Inhibition of the DNA-binding activity of NF-κB by gold compounds in vitro

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Received 9 January 1995; revised version received 8 February 1995

Abstract **Nuclear factor κB (NF-κB) is a transcription factor that is critical for the inducible expression of multiple cellular and viral genes. DNA binding activity is essential for its function. Here, we report that gold compounds, especially aurothioglucose (AuTG), have a strong inhibitory effect on NF-κB–DNA binding. Our finding also reveals that Zn^{2+} is a necessary component of NF-κB for its DNA binding activity and that gold ion can efficiently block NF-κB–DNA binding, presumably through oxidation of the cysteins associated with zinc. This redox mechanism may provide an explanation for the observed efficacy of gold compounds in the treatment of rheumatoid arthritis.**

Key words: NF-κB; Gold; Zinc; DNA binding; Rheumatoid arthritis

1. Introduction

Nuclear factor κB (NF-κB) is an inducible cellular transcription factor that regulates a wide variety of cellular and viral genes [1,2]. These include genes for several cytokines, some cell adhesion molecules (CAMs), major histocompatibility antigen, serum amyloid A protein and a variety of viruses including human immunodeficiency virus (HIV) [3]. Although NF-κB is by no means the sole determinant for inducible expression of these genes, it has been shown to play a significant role [4]. Previous studies have demonstrated that NF-κB binding to the specific DNA sequence (called the κB motif) is essential for the transcriptional activity of these genes [5]. The induction of NF-κB binding is independent of new protein synthesis and involves dissociation of NF-κB from a cytosolic anchoring protein, IκB, followed by translocation of NF-κB to the nucleus where it activates the target genes [2]. Most of the genes known to be activated by NF-κB are involved in the immune and inflammatory responses [4,6]. In recent years, we have demonstrated that the DNA binding activity of NF-κB is regulated by an oxido-reductive mechanism (redox regulation). We found that oxidation of NF-κB abolished the DNA binding activity and that the subsequent reduction completely restored the activity [7,8]. These observations have indicated that development of a novel therapeutic strategy might be feasible by using chemical reagents that could modulate the redox status of NF-κB.

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Abbreviations: NF-κB, nuclear factor κB; CAMs, cell adhesion molecules; RA, rheumatoid arthritis; oPA, 1,10-orthophenanthroline; DTT, dithiothreitol; AuTG, aurothioglucose; DPA, D-penicillamine; AuTM, aurothiomalate; EMSA, electrophoretic mobility shift assay.

In rheumatoid arthritis (RA), the involvement of constitutive production of high levels of several cytokines, such as interferon β (IFN-β), TNF-α, IL-2, IL-6, IL-8 and granulocyte-macrophage colony stimulating factor (GM-CSF), as well as expression of some CAMs, including intercellular adhesion molecule-1 (ICAM-1), mostly under the control of NF-κB, has been implicated [9,10]. Gold compounds, containing elemental aurous gold cation, Au(I), combined with a sulfur-containing ligand have been widely used in the treatment of RA, although the precise mechanism of their actions are largely unknown [11]. These findings led us to investigate whether these anti-RA drugs might have an effect on the action of NF-κB.

In this paper, we examined the inhibitory effects of gold compounds, together with related reagents, on the DNA binding activity of NF-κB in vitro. The possible mechanism for such activity is discussed.

2. Materials and methods

2.1. Reagents

DEAE-Sepharose CL-6B, Heparin Sepharose CL-6B and poly(dI–dC) were purchased from Pharmacia (Uppsala, Sweden). 1,10-Orthophenanthroline (oPA), zinc chloride, cadmium chloride, copper chloride, gold chloride, and aurothioglucose (AuTG) were supplied by Sigma. Auranofin (SmithKline Beecham Pharmaceuticals, Philadelphia, PA), aurothiomalate (AuTM) (Shionogi Pharmaceutical Co., Tokyo) and D-penicillamine (DPA) (Tokyo Kasei Co., Tokyo) were obtained from the respective pharmaceutical companies.

2.2. Purification of NF-κB

NF-κB was prepared from the nuclei of human primary lymphocytes as reported previously [7]. Briefly, the crude nuclear extract was fractionated on heparin-Sepharose and DEAE-Sepharose columns. Protein was eluted from DEAE-Sepharose columns by a continuous 0.05–1.0 M KCl gradient using the Econo System (Bio-Rad, Hercules, CA). The fractions with the κB DNA binding activity were pooled and concentrated. The specificity of NF-κB was examined by immunoblotting using specific antiserum [12] and electrophoretic mobility shift assay (EMSA) using the κB DNA probe [7,8].

2.3. Electrophoretic mobility shift assay (EMSA)

The DNA binding activity was examined by EMSA using the specific κB DNA probe as previously described [7,8]. The κB sequence was taken from the human immunodeficiency virus long terminal repeat (HIV-LTR) [8]. The sequences of the κB wild-type and mutant oligonucleotides are as follows:

Wild type κB (wκB):

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5' TTTCTAGGGACTTTCCGCCTGGGGACTTTCCAG 3'    (plus strand)
3'    GATCCCTGAAAGGCGGACCCCTGAAAGGTCTTT 5' (minus strand)
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Mutant κB (mκB):

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5' TTTCTACTCACTTTCCGCTGCTCACTTTCCAG 3'     (plus strand)
3'    GATGAGTGAAAGGCGACGAGTGAAAGGTCTTT 5' (minus strand)
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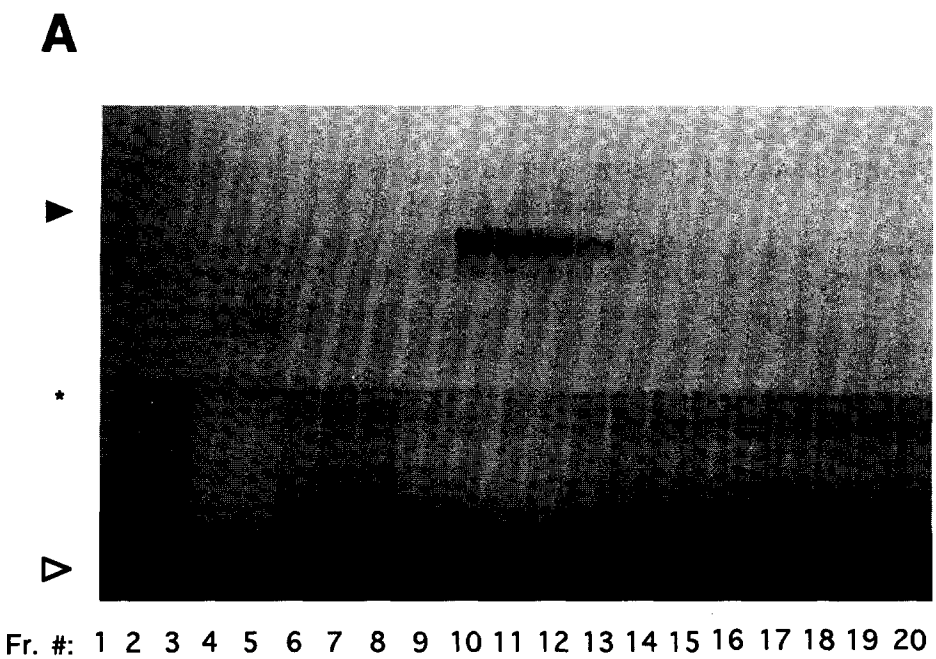
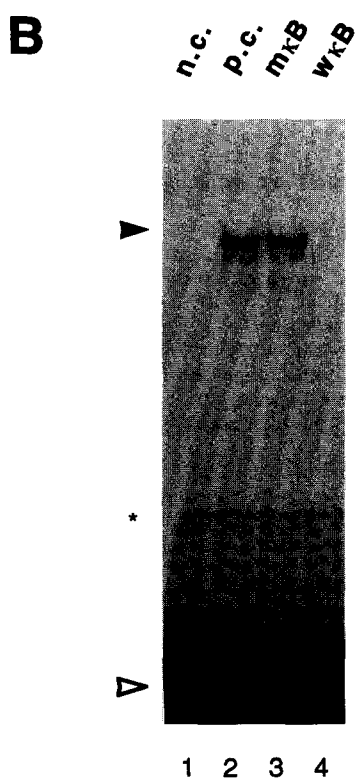
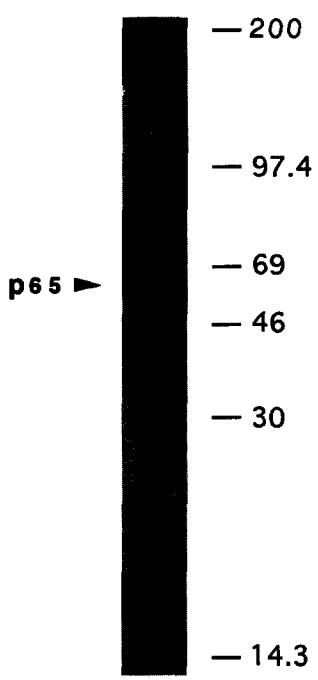






The three T's were added to each oligonucleotide sequence for end labeling by Klenow DNA polymerase (Takara Biomedicals, Japan) and [α-^{32}P]dATP (3000 Ci/mmol, ICN Pharmaceuticals Inc., USA). The labeled DNA probe was purified by passing it through a gel-filtration column (Quick Spin Column Sephadex G-25; Boehringer Mannheim, Germany) according to the instructions provided by the manufacturer.

Binding reactions of the DNA probe with protein were performed at 30°C for 10 min in a total volume of 10 μl of buffer containing 22 mM HEPES-KOH (pH 7.9), 80 mM KCl, 5% (v/v) glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethysulfonyl fluoride (PMSF), 1 μg poly(dI–dC), 2 μg tRNA and 0.1 ng (20,000–30,000 cpm) of the labeled κB DNA probe. The DNA–protein complexes were resolved in non-denaturing 6% polyacrylamide gels. Electrophoresis was performed in 0.5× TBE buffer (4.5 mM Tris, 4.5 mM boric acid, 0.1 mM EDTA, pH 8.0) at 4°C.

For the competition experiment, 50-fold molar excess of unlabeled

Fig. 1. (A) NF-κB–DNA binding measured by EMSA. Fractions eluted from a DEAE column were incubated with 10 μl buffer containing radiolabeled HIV-LTR oligonucleotide. The samples were analyzed by 6% non-denaturing PAGE. The filled arrowhead indicates the NF-κB–DNA complexes, while the open arrowhead indicates the free probe. *Indicates unspecific band. (B) Mutant (lane 3) and wild-type (lane 4) κB oligonucleotide in the presence of 50-fold excess labeled DNA were preincubated with purified protein on ice for 5 min before adding reaction buffer. The wild-type κB oligonucleotide, but not the mutant type, abolished the NF-κB–DNA binding, indicating that the band represents sequence-specific binding. Lane 1, distilled water was added instead of mκB and wκB. (C) Western blotting assay. A protein sample was separated by 5–20% SDS-PAGE and was transferred overnight at 4°C to a polyvinylidene difluoride (PVDF) membrane using buffer containing 20 mM Tris (pH 7.9), 15% methanol and 0.05% SDS. After washing with TBS (0.05% Tween 20), the membrane was blocked with 80% BlockAce (Yukijirushi Inc., Sapporo, Japan) and 1% gelatin for 8 h at 4°C, and subsequently incubated with anti-NF-κB antibody (1 : 500) reconstituted with 10% BlockAce for 2 h at room temperature and 1 h at 4°C with gently shaking. After washing with TBS, the blot was incubated with donkey anti-rabbit PHA (1 : 500) for 1 h at room temperature. The specific antigen–antibody reaction was visualized using the ECL detection system (Amersham, Sweden). For the determination of molecular weight a rainbow marker was used.
←

HIV wild-type κB or its mutant κB DNA was preincubated with the protein on ice for 5 min before adding the radioactive probe. After electrophoresis, gels were dried under vacuum and autoradiographed with Kodak T-Mat films (Eastman Kodak Co., Rochester, NY) at −80°C. Radioactive bands were quantified on an AMBIS 4000 system (AMBIS Inc., San Diego, CA).

For the treatment of NF-κB with heavy metals and chelating agents, the protein was incubated with the test reagent in the binding buffer on ice for 20 min before adding the radioactive probe. Stock solutions of oPA (100 mM) or auranofin (5 mM) were prepared in ethanol and diluted with double-distilled water just before use. Control samples for these two reagents were likewise prepared in the same concentration of ethanol. All tests were performed at least three times which revealed consistent results.

3. Results

3.1. Preparation and identification of the nuclear NF-κB

Protein was purified as described in section 2. Nuclear NF-κB–DNA binding activity was detected by EMSA using the radiolabeled κB DNA probe (Fig. 1A). The specificity of NF-κB activity was confirmed by DNA competition experiments using excess amount of unlabeled double-stranded κB DNA (both wild-type and mutant) (Fig. 1B). The Western blotting technique was applied to identify NF-κB protein using a specific antibody [12] (Fig. 1C). From these observations, we con-

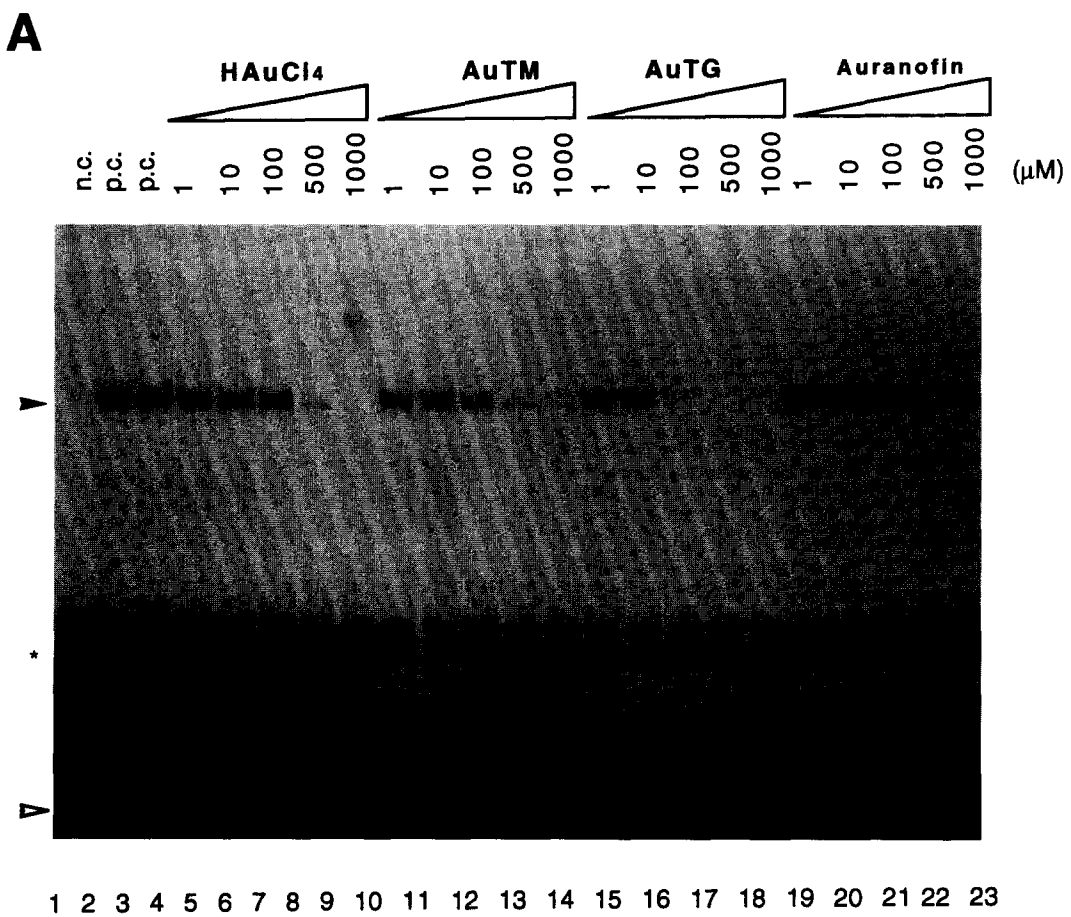
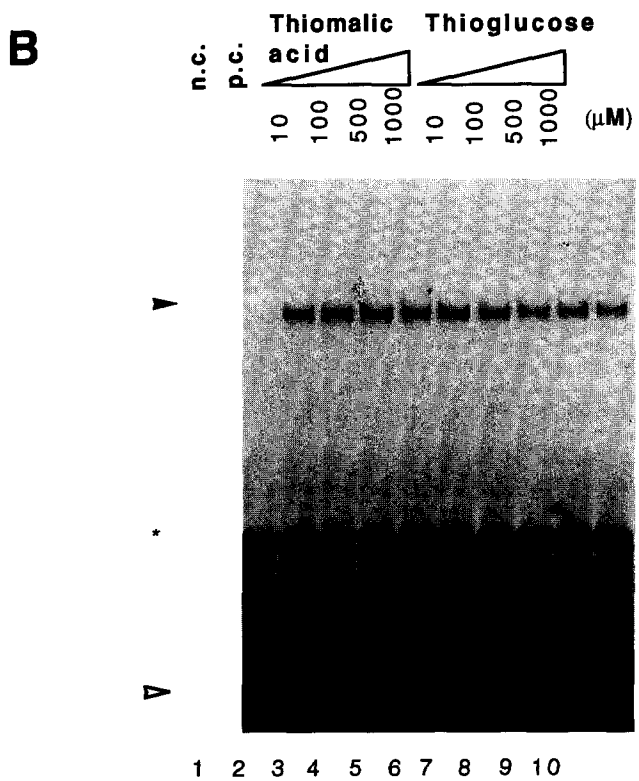


Fig. 2. Nuclear extract was preincubated with (A) gold compounds, (B) counter anions, and (C) other heavy metals, before being incubated with radiolabeled κB. Samples were analyzed by EMSA at the final concentrations of heavy metals and counter anions indicated.



firmed that the κB–DNA binding was due to the activity of NF-κB.

3.2. Effects of gold compounds on DNA binding of NF-κB

NF-κB was preincubated with various concentrations of gold compounds prior to addition of the radiolabeled κB probe. Among the gold compounds tested, AuTG showed the most effective inhibition of the protein–DNA binding while other gold compounds exhibited less inhibition in vitro (Fig. 2A). Au(I) appeared to have higher reactivity than Au(III). Although auranofin contains Au(I), it did not efficiently inhibit the binding, probably because of its lower solubility in water. Since treatment with counter anions alone, thiomalate or thioglucose, did not show such inhibition (Fig. 2B), this activity is considered to be due to the action of gold ion. This effect of Au(I) was obtained in the presence of 1 mM DTT, suggesting that oxidation by gold predominantly occurs at the thioate group of NF-κB rather than DTT.

We then investigated whether other heavy metals have the same effects as that of gold. Results in Fig. 2C show that, although Zn^{2+} could slightly increase the binding activity at 100 μM, it inhibited the binding at concentrations higher than 500 μM, presumably due to the binding of excess zinc ions to other sensitive cysteins in the protein (Fig. 2C, lanes 3–6). A similar effect of zinc was also described in Zabel et al. [13]. On the other hand, Mg^{2+} and Mn^{2+} appeared to have little effect on the binding (Fig. 2C). It is worth noting that the order of such effects corresponded to the affinities for thiols, or oxidation potentials, of these heavy metals [14,15].

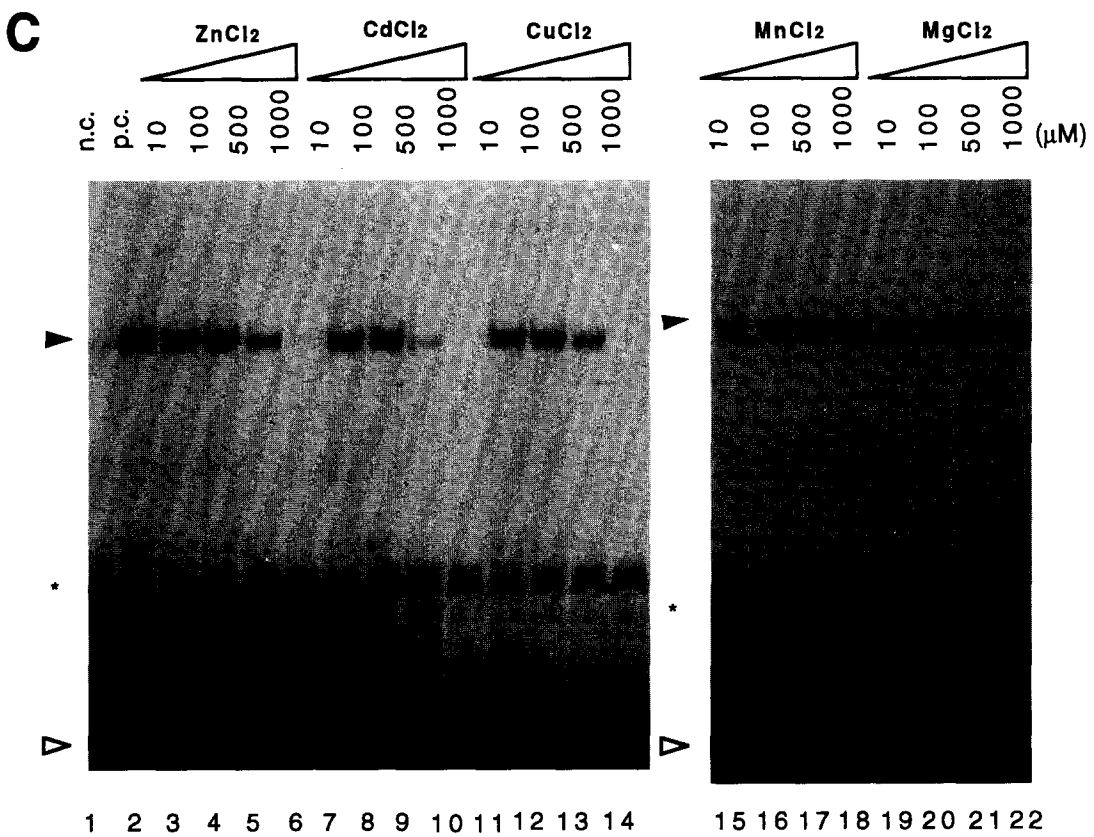
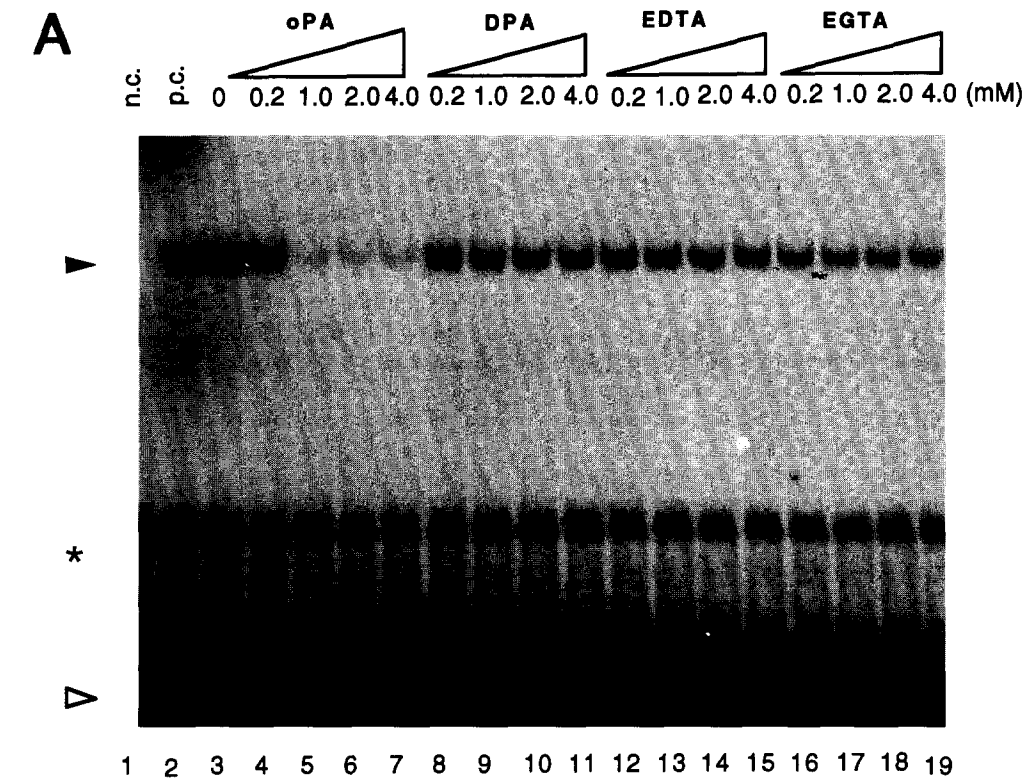


Fig. 2 (*continued*).



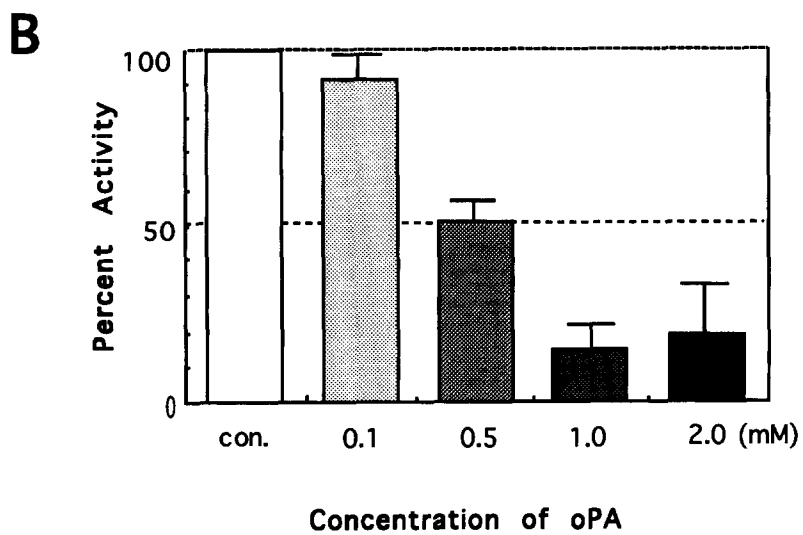


Fig. 3. (A) oPA, EDTA, EGTA and DPA were preincubated with protein on ice for 20 min before adding radiolabeled κB. Samples were analyzed by EMSA. (B) The gel was dried and quantified by STL-4000 and plotted as percent activity of protein–DNA binding of the control lane. Values represent the mean of three independent experiments.

3.3. *Combination of AuTG, Zn^{2+} and oPA at limited concentrations can restore NF-κB–DNA binding activity*

It was previously reported that the DNA binding of NF-κB requires Zn^{2+} [13], although NF-κB is not known to contain a typical zinc finger motif [16]. We first examined effects of the different chelating agents on the protein–DNA interaction. Among the chelators examined, only oPA showed a concentration-dependent inhibition of NF-κB–DNA binding activity (Fig. 3B). Other chelators, even at a concentration of 4.0 mM, failed to show such an inhibitory effect (Fig. 3A).

We then asked if gold inhibits the NF-κB activity by replacing with zinc ion or by oxidizing the thiolates on the NF-κB molecule without binding to them. The following experiments were thus carried out in an attempt to distinguish between these two possibilities. In Fig. 4A, NF-κB was preincubated with both oPA and AuTG and subsequently examined for the effect

of gold. As shown in Fig. 4A, AuTG could restore the binding at a concentration equivalent to oPA. These findings suggest that Au(I) could replace Zn^{2+} associated with oPA and the released Zn^{2+} could then reassociate with NF-κB. Therefore, it is likely that gold would not associate with NF-κB but rather oxidize the thiolate ions on NF-κB into disulfides (see section 4 for the possible mechanism). In Fig. 4B, NF-κB was preincubated with both oPA and $ZnCl_2$. The results showed a similar pattern as in Fig. 4A, although the restoration of binding by Zn^{2+} appeared to be more efficient. For example, the binding activity upon treatment with oPA (higher than 0.5 mM) and Zn^{2+} was even enhanced as much as two times (Fig. 4B, lanes 10, 15, 20). These results again confirmed that Zn^{2+} is an essential component of NF-κB activity. At higher concentrations of AuTG or $ZnCl_2$ the DNA binding activity was suppressed (Fig. 4A, lanes 6, 11, 16 and 21; Fig. 4B, lanes 6, 11, 16 and 21) as also noticed in Fig. 2. In Fig. 4C, NF-κB was preincubated with both DPA and AuTG, but no effect was observed. DPA appeared to block the inhibitory effect of AuTG on NF-κB activity, indicating the possible interaction between Au(I) and DPA.

4. Discussion

The results presented here demonstrate that gold compounds can inhibit the DNA binding of NF-κB in vitro. Among the various gold compounds, AuTG showed the most effective inhibition of the DNA binding of NF-κB. It is known that Au(I) is the therapeutically active component of gold compounds in the treatment of RA and that the counter anion might provide some additional benefits [17]. It is suggested that the different inhibitory action of gold compounds on NF-κB (Fig. 2A) might be due to the difference in the ability of their counter anions to release Au(I) since counter anions alone did not show any effect. Similar effects of gold compounds in inhibiting DNA binding of glucocorticoid receptor and progesterone receptor have been reported elsewhere [18,19]. Zabel et al. [13] reported that NF-κB specifically requires Zn^{2+} for optimal DNA binding. Our observations revealed that addition of Zn^{2+} facilitated the DNA binding (Fig. 2C, lane 4). We also observed that the effects of heavy metals in inhibiting the NF-κB DNA binding were in accord with the rank order of oxidation potential of these metal ions [14,15] (Fig. 2A and B). We observed that Au(I) inhibited the DNA binding activity of NFκB by oxidizing the Zn^{2+}-associated thiols and not by replacing them; this was clearly demonstrated in Fig. 4A in which withdrawal of Zn^{2+} abolished the DNA binding, and the subsequent addition of AuTG restored the binding. It is conceivable that the stability of the Au–oPA complex is much higher than that of Zn–oPA [15]; therefore, addition of a small amount of Au(I) could release Zn^{2+} from the Zn–oPA complex, thus generating the active form of NF-κB, the NF-κB–Zn complex. Therefore, the actions of gold can be explained by its strong oxidative potential over the zinc ions associated with NF-κB.

The redox regulation of NF-κB of its DNA binding activity

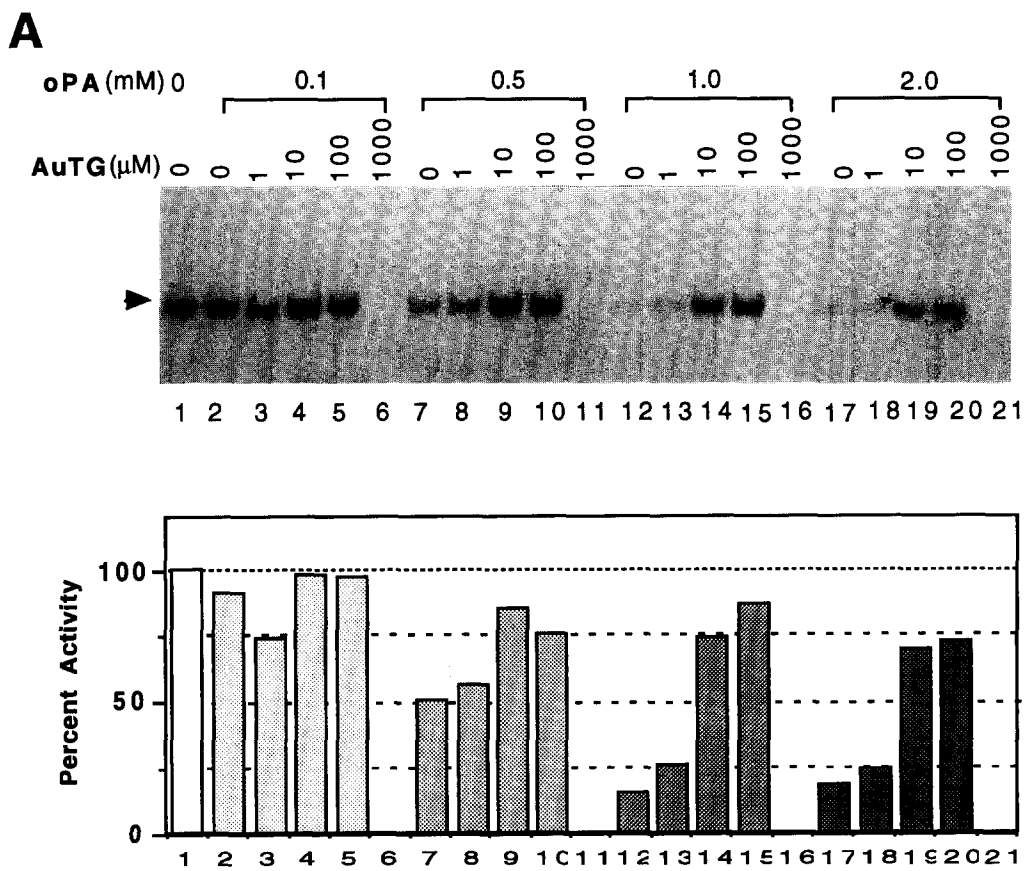
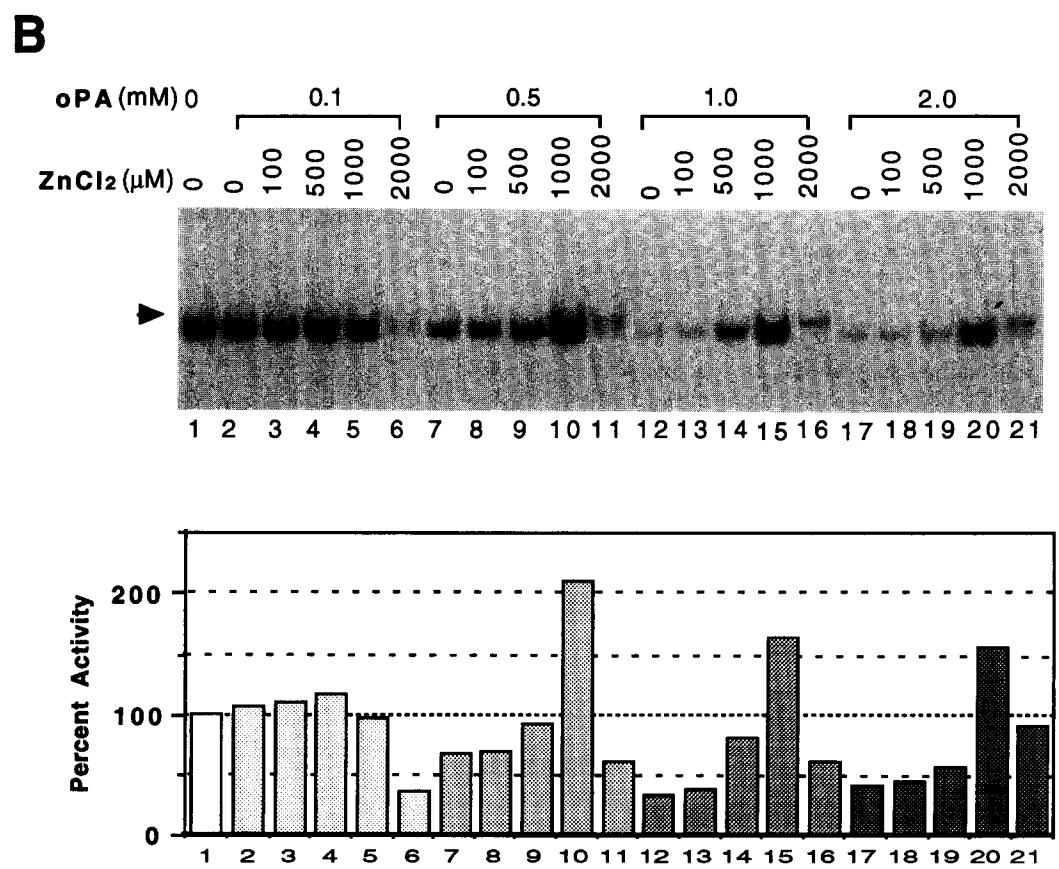


Fig. 4. Nuclear extract was preincubated with both (A) oPA and AuTG, (B) oPA and $ZnCl_2$, or (C) DPA and AuTG, at the indicated concentrations and subjected to gel-retardation with radiolabeled κB. After autoradiography, the radioactivity was quantified by STL-4000 and plotted as percent activity of NF-κB-DNA binding of the control lane.



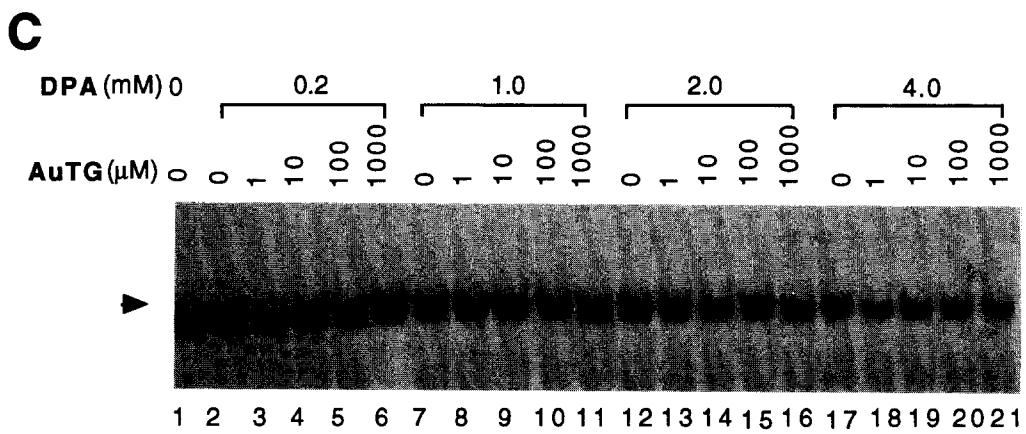


Fig. 4 (*continued*).

has been demonstrated [7,20]. When the cysteineresidues of NF-κB were oxidized by diamide or modified by *N*-ethylmaleimide (NEM), the DNA binding activity of NF-κB was abrogated [7,20]. Involvement of cysteine residues in the NF-κB–DNA binding was confirmed by the site-directed mutagenesis experiment in which Cys^{62} of p50 was shown to be critical [20]. It was previously postulated that the therapeutic effects of gold salts might be due to the inhibition of sulfhydryl systems [17]. Together with the present results, we assume that the redox mechanism might also be involved in the inhibitory effects of gold compounds on NF-κB–DNA binding. Since gold ion did not appear to exert the NF-κB inhibition by direct binding (Fig. 4), it is likely that Au(I) can oxidize the thiolate anions on the NF-κB molecule into disulfides and thus abrogate the DNA binding activity, considering the higher oxidation potential of Au(I) over Zn^{2+} (Fig. 5).

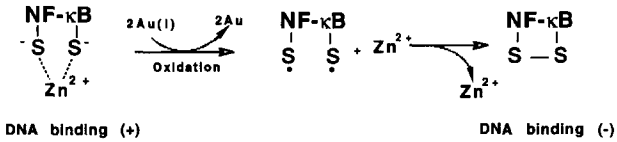


Fig. 5. Oxidation of NF-κB by gold cation. The active DNA binding form of NF-κB is hypothesized to contain zinc ions. When gold compound is added, Au(I) can take the electron from the thiolate anions due to its higher oxidation potential compared to that of Zn^{2+}. Thus, Au(I) eventually oxidizes the thiolate anions of NF-κB into disulfide. The oxidation of NF-κB abolishes the DNA binding activity [7,8].

In conclusion, our findings suggest that Zn^{2+} is an essential component for the NF-κB activity, and that one of the mechanisms of gold compounds might be through the inhibition of the DNA binding activity of NF-κB by redox mechanism. The observed concentrations of AuTG required for in vitro inhibition of NF-κB–DNA binding (less than 100 μM) are comparable to the peak serum gold concentration in treated patients (30–40 μM) [11]. It should be noted that NF-κB plays a pivotal role in the cytokine network involved in the pathogenesis of RA, and even minimal or partial inhibition of its activity might elicit a substantial effect on inflammatory processes. It is also possible that gold compounds affect multiple transcription factors in addition to NF-κB [19]. Our attempt to examine the effects of gold compounds in cell culture systems is underway.

Acknowledgements: We thank Drs. Shinsaku Sakurada and Hirotoshi Tanaka for helpful comments, and Dr. Librado A, Santiago for critical reading of this manuscript. J.-P.Y. is the recipient of a Sasagawa Medical fellowship. This work was supported by grants-in-aid from the Ministry of Health and Welfare and the Ministry of Education, Science and Culture of Japan.

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