuclear translocation of Nrf2. Further study is required to understand the mechanism of auranofin-mediated Keap1 modifications.

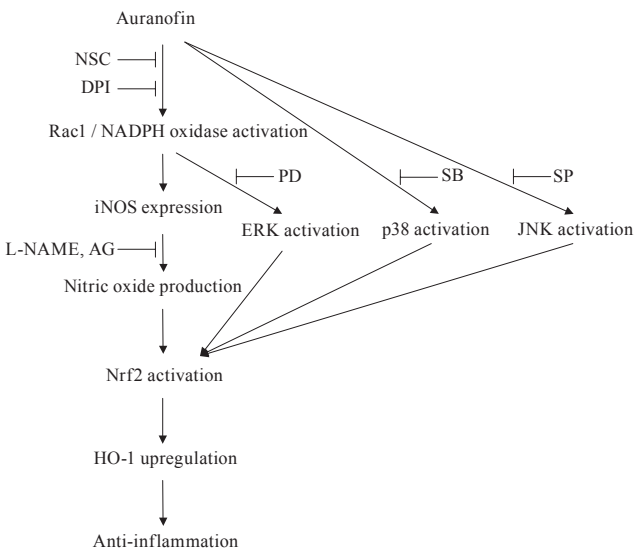

Fig. 7. Proposed mechanism underlying the auranofin-mediated upregulation of Nrf2 and HO-1, involving Rac1 / NADPH oxidase / iNOS signaling and activation of MAPKs.

The present study clearly demonstrated that auranofin could activate the Nrf2/HO-1 in both the synoviocytic cells derived from a patient with rheumatoid arthritis and monocytic cells. It suggests that the upregulation of Nrf2/HO-1 may be a pivotal event in the mechanism for the antirheumatic property of auranofin. In addition, the pharmacologic activity of auranofin on Nrf2/HO-1 activation might be applicable to various therapeutic strategies for cancers and inflammatory disorders, in which the Nrf2-dependently elevated antioxidant system plays an important role.

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