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Gold compound auranofin inhibits IκB kinase (IKK) by modifying Cys-179 of IKKβ subunit

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Abbreviations: $15dPGJ_2$, 15-deoxy-Δ^{12-14}-prostaglandin J_2; GST, glutathione *S*-transferase; HA, hemagglutinin; IKK, IκB kinase; NIK, NF-κB-inducing kinase

Abstract

Antirheumatic gold compounds have been shown to inhibit NF-κB activation by blocking IκB kinase (IKK) activity. To examine the possible inhibitory mechanism of gold compounds, we expressed wild type and mutant forms of IKKα and β subunits in COS-7 cells and determined the effect of gold on the activity of these enzymes both *in vivo* and *in vitro*. Substitution of Cys-179 of IKKβ with alanine (C179A) rendered the enzyme to become resistant to inhibition by a gold compound auranofin, however, similar protective effect was not observed with an equivalent level of IKKα (C178A) mutant expressed in the cells. Auranofin inhibited constitutively active IKKα and β and variants; IKKα (S176E, S180E) or IKKβ (S177E, S181E), suggesting that gold directly cause inhibition of activated enzyme. The different inhibitory effect of auranofin on IKKα (C178A) and IKKβ (C179A) mutants indicates that gold could inhibit the two subunits of IKK in a different mode, and the inhibition of NF-κB and IKK activation induced by inflammatory signals in gold-treated cells appears through its interaction with Cys-179 of IKKβ.

Keywords: antirheumatic agent gold; auranofin; cysteine; mutagenesis; NF-κB; protein-serine-threonine kinases

Introduction

The transcription factor NF-κB plays a critical role in the regulation of genes involved in chronic inflammatory diseases (Barnes and Karin, 1997). In unstimulated cells, NF-κB is sequestered in the cytoplasm through interaction with inhibitory proteins called IκB (IκBα, β and ε). NF-κB is activated in response to many signals including proinflammatory cytokines such as IL-1 and TNF, bacterial LPS, virus, ultraviolet light, and oxidants (Baeuerle and Henkel, 1994; Barnes and Karin, 1997). These signals cause phosphorylation and subsequent degradation of IκB proteins, and the released NF-κB enters the nucleus to induce expression of specific target genes. In the signal pathway for NF-κB activation, phosphorylation of IκB is likely to be the central point of regulation. Previous studies identified an IκB kinase (IKK) complex of 700 kDa, which is induced by inflammatory signals and able to phosphorylate two conserved N-terminal serine residues of IκBα and IκBβ (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997). IKK complex is composed of two catalytic subunits, IKKα (or IKK1) and IKKβ (IKK2), and a regulatory subunit, IKKγ/NEMO/IKKAP1 (reviewed in May and Ghosh, 1998; Zandi and Karin, 1999). IKKα and IKKβ are Ser/Thr kinases of similar structure which can form homodimers and heterodimers *in vitro*, and purified recombinant form of each can directly phosphorylate IκBα and β at proper sites (Lee *et al.*, 1998; Li *et al.*, 1998; Zandi *et al.*, 1998). The activity of IKKα and IKKβ was shown to depend on their phosphorylation at specific serine residues (Ser-176/180 of IKKα and Ser-177/181 of IKKβ) located in the T (activation) loop of the kinase domain, and substitution of these sites with glutamate, which mimic the effect of phosphoserine, generated constitutively activated form of IKKα and IKKβ (Mercurio *et al.*, 1997; Ling *et al.*, 1998). Although the similar structures of IKKα and IKKβ suggest their common functional role in NF-κB activation, studies with animals deficient in each IKK subunit revealed that IKKα plays a role during embryonic development of the skin and skeletal system, while IKKβ is essential for the activation of IKK in response to TNF and other proinflammatory stimuli (Gerondakis *et al.*, 1999; reviewed in Zandi and Karin, 1999).

Gold compounds, comprised of elemental Au(I) and a sulfur-containing ligand, have been used for the treatment of rheumatoid arthritis, inducing improvement of clinical conditions in a majority of patients

(Research Subcommittee of the Empire Rheumatism Council, 1960; Champion *et al*., 1990). Administration of a gold compound aurothiomalate to rheumatoid arthritis patients was shown to reduce expression of inflammatory cytokines, IL-1, IL-6 and TNF, in the synovial membrane (Yanni *et al*., 1994). In LPS-stimulated monocytes and macrophages, a lipid-soluble gold compound auranofin inhibited production of IL-1 and TNF by reducing their mRNA levels, suggesting a transcriptional regulation of IL-1 and TNF expression by auranofin (Bondeson and Sundler, 1995). Our previous study showed that auranofin and other thiol-reactive metal ions inhibit NF-κB activation by blocking IKK activation in LPS-stimulated macrophages (Jeon *et al*., 2000). These metal compounds and other thiol-modifying agents such as *N*-ethylmaleimide and *p*-hydroxymercuribenzoate directly suppressed IKK activity *in vitro*, indicating that these agents inhibit IKK by modifying a critical cysteine thiol group of the enzyme molecule. Recently, other thiol-reactive agents, including cyclopentenone PG [PGA_1 and 15-deoxy-$\Delta^{12\text{-}14}$-PGJ_2 (15dPGJ_2)] and arsenite anion (AsO_3^{3-}) were also reported to inhibit NF-κB and IKK activation in cells stimulated with TNF, IL-1, or phorbol esters (Kapahi *et al*., 2000; Rossi *et al*., 2000). The inhibitory effect of 15dPGJ_2 and arsenite was not observed in cells expressing mutant IKKβ in which Cys-179 in the activation loop is replaced with alanine, indicating that they inhibit IKK by modifying Cys-179 of IKKβ. These results suggest that gold and other thiol-reactive agents can affect IKK activity reacting with Cys-179 of IKKβ or Cys-178 of IKKα.

Here, we examined the effect of gold and other thiol-reactive agents on the activity of various IKKα and IKKβ mutants expressed in COS-7 cells. IKKβ mutant in which Cys-179 was substituted to alanine was resistant to inhibition by auranofin, while an equivalent IKKα mutant, IKKα (C178A), was still susceptible to gold. We also show that various thiol-reactive agents inhibit constitutive-active forms of IKKα and IKKβ as effectively as wild type enzymes. Our results reveal that gold blocks IKK activity by reacting with different sites of IKKα and IKKβ.

Materials and Methods

Materials and cell culture

Antibodies to IKKα (M-280), IKKβ (H-4), and hemagglutinin (HA) tag (Y-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag (M2) antibody was from Stratagene (La Jolla, CA). Auranofin was provided by Yukyung Medical (Seoul, South Korea). 15dPGJ_2 was from Biomol (Plymouth Meeting, PA) and dissolved in ethanol. Other chemicals were obtained from Sigma (St. Louis, MO). Recombinant glutathione S-transferase (GST)-IκBα containing N-terminal 54 residues of IκBα was prepared by expression in *Escherichia coli* as described previously (Jeon *et al*., 2000). COS-7 monkey kidney cells were obtained from the American Type Culture Collection (ATCC, Manassa, VA). Cells were grown and maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Expression of IKKα and IKKβ in COS-7 cells and IKK assay

The expression plasmids pRcβActin-3xHA-IKKα encoding wild-type human HA-IKKα was kindly provided by Dr. M. Karin (University of California, San Diego, CA), and described previously (DiDonato *et al*., 1997). pFlagCMV-IKKβ encoding Flag-IKKβ, and the constructs for constitutively active mutants of IKKα and IKKβ were kind gifts of Dr. F. Mercurio (Signal Pharmaceuticals, San Diego, CA) (Mercurio *et al*., 1997). Wild-type NF-κB-inducing kinase (NIK) construct was a gift from Dr. J.-H. Kim (Korea University, Seoul, South Korea). The cDNA for HA-IKKα was subcloned between *Hin*dIII and *Xho*I sites of pCR3.1 (Invitrogen, Groningen, Netherlands). Expression vectors encoding IKKα (C178A) and IKKβ (C179A) mutants with alanine instead of cysteine at appropriate sites were prepared by site-directed mutagenesis using an overlap extension method (Ho *et al*., 1989). Mutations were verified by DNA-sequencing analysis. COS-7 cells grown in 12-well plates were transfected with plasmid vectors and expressed IKK was isolated by immunoprecipitation as described previously (Jeon *et al*., 2000). *In vitro* kinase assay was performed with immune complexes in kinase buffer containing 2-5 μCi of [γ-^{32}P]ATP and GST-IκBα (2 μg). Samples were analyzed by SDS-PAGE on a 12.5% gel and electrophoretically transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA). Phosphorylated GST-IκBα was visualized by autoradiography and quantitated in a phosphor image analyzer (BAS-2500; Fujifilm, Tokyo, Japan). The membrane was subsequently probed with appropriate antibodies to determine the amount of IKKα or IKKβ.

Results

Inhibition of IKKβ by gold occurs through modification of Cys-179

Previously, thiol-reactive 15dPGJ_2 and arsenite were shown to inhibit IKK by complexing Cys-179 of IKKβ (Kapahi *et al*., 2000; Rossi *et al*., 2000). To determine whether gold compounds inhibit IKK also by reacting with Cys-179 of IKKβ (and equivalent Cys-178 of IKKα), COS-7 cells were transfected with expression

constructs for wild type HA-IKKα and Flag-IKKβ and mutants in which the cysteine residues were substituted with alanine. After treatment with various doses of auranofin, cells were lysed and IKK was isolated by immunoprecipitation using anti-tag antibodies. The immune complexes were used to determine kinase activity with GST-IκBα as a substrate (Figure 1). Because IKKα and Cys→Ala variants of IKKα and IKKβ expressed alone in the absence of stimuli showed low IκB kinase activity (Jeon *et al.*, 2000; data not shown), an IKK-activating kinase, NIK, was coexpressed with IKKα or IKKβ. Our result revealed that auranofin inhibited both wild type HA-IKKα and mutant HA-IKKα (C178A) in a similar way (Figure 1A). By contrast, a remarkable difference in their susceptibility to auranofin was detected between wild type Flag-IKKβ and C179A mutant of IKKβ (Figure 1B). The wild type IKKβ-Flag was inhibited by auranofin in a dose-dependent manner, and enzyme activity was reduced by more than 80% at 30 μM auranofin. However, no inhibitory affect was seen in cells expressing Flag-IKKβ (C179A) treated with 10 and 30 μM auranofin.

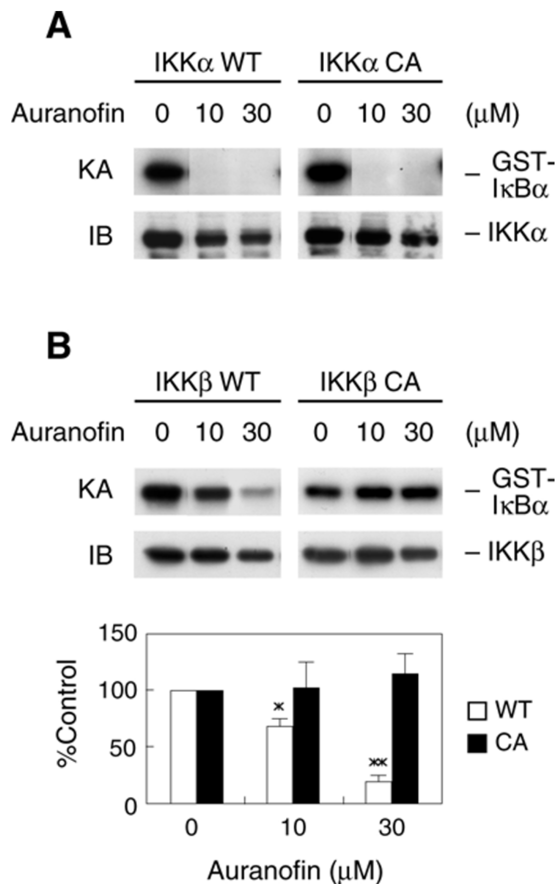

Figure 1. Gold inhibits IKKβ through modification of Cys-179 in its activation loop. (A) COS-7 cells were transiently transfected with wild type HA-IKKα (IKKα WT) or HA-IKKα (C178A) (IKKα CA) expression vectors along with NIK expression vector. After 46 h, the cells were treated for 2 h with 0-30 μM auranofin. Kinase assay (KA) was performed with immune complex obtained from cell lysate (120 μg protein), and the expression level of enzyme was measured by immunoblotting (IB) with anti-HA antibody. (B) COS-7 cells were transfected with wild type Flag-IKKβ (IKKβ WT) or Flag-IKKβ (C179A) (IKKβ CA) expression vectors together with NIK expression vector, and treated with auranofin as in (A). Immune complex prepared from cell lysate (20 μg protein) was used for kinase assay and immunoblotting analysis. IKK activity was determined by measuring the radioactivity of GST-IκBα by phosphor image analysis and calculated as a percent of control (lower histogram). The results are presented as mean±SD (n = 3), and the statistical significance of difference was determined by Student's t-test. *$P < 0.05$; **$P < 0.01$.

Previous studies showed that gold and other thiol-reactive agents directly inhibit IKK complex *in vitro* (Jeon *et al.*, 2000; Kapahi *et al.*, 2000; Rossi *et al.*, 2000). We thus examined whether Cys-178 of IKKα and Cys-179 of IKKβ are involved in inhibition of IKK after exposure of enzyme to gold compounds and other thiol-reactive agents *in vitro*. Wild type and C178A and C179A mutants of IKKα-HA and IKKβ-Flag, respectively, were expressed in COS-7 cells, and isolated by immunoprecipitation. The immune complex was used to determine IKK activity in the presence or absence of various concentrations of hydrophilic gold compound, aurothiomalate, a reducing agent dithiothreitol, and other thiol-blocking agents such as *N*-ethylmaleimide and 15dPGJ$_2$ (Figure 2). Addition of thiol-modifying agents, aurothiomalate, *N*-ethylmaleimide and 15dPGJ$_2$ inhibited IKKα and β activity dose-dependently, while decreasing concentrations of dithio-

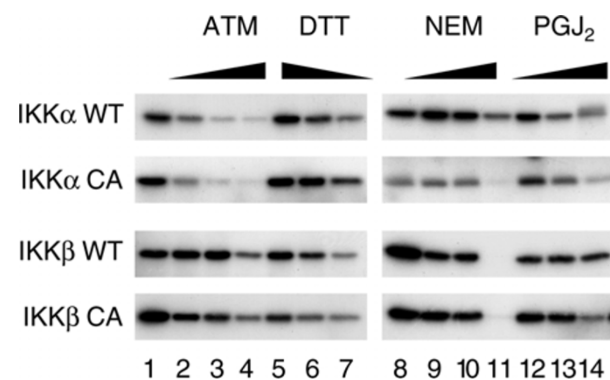

Figure 2. Inactivation of IKKβ (C179A) as well as IKKα (C178A) by *in vitro* exposure to thiol-reactive agents. COS-7 cells were transiently transfected with expression vectors for wild type HA-IKKα (IKKα WT), HA-IKKα (C178A) (IKKα CA), wild type Flag-IKKβ (IKKβ WT), or Flag-IKKβ (C179A) (IKKβ CA), together with NIK expression vector. After 48 h, cells were lysed, immunoprecipitated with anti-tag antibodies, and equal aliquots of immune complex were assayed for IKK activity in the absence (lanes 1 and 8) or presence of various concentrations of aurothiomalate (ATM, 10, 30, and 100 μM, lane 2-4), dithiothreitol (DTT, 300, 100, and 0 μM, lane 5-7), *N*-ethylmaleimide (NEM, 0.1, 0.3, and 1 mM, lane 9-11), and 15dPGJ$_2$ (PGJ$_2$, 3, 10, and 30 μM, lane 12-14). The data represent two experiments.

threitol in the reaction mixture reduced enzyme activity. $15dPGJ_2$ inhibited IKKβ to 35% of control at 3 μM concentration and no additional inhibition was observed at increasing concentrations of $15dPGJ_2$. Mutant enzymes IKKα (C178A) and IKKβ (C179A) were also sensitive to inhibition caused by addition of thiol-reactive agents or depletion of reducing agent in the reaction mixture.

Effect of thiol-reactive agents on constitutively active variants of IKKα and β

The IKKα and IKKβ are activated by phosphorylation of serine residues in their activation loops by upstream kinases and autophosphorylation (Mercurio *et al.*, 1997; Ling *et al.*, 1998; Delhase *et al.*, 1999). These serine residues are located near the Cys-178 and Cys-179 of IKKα and β, respectively, indicating a possibility that thiol-reactive agents inhibit IKK activation by interfering with phosphorylation of these serine residues. To test this possibility, we used constitutively active IKKα and β variants, HA-IKKα (S176E, S180E) and Flag-IKKβ (S177E, S181E), in which the two phospho-acceptor serine residues in the activation loop were substituted with glutamate. COS-7 cells transfected with wild type or constitutively active mutants of HA-IKKα and Flag-IKKβ were treated with auranofin for 2 h, and IKK activity of immunoprecipitated enzyme was measured (Figure 3). Auranofin inhibited both wild type and constitutively active forms of HA-IKKα and Flag-IKKβ.

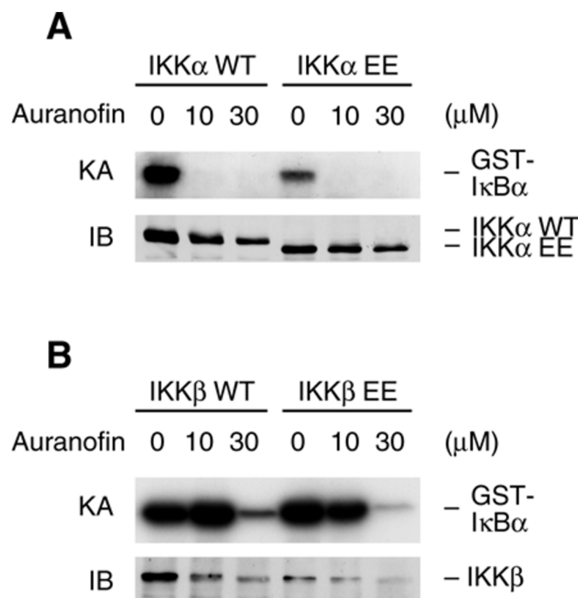

Figure 3. Constitutively active IKKα and IKKβ are inhibited by gold. (A) COS-7 cells were transiently transfected with wild type HA-IKKα (IKKα WT) or HA-IKKα (S176E, S180E) (IKKα EE) expression vectors along with NIK expression vector and treated with auranofin as described in Figure 1. Kinase assay (KA) was performed with immune complex obtained from cell lysate (120 μg protein), and the expression level of enzyme was measured by immunoblotting (IB) with anti-HA antibody. (B) COS-7 cells were transfected with wild type Flag-IKKβ or Flag-IKKβ (S177E, S181E) expression vectors together with NIK expression vector, and treated with auranofin as in (A). Immune complex prepared from cell lysate (20 μg protein) was used for kinase assay and immunoblotting analysis.

Discussion

In this study, we examined whether the inhibitory effect of gold compounds on IKK activity occurs through modification of Cys-178 and Cys-179 of IKKα and β, respectively. Previously, Cys-179 of IKKβ has been shown to be involved in inhibition of IKK by cyclopentenone PGs and arsenite (Kapahi *et al.*, 2000; Rossi *et al.*, 2000). Our result showed that when an IKKβ mutant that contains alanine instead of Cys-179 was expressed in COS-7 cells, it was resistant to inhibition by a cell-permeable gold compound, auranofin, indicating that gold inhibits IKK by modifying Cys-179 of IKKβ. Our result agrees with the previous results obtained with cyclopentenone PGs and arsenite, confirming a role of Cys-179 of IKKβ as a common target for various thiol-reactive agents. By contrast, this protective effect of Cys→Ala mutation was not observed in cells expressing an equivalent IKKα mutant, IKKα (C178A). These results demonstrate a critical difference in catalytic and regulatory mode of enzyme activity between IKKα and IKKβ, despite their identical substrate specificity and homologous structures. Genetic analysis of IKK function revealed that IKKα-deficient mice display abnormalities in skin and limb development, whereas deletion of IKKβ gene causes embryonic death due to massive liver apoptosis and IKKβ-deficient cells are sensitive to TNF-induced apoptosis (reviewed in Gerondakis *et al.*, 1999; Zandi and Karin, 1999). Moreover, studies with these knockout mice showed that NF-κB and IKK activation in response to TNF and IL-1 are severely impaired in IKKβ-deficient cells, whereas inactivation of IKKα gene had less effect. Taken together, these results indicate an essential role of IKKβ in IKK and NF-κB activation by proinflammatory signals, and suggest that anti-inflammatory effect of gold compounds appear mainly through modification of Cys-179 of IKKβ.

We observed that IKKα (C178A) mutant was inhibited by auranofin as effectively as wild type IKKα. This result suggested that gold compounds inhibit IKKα by reacting with group(s) other than Cys-178 in IKKα. Our result also showed that *in vitro* exposure of mutant enzymes, including IKKβ (C179A), to thiol-reactive agents reduced enzyme activity in a similar way to wild type enzymes. This result also suggested

the presence of group(s) in the IKKβ molecule other than Cys-179, which is(are) sensitive to oxidation or modification by reactive agents. This sensitive site of IKKβ (C179A) seems to be protected from inhibition by gold inside the cell, where redox balance maintains a more reduced state and competing thiols are more abundant than *in vitro* assay system. In a previous study, *in vitro* incubation of protein phosphatase 1B with reactive oxygen species, such as H_2O_2 or superoxide anion (O_2^-), has been shown to induce oxidation at multiple sites in addition to Cys-215, which acts as a specific regulatory site for reactive oxygen species in the cell (Barrett, 1999).

Cys-179 is critically positioned within the activation loop of IKKβ, suggesting that modification of this residue can interfere phosphorylation of adjacent Ser-177 and Ser-181 and thus activation of enzyme. We tested this possibility by employing constitutively active versions of IKKα and IKKβ, in which the two serine residues were replaced with acidic glutamate residues. Our result showed that the mutant enzyme of IKKα and IKKβ were inhibited by auranofin in a similar degree with wild type enzymes (Figure 3). Gold compounds and other thiol-reactive agents were also shown to directly inhibit activated IKK isolated from cells stimulated with LPS or TNF (Jeon *et al*., 2000; Kapahi *et al*., 2000; Rossi *et al*., 2000). These results suggest that the modification of Cys-179 by gold and other thiol-reactive agents interfere formation of active conformation of IKK even when the two serine residues are in phosphorylated state and Cys-179 plays a pivotal role in the conversion of inactive enzyme to an active form.

In summary, our results show that antirheumatic gold compounds inhibit NF-κB activation by modifying Cys-179 of IKKβ, and the effect appears through direct inhibition of activated enzyme. NF-κB controls the expression of diverse inflammatory and immune response mediators, and the abnormal, constitutive activation of NF-κB has been associated with numerous chronic inflammatory diseases, including rheumatoid arthritis, asthma, inflammatory bowel disease, ulcerative colitis and atherosclerosis (Barnes and Karin, 1997). Understanding the inhibitory mode gold compounds in IKK activation will benefit development of novel strategies to treat these diseases.

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