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Inhibition of TPA-induced NF-κB nuclear translocation and production of NO and PGE_2 by the anti-rheumatic gold compounds

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Abstract

Auranofin, aurothioglucose and aurothiomalate (10 μM each) inhibited 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 16.2 nM)-induced nuclear translocation of nuclear factor-kappa B (NF-κB), and production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in rat peritoneal macrophages when the cells were pre-incubated with each gold compound for 20 h. Without pre-incubation for 20 h, aurothioglucose and aurothiomalate, but not auranofin, failed to inhibit the TPA-induced NF-κB nuclear translocation and production of NO and PGE_2. Auranofin, aurothioglucose and aurothiomalate did not affect the direct binding of NF-κB to the DNA probe. It was suggested that these gold compounds inhibit the TPA-induced production of NO and PGE_2 by inhibiting the NF-κB nuclear translocation.

Introduction

The clinical effects of gold compounds on rheumatoid arthritis have been known for more than 60 years (Forestier 1935). The classic hydrophilic gold salts, aurothioglucose ((1-thio-D-glucopyranosato)-gold) and aurothiomalate (a mixture of monogold monosodium monohydrogen sulfidobutanedioate and monogold disodium sulfidobutanedioate), are administered to patients by intramuscular injection, but the lipophilic gold compound auranofin ((1-thio-β-D-glucopyranosato)(triethylphosphine)gold 2,3,4,6-tetraacetate) (Finkelstein et al 1976) is effective via oral administration (Ward et al 1983; Wenger et al 1983). It is reported that auranofin inhibits production of pro-inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α (Bondeson & Sundler 1995; Bondeson 1997), aurothioglucose inhibits IL-6 and IL-8 production (Yoshida et al 1999) and aurothiomalate inhibits TNF-α production (Mangalam et al 2001).

In cultures of rat peritoneal macrophages, we previously reported that auranofin, at 1–10 μM, inhibits prostaglandin E_2 (PGE_2) production induced by the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Yamashita et al 1997b). We also reported that auranofin inhibits the induction of cyclooxygenase-2 (COX-2) protein in TPA-stimulated rat peritoneal macrophages by lowering the level of COX-2 mRNA (Yamada et al 1997; Yamashita et al 1999). In addition, auranofin, at 0.3–3 μM, also inhibits nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated murine macrophage-like cell line RAW 264.7 cells by suppressing levels of inducible NO synthase (iNOS) protein and its mRNA (Yamashita et al 1997a). Other gold compounds, aurothioglucose and aurothiomalate, neither inhibited PGE_2 production nor NO production when the cells had not been pre-incubated with aurothioglucose and aurothiomalate (Yamashita et al 1997a, b). Because auranofin does not directly inhibit the activity of COX (Yamada et al 1997) and NOS (Yamashita et al 1997a), it was suggested that auranofin inhibits the inflammatory signal at the level of mRNA regulation.

The mRNA level is regulated by the balance of DNA transcription to mRNA and mRNA degradation. DNA transcription is regulated by a group of proteins called transcription factors. NF-κB is one of the transcription factors, which is ordinarily in

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cytosol and forms a complex with the inhibitory protein IκB (Neuveut et al 1991). After inflammatory stimulation such as by IL-1β, TNF-α, TPA, lipopolysaccharide (LPS) and so on, IκB is phosphorylated by IκB kinase (IKK) (Mercurio et al 1997; Woronicz et al 1997) and subsequently dissociates from the complex. The free NF-κB then translocates into the nucleus and binds to the specific DNA sequence. An observation that anti-inflammatory steroids inhibit NF-κB nuclear translocation (Brasier et al 1990) provided a new role for NF-κB as an anti-inflammatory effect. NF-κB is also involved in the regulation of COX-2 gene expression in man (Kosaka et al 1994) and rats (Feng et al 1993) and iNOS gene expression in man (Chartrain et al 1994; Nunokawa et al 1994), rats (Nunokawa et al 1993) and RAW 264.7 macrophages (Kim et al 1997).

In this study, we examined the effect of the three gold compounds, auranofin, aurothioglucose and aurothiomalate, on TPA-induced NF-κB nuclear translocation and production of NO and PGE_2.

Materials and Methods

Drugs

The drugs used were auranofin (BIOMOL Research Labs, USA), aurothioglucose (Sigma Chemical Co., USA), aurothiomalate (Shionogi & Co., Japan), 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Sigma). Auranofin and TPA were dissolved as 2000-times concentrated ethanol solution. Aurothioglucose and aurothiomalate were dissolved in medium as 2000-times concentration. The concentrated solutions were added to the medium (Eagle's minimal essential medium (EMEM; Nissui, Japan) containing 10% calf serum (Dainippon Pharmaceutical, Japan), penicillin G potassium ($18\,\mu g\,mL^{-1}$) and streptomycin sulfate ($50\,\mu g\,mL^{-1}$) (Meiji Seika, Japan)). Ethanol was added to the control medium, medium with TPA or auranofin alone, and medium with aurothioglucose and aurothiomalate, to adjust the final concentration of ethanol to 0.1% (v/v). Then the medium was well mixed and poured into wells or dishes.

Viability of the cells was over 95% in every group after treatment with drug, as evaluated by the trypan blue exclusion test (Yamashita et al 1997b).

Preparation of rat peritoneal macrophages

A solution of soluble starch (Wako Pure Chemical Ind., Japan) and Bactopeptone (Difco Laboratory, USA), 5% each, was injected intraperitoneally into male Sprague-Dawley rats (300–350 g, specific pathogen-free; Charles River Japan, Japan) at a dose of 5 mL per 100 g body weight. Four days later, the rats were sacrificed by cutting the carotid artery under pentobarbital sodium (Nembutal; Dainippon Pharmaceutical Co., Japan) anaesthesia and peritoneal cells were harvested (Ohuchi et al 1985). The experiments were carried out in accordance with the procedures approved by the Committee of Animal Experiments at Tohoku Pharmaceutical University, Sendai, Japan. The peritoneal cells were suspended in medium at a density of 1.5×10^6 cells/mL. For the determination of NO and PGE_2, 1 mL of the cell suspension was poured into each well of a 12-well tissue culture plate (Asahi Techno Glass, Japan), and for the determination of NF-κB nuclear translocation, 10 mL of the cell suspension was poured into 100-mm tissue culture dish (Asahi Techno Glass), and incubated for 2 h at 37 °C. The dishes were then washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells, and the adherent cells used for the following experiments. More than 95% of the adherent cells were identified as macrophages (Ohuchi et al 1985).

Determination of NO and PGE_2

One set of the adherent cells was further incubated for 20 h in medium containing no gold compounds, washed 3 times with PBS and incubated for 4 h for the determination of NO, and for 20 h for the determination of PGE_2 in medium containing TPA (16.2 nM) in the presence or absence of auranofin, aurothioglucose and aurothiomalate, each at 10 μM. Another set of the adherent cells was incubated for 20 h in medium in the presence of auranofin, aurothioglucose and aurothiomalate, each at 10 μM, washed 3 times with PBS and incubated for 4 h for the determination of NO and for 20 h for the determination of PGE_2 in medium containing TPA (16.2 nM) in the presence of the corresponding gold compounds, each at 10 μM. NO and PGE_2 in the conditioned medium were determined using Griess reagent (Yamashita et al 1997a) and by radioimmunoassay (Yamashita et al 1997b), respectively.

Preparation of nuclear protein

Nuclear protein was extracted according to the method described by Dignam et al (1983). After incubation, the cells were washed 3 times with ice-cold PBS, scraped off the dish using a silicone scraper and centrifuged at 500 *g* and 4 °C for 5 min. Supernatant was removed and 400 μL of cell-degradation buffer (20 mM Tris-HCl (pH 7.8), 50 mM KCl, 1.2% tergitol NP-40 (Sigma), 0.1% protease inhibitor cocktail (Sigma) and 100 μM dithiothreitol (Wako)) was added to the precipitate. The precipitate was vigorously mixed for 10 s, left on ice for 15 min and centrifuged at 5000 *g* and 4 °C for 3 min to obtain the nuclear fraction. Protein was extracted from the nuclear fraction with 25 μL of extraction buffer (20 mM Tris-HCl (pH 7.8), 500 mM KCl, 0.1% protease inhibitor cocktail and 100 μM dithiothreitol) by vigorous mixing for 10 s. After keeping on ice for 15 min, the solution was centrifuged at 20 000 *g* and 4 °C for 20 min. The supernatant was obtained and used as the nuclear protein. NF-κB in the nuclear protein was analysed by electrophoretic mobility shift assay (EMSA) as described below.

[^{32}P]-Labelling of DNA probe

The κB consensus double-strand DNA probe (sense: 5′-AGTTGAGGGGACTTTCCCAGGC-3′, antisense: 5′-GCCTGGGAAAGTCCCCTCAACT-3′) (Fasmac Co.,

Japan) was obtained according to the information in Lenardo & Baltimore (1989). The DNA probe was incubated for 30 min at 37 °C with 18.5 kBq of [γ-^{32}P]-ATP (specific activity 111 TBq mmol^{-1}; NEN Life Science Products, USA) and 10 U of T4 polynucleotide kinase (Takara Shuzo Co., Japan) and the 5′-terminal of the DNA probe was end-labelled. The [^{32}P]-labelled DNA probe was purified by a NICK spin column (Amersham Pharmacia Biotech, UK).

Determination of NF-κB nuclear translocation

After incubation (1 h) of rat peritoneal macrophages in the presence or absence of TPA (16.2 nM) and the indicated concentration of auranofin, aurothioglucose or aurothiomalate, 4 μg of nuclear protein was incubated in 10 μL of binding buffer (20 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 10% glycerol, 400 μg mL^{-1} bovine serum albumin, 33.3 μg mL^{-1} poly(dI-dC)-poly(dI-dC) (Amersham Pharmacia) and 400 μM dithiothreitol) for 30 min at 4 °C. Then, 0.3 kBq of [^{32}P]-labelled κB consensus DNA probe was added and incubated for 20 min at room temperature. The reaction mixture was applied to a 4% polyacrylamide gel in 0.5 × TBE buffer (23 mM Tris (pH 8.0), 450 mM boric acid and 500 μM EDTA), then electrophoresed at 300 V for 70 min. The gel was dried and autoradiographed at −70 °C.

Autoradiographed X-OMAT film (Eastman Kodak Co., USA) was processed and the band density was read using an optical flat bed scanner (Canon, Japan).

Determination of the effects of gold compounds on direct binding of NF-κB to consensus DNA probe

Four micrograms of nuclear protein extracted from rat peritoneal macrophage that had been incubated for 1 h in the presence of TPA (16.2 nM) was supplemented with 10 μL of binding buffer (20 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 10% glycerol, 400 μg mL^{-1} bovine serum albumin, 33.3 μg mL^{-1} poly(dI-dC)-poly(dI-dC) (Amersham Pharmacia) and 400 μM dithiothreitol), and incubated for 30 min at 4 °C with or without 10 μM each of auranofin, aurothioglucose or aurothiomalate. Then, 0.3 kBq of [^{32}P]-labelled κB consensus DNA probe was added and incubated for 20 min at room temperature. After electrophoresis, the density of the band of NF-κB–DNA probe complex was determined as described above.

Statistical analysis

Results obtained by radioimmunoassay for PGE_2 and by Griess assay for nitrite were analysed by one-way analysis of variance followed by Dunnett's test for multiple comparison.

Results and Discussion

Identification of the NF-κB band on EMSA

After incubation (1 h) of rat peritoneal macrophages in medium with or without TPA (16.2 nM, 10 ng mL^{-1}), nuclear protein was extracted. There were 3 major bands on the autoradiographed film in non-treated (Figure 1, lane 1) and TPA-treated cells (lane 2) detected by EMSA. The bottom band is the free [^{32}P]-labelled DNA probe. The top band in lanes 1 and 2 disappeared in the presence of a 40-times-excess non-labelled DNA probe (lane 4), and was supershifted using anti-p50 antibody (lane 3), indicating that the top band in lanes 1 and 2 is NF-κB.

The NF-κB band was markedly increased when rat peritoneal macrophages were incubated with TPA (16.2 nM) for 1 h (lane 2), compared with the cells incubated without TPA (lane 1). Nelsen et al (1988) reported that TPA at 162 nM (100 ng mL^{-1}) enhances NF-κB-dependent transcription in human cervical carcinoma HeLa cells transfected with NF-κB binding site. We previously reported that TPA (16.2 nM) induces COX-2 protein (Yamada et al 1997; Yamashita et al 1997b), COX-2 mRNA (Yamashita et al 1999) and iNOS mRNA (Yamashita et al 1997a) in rat peritoneal macrophages after 4 h incubation. Together, these findings suggest that COX-2 and iNOS proteins are induced by NF-κB nuclear translocation in TPA-stimulated rat peritoneal macrophages.

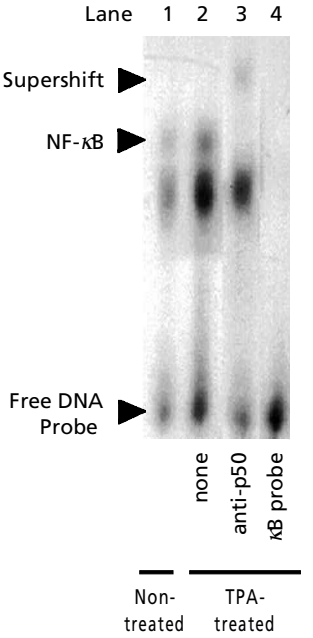

Figure 1 Identification of the NF-κB band. Rat peritoneal macrophages (1.5×10^7 cells) were incubated for 1 h at 37 °C in medium with (lanes 2–4) or without (lane 1) TPA (16.2 nM). Nuclear protein (4 μg) was incubated for 30 min at 4 °C in the buffer (lanes 1 and 2) containing 4 μg of anti-p50 (lane 3) or 40-times excess of non-radioactive κB probe (lane 4). Then, 0.3 kBq of [^{32}P]-labelled κB consensus DNA probe was added and further incubated for 20 min at room temperature. The reaction mixture was applied on a 4% polyacrylamide gel in 0.5 × TBE buffer, electrophoresed at 300 V for 70 min and the gel was autoradiographed.

Effects of gold compounds on the direct binding of NF-κB to DNA probe

The nuclear protein extracted from TPA-treated macrophages was incubated with [^{32}P]-labelled κB consensus DNA probe in the presence or absence of auranofin, aurothioglucose or aurothiomalate, each at 10 μM, to examine whether these gold compounds inhibit the binding of NF-κB to the consensus DNA probe. Auranofin (Figure 2, lane 2), aurothioglucose (lane 3) and aurothiomalate (lane 4) did not inhibit the binding of NF-κB to the DNA probe.

Yang et al (1995) reported that auranofin and aurothiomalate at > 100 μM, and aurothioglucose at > 10 μM directly inhibit NF-κB binding to DNA. They suggested that a higher concentration of gold ion replaces the intramolecular zinc ion of NF-κB, and directly lowers the binding ability of NF-κB to DNA. It is reported that the mean gold concentration in the serum is 0.67 μg mL^{-1} (3.4 μM) in patients suffering from rheumatoid arthritis who have been given 3 mg of auranofin (twice daily) for more than 9 weeks (Homma et al 1982). According to Gottlieb (1982), the gold concentration in serum from patients who had been given 2–9 mg auranofin daily for 12 weeks was 30–100 μg dL^{-1} (1.52–5.08 μM), and that in synovial fluid was 5–45 μg dL^{-1} (0.25–2.28 μM). The concentration of auranofin in the medium in this study (1–10 μM) is close to that in the serum or synovial fluid from patients treated with auranofin. The effective concentration of auranofin in the study of Yang et al (1995) was much higher than that in our study (Figure 2) and the serum concentration of gold compounds in clinical use, suggesting that the direct inhibition of NF-κB binding to DNA does not participate in the action mechanism of auranofin for the inhibition of PGE_2 production and NO production (Yamashita et al 1999).

Effects of gold compounds, without pre-incubation, on TPA-induced NF-κB nuclear translocation and production of NO and PGE_2

Rat peritoneal macrophages were incubated for 1 h in medium containing TPA (16.2 nM) and the indicated concentration of each gold compound. TPA induced nuclear translocation of NF-κB (Figure 3, lane 2), and auranofin, at 1–10 μM, inhibited the TPA-induced nuclear translocation of NF-κB in a concentration-dependent

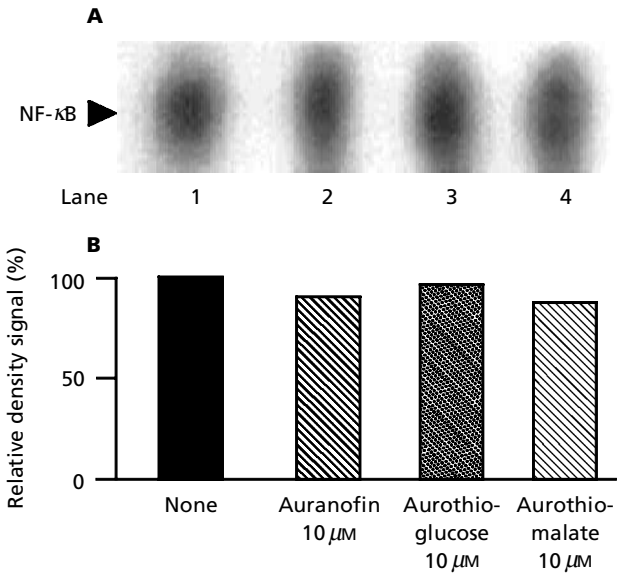

Figure 2 Effect of auranofin, aurothioglucose and aurothiomalate on the direct binding of NF-κB to the DNA probe. Nuclear protein (4 μg) from 1.5 × 10^7 cells of rat peritoneal macrophages that had been incubated at 37 °C for 1 h in medium containing TPA (16.2 nM) was incubated for 30 min at 4 °C in the buffer containing 0 μM (none, lane 1) or 10 μM of auranofin (lane 2), aurothioglucose (lane 3) or aurothiomalate (lane 4). Then, 0.3 kBq of [^{32}P]-labelled κB consensus DNA probe was added and further incubated for 20 min at room temperature. Reaction mixture was applied on a 4% polyacrylamide gel in 0.5 × TBE buffer, electrophoresed at 300 V for 70 min and the gel was autoradiographed (A). NF-κB bands were quantified densitometrically and the density of lane 1 is set to 100% (B).

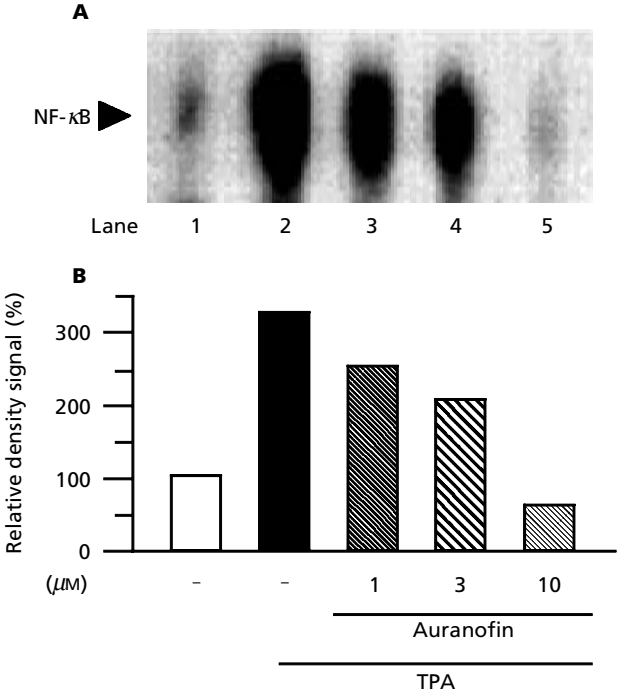

Figure 3 Effect of auranofin on NF-κB nuclear translocation. Rat peritoneal macrophages (1.5 × 10^7 cells) were pre-incubated in the absence of auranofin for 20 h at 37 °C. After three washes with PBS, the cells were further incubated at 37 °C for 1 h in medium in the presence (lanes 2–5) or absence (lane 1) of TPA (16.2 nM) and the indicated concentration of auranofin. Nuclear protein (4 μg) from rat peritoneal macrophages was incubated for 20 min at room temperature with 0.3 kBq of [^{32}P]-labelled κB consensus DNA probe. The reaction mixture was applied on a 4% polyacrylamide gel in 0.5 × TBE buffer, electrophoresed at 300 V for 70 min and the gel was autoradiographed (A). NF-κB bands were quantified densitometrically and the density of lane 1 is set to 100% (B).

manner (lanes 3–5); the nuclear translocation was almost completely inhibited by auranofin at 10 μM.

In 293.27.2 human kidney cell line, auranofin, at 1 μM, inhibited NF-κB nuclear translocation induced by TPA but not by TNF-α (Daniel et al 1995). Inhibition of NF-κB nuclear translocation by auranofin is also observed in IL-1β-stimulated human synoviocytes (Yamada et al 1999), LPS-stimulated RAW 264.7 (Jeon et al 2000) and TNF-α-stimulated human umbilical vein endothelial cells (Bratt et al 2000). Jeon et al (2000) reported that the inhibition of NF-κB nuclear translocation by auranofin is due to the inhibition of the phosphorylation of IκBα by IKK. If the effect on NF-κB is the specific property of auranofin, inhibition of NF-κB nuclear-translocation could be independent of the anti-rheumatic effect, against the former evidence.

Effects of gold compounds, with pre-incubation, on TPA-induced NF-κB nuclear translocation and production of NO and PGE_2

Herringer et al (1982) demonstrated that the gold of auranofin penetrates cell membranes of erythrocytes and granulocytes more easily than that of aurothiomalate, and Snyder et al (1986) also reported the association of radiolabelled auranofin with RAW 264.7 cells within 10 min. Therefore, we hypothesised that auranofin in the medium is expected to accumulate in macrophages more immediately than aurothioglucose and aurothiomalate. Our preliminary experiments revealed that aurothioglucose and aurothiomalate inhibit TPA-induced production of NO and PGE_2, depending on the length of the pre-incubation period with aurothioglucose and aurothiomalate, from 8 to 20 h (data not shown).

The cells were treated with auranofin, aurothioglucose or aurothiomalate, each at 10 μM, for 20 h and subsequently treated with TPA (16.2 nM) in the continued presence of the corresponding gold compound for 1 h for NF-κB nuclear translocation, 4 h for NO production and 20 h for PGE_2 production.

The TPA-induced NF-κB nuclear translocation (Figure 4A, B), NO production (Figure 4C) and PGE_2 production (Figure 4D) were inhibited by pre-incubation for 20 h with auranofin, aurothioglucose or aurothiomalate (lanes 4, 6, 8). Inhibition of TPA-induced NF-κB nuclear translocation by auranofin (lane 3), and no inhibition by aurothioglucose or aurothiomalate (lanes 5, 7) paralleled the effects on NO production and PGE_2 production.

These observations suggest that pre-incubation for 20 h with auranofin, aurothioglucose and aurothiomalate inhibits the production of NO and PGE_2 and that this is highly contributed to by inhibition of the nuclear translocation of NF-κB, with the evidence that NO and PGE_2 are modulated by NF-κB (Feng et al 1993; Nunokawa et al 1993, 1994; Chartrain et al 1994; Kosaka et al 1994). Yoshida et al (1999) observed that pre-incubation of rheumatoid synovial fibroblasts with 20 μM of aurothiomalate for 12 days inhibited the IL-1β-initiated

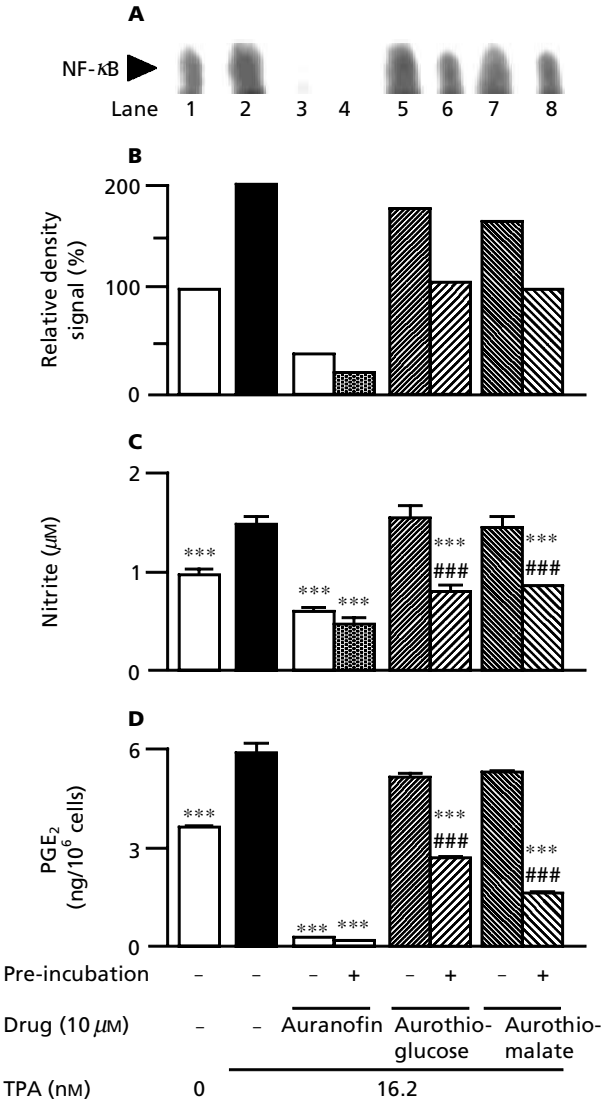

Figure 4 Effect of pre-incubation for 20 h with gold compounds on TPA-induced NF-κB nuclear translocation and production of NO and PGE_2 in rat peritoneal macrophages. Rat peritoneal macrophages (1.5×10^6 cells for NO and PGE_2, 1.5×10^7 cells for NF-κB) were pre-incubated for 0 h (−) or 20 h (+) at 37 °C in the presence or absence of 10 μM of auranofin, aurothioglucose or aurothiomalate. After three washes with PBS, the cells were further incubated for 1 h for NF-κB nuclear translocation (A, B), 4 h for NO production (C) and 20 h for PGE_2 production (D) at 37 °C in the presence of TPA (16.2 nM) and the corresponding gold compound (10 μM). Nuclear protein (4 μg) from rat peritoneal macrophages incubated for 1 h in the presence of TPA (16.2 nM) was incubated for 20 min at room temperature with 0.3 kBq of [^{32}P]-labelled κB consensus DNA probe. The reaction mixture was applied on a 4% polyacrylamide gel in 0.5 × TBE buffer, electrophoresed at 300 V for 70 min and the gel was autoradiographed (A). NF-κB bands were quantified densitometrically and the density of lane 1 is set to 100% (B). Values are the means, with s.e.m. shown by the vertical bars, from four wells (C and D). ***$P < 0.001$ vs TPA control, ###$P < 0.001$ vs corresponding non-pre-incubation group.

production of IL-6 and IL-8, both of which are known to be induced through NF-κB nuclear translocation, but they did not examine whether the pre-incubation with aurothiomalate inhibits NF-κB nuclear translocation.

von Knethen et al (1999) reported that superoxide donor 2,3-dimethoxy-1,4-naphtoquinone (DMNQ) induced NF-κB, and that NF-κB decoy oligonucleotide (3 μM) added 24 h before DMNQ addition inhibited the DMNQ-induced transcription of 5′-flanking lesion of COX-2 and the DMNQ-induced COX-2 protein. They also observed the DMNQ-induced mitogen-activated protein kinase (MAPK)-dependent induction of activating protein (AP)-1 transcription factor, the binding site of which appears in the 5′-flanking lesion of COX-2 gene annotated by Lukiw et al (1998), and the transient transfection of dominant-negative mutant of c-*jun*, the AP-1 component, also abrogated DMNQ-induced COX-2 protein expression. Their observation suggests the importance of NF-κB in COX-2 induction, and they also suggest that the NF-κB inhibition may not be the only mechanism for the inhibition of COX-2 induction. Aurothioglucose (Williams et al 1992) and aurothiomalate (Handel et al 2000) are reported to inhibit the activation of AP-1, with 50% inhibitory concentrations of 30 μM and 5 μM, respectively. The effect of gold compounds on AP-1 and the other transcription factors and signal transduction pathways must be further discussed.

Conclusion

Auranofin, aurothioglucose and aurothiomalate, at 10 μM, showed no inhibition of the direct binding of NF-κB to the DNA consensus probe. Aurothioglucose and aurothiomalate did not inhibit the TPA-induced NF-κB nuclear translocation and production of NO and PGE_2 without pre-incubation, but pre-incubation for 20 h with aurothioglucose or aurothiomalate inhibited the TPA-induced NF-κB nuclear translocation and production of NO and PGE_2. In contrast, auranofin inhibited the TPA-induced NF-κB nuclear translocation without pre-incubation in the synchronous range of concentrations at which auranofin inhibited TPA-induced production of NO and PGE_2. These findings suggest that one of the common fundamental mechanisms of action of the three gold compounds for the suppression of TPA-induced production of NO and PGE_2 is the suppression of NF-κB nuclear translocation.

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