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Auranofin inhibits interleukin-1β-induced transcript of cyclooxygenase-2 on cultured human synoviocytes

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Abstract

The aim of this study was to characterize the effects of auranofin (2,3,4,6-tetra-*O*-acetyl-l-thio-β-D-gluco-pyranosato-*S*) on cyclooxygenase expression and prostaglandin E_2 synthesis on cultured human synovial fibroblast-like cells (synoviocytes). Synoviocytes were treated with auranofin in the presence or absence of interleukin-1β. Cultured supernatants were harvested for prostaglandin E_2 synthesis. Cyclooxygenase-1 and -2 expression was analyzed with Western and Northern blotting. Translocation of nuclear factor-κ B p65 was determined by immunostaining. Cytotoxicity was measured with ^{51}Cr release assay. Auranofin attenuated interleukin-1β-induced prostaglandin E_2 production of the cells in a dose-dependent fashion. Auranofin selectively suppressed interleukin-1β-induced cyclooxygenase-2 mRNA and protein expression of the cells without alteration of cyclooxygenase-1 expression. Also, auranofin interfered with interleukin-1β-induced translocation of nuclear factor-κ B. These inhibitory effects did not originate in the cytotoxicity of the agent. Our data indicate that auranofin inhibits interleukin-1β-induced prostaglandin E_2 synthesis and cyclooxygenase-2 expression via suppression of nuclear factor-κ B activation on synoviocytes.

Keywords: Synovial fibroblast-like cell; Auranofin; Prostaglandin E_2; Cyclooxygenase-2; Nuclear factor-κ B

1. Introduction

Rheumatoid arthritis is characterized by synovial hyperplasia and inflammatory cell infiltration (Harris, 1990). Local proliferation and monoclonal expansion of synovial fibroblast-like cells (synoviocytes) contributes to the hyperplasia of synovium that exhibits tumor-like proliferation and invasion to juxtaarticular bone and cartilage, that results in joint destruction (Qu et al., 1994; Imamura et al., 1998). Moreover, the synoviocyte is one of the major sources of prostaglandin E_2 that is induced by monocyte-derived cytokines such as interleukin-1β in the inflamed rheumatoid synovium (Arend and Dayer, 1990). Prostaglandin E_2 plays main roles in the angiogenesis of synovium through vascular endothelial growth factor expression (Ben-Av et al., 1995), synovial inflammation and the joint erosion of rheumatoid arthritis (Robinson et al., 1975).

In the pathway of prostaglandin biosynthesis, cyclooxygenase is the key enzyme that catalyzes the conversion of arachidonic acid to prostaglandin E_2. Recently, the inducible isoform of cyclooxygenase, cyclooxygenase-2, has been identified (Hla and Neilson, 1992). It is generally accepted that prostaglandin E_2 at the inflammatory sites is produced by cyclooxygenase-2, and that another constitutive isoform, cyclooxygenase-1, is relevant to the production of prostaglandins that regulate normal cellular processes such as vascular homeostasis regulation, gastric mucosal protection and renal integrity maintenance (Smith et al., 1994; Vane et al., 1998). Cyclooxygenase-2 is also expressed in the synovium of rheumatoid arthritis patients and is likely to contribute to prostaglandin E_2 biosynthesis in affected joints (Sano et al., 1992; Crofford et al., 1994;

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Siegle et al., 1998). Since non-steroidal anti-inflammatory drugs mainly attenuate prostaglandin E_2 production by inhibiting cyclooxygenase activity (Vane, 1971) and since the adverse effects of non-steroidal anti-inflammatory drugs are suggested to be due to the inhibition of cyclooxygenase-1 (Vane et al., 1998), the development of specific cyclooxygenase-2 inhibitors is now completed. Furthermore, it is reported that newly developed cyclooxygenase-2 inhibitors ameliorate the joint inflammation of the disease without serious gastrointestinal adverse events (Furst, 1997; Lipsky and Isakson, 1997).

Gold compounds, which are disease-modifying anti-rheumatoid drugs, have been empirically applied to the management of rheumatoid arthritis, but their mechanism of action is obscure. It has been proposed that they inhibit monocyte functions and thus possess immunosuppressive activity (Lipsky and Ziff, 1977; Scheinberg et al., 1982), and suppress T cell proliferation (Hashimoto et al., 1994), T cell function (Hashimoto et al., 1992), B cell activation (Hirohata, 1996), chemotaxis (Elmgreen et al., 1989), and phagocytosis of leukocytes (Dimartino and Waltz, 1977; Davis et al., 1983). As for the effect of gold compounds on arachidonic acid metabolism, previous results have sometimes conflicted in that some studies have shown stimulatory effects (Peters-Golden and Shelly, 1988; Klaushofer et al., 1989; Bondeson and Sundler, 1993), whereas others have exhibited inhibitory effects (Bondeson and Sundler, 1995; Yamada et al., 1997; Yamashita et al., 1997).

Thus, the synoviocyte is one of the most useful therapeutic targets of rheumatoid arthritis treatment and gold compounds may affect prostaglandin E_2 production. However, while gold sodium thiomalate has been reported to inhibit the proliferation of synovial fibroblastic cells (Matsubara et al., 1988) and prostaglandin E_2 production (Seitz et al., 1997), the direct effect of an orally available gold compound, auranofin (2,3,4,6-tetra-*O*-acetyl-l-thio-β-D-gluco-pyranosato-*S*), on synoviocytes has not been documented.

In this study, we investigated the affect of auranofin on cyclooxygenase expression and prostaglandin E_2 production of cultured human synoviocytes.

2. Materials and methods

2.1. Reagents

Auranofin, obtained from SmithKline and Beecham (Tokyo, Japan), was diluted in ethanol and the final concentration of ethanol was adjusted at 0.1% in all experiments. Indomethacin and dexamethasone were purchased from Wako Pure Chemical (Osaka, Japan). Human recombinant interleukin-1β was a kind gift from Otsuka Pharmaceutical (Tokyo, Japan). Anti-cyclooxygenase-2 was a mouse monoclonal antibody (Transduction Laboratories, Lexington, KY) that was shown to be highly specific and did not cross-react with human cyclooxygenase-1 (Niiro et al., 1997). Anti-cyclooxygenase-1 was a mouse monoclonal antibody (Cayman Chemical, Ann Arbor, MI) which did not recognize human cyclooxygenase-2 (Macchia et al., 1997). Horseradish peroxidase-linked goat anti-mouse immunoglobulin G (IgG), enhanced chemiluminescence (ECL) system, Hyperfilm, Hybond N+, and Megaprime labeling kit were from Amersham Life Science (Buckinghamshire, England).

2.2. Patients

Synovial tissues were obtained from elbow, knee and hip joints at the time of total joint replacement surgery from eight patients with rheumatoid arthritis. All patients were female, aged 21 to 65 and fulfilled the 1987 American College of Rheumatology revised criteria for rheumatoid arthritis (Arnett et al., 1988) stages III to IV. None of the patients had received chrysotherapy. Consent for the study was obtained from each patient.

2.3. Preparation of cultured synovial fibroblast-like cells (synoviocytes)

Synoviocytes were isolated by enzymatic digestion of synovial tissues and cultured as previously described (Hashiramoto et al., 1999). Confluent cultures with a homogeneous population of synovial fibroblast-like cells (synoviocytes) from passages three to eight were used for the experiments.

2.4. Determination of prostaglandin E_2 concentration

Synoviocytes were cultured in 24-well plates at a density of 4×10^4 cells/well in 1 ml of conditioned media. Then, the cells were incubated with 1 μM of auranofin for 2, 4, 8, 16, 24 h. Thereafter, the supernatants were discarded, interleukin-1β (1 ng/ml) was or was not added, and incubation continued for a further 6 h. Then the supernatants were replaced by fresh media and incubation continued for 4 h. Supernatants (100 μl) were immediately used for measurement of prostaglandin E_2 concentration by enzyme-linked immunosorbent assay (ELISA) using a commercially available prostaglandin E_2 enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) according to manufacturer's instructions.

In order to examine the dose-dependent effects of auranofin, the cells were incubated with 0.1, 1, 3, 10 μM of auranofin or 1 μM of dexamethasone for 4 h. Dexamethasone was used as a cyclooxygenase-2 inhibitor to demonstrate the inhibition of stimulus-induced prostaglandin E_2 production by the cells. After the supernatants were discarded, interleukin-1β (1 ng/ml) was added and further

incubated for 6 h. Then the supernatants were replaced by fresh media, the cells were incubated for a further 4 h, and the supernatants were used for ELISA.

To examine the effect of auranofin on the prostaglandin E_2 production synthesized from exogenous arachidonic acid under unstimulated conditions, the cells were incubated with various concentrations of auranofin (0.1, 1, 3, 10 μM) or 5 μM of indomethacin for 4 h. Indomethacin was used as a cyclooxygenase-1 inhibitor to demonstrate the inhibition of prostaglandin E_2 production under basal conditions. After incubation, 30 μM of arachidonic acid was or was not added and incubation continued for a further 20 min and the supernatants were used for ELISA. Prostaglandin E_2 production was evaluated in triplicate and correlated to a standard curve of prostaglandin E_2.

2.5. Western blot analysis

Synoviocytes were incubated at various concentrations (0.1, 1, 10 μM) of auranofin or 1 μM of dexamethasone for 4 h and further incubated with or without 1 ng/ml of interleukin-1β for 8 h. Dexamethasone was used as a cyclooxygenase-2 inhibitor. Then the cells were collected and lysed in 200 μl of solubilization buffer (62.5 mM Tris–HCl, 25% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol) at room temperature. Cell lysates were then centrifuged for 15 min and supernatants were harvested and boiled at 95°C. Aliquots of protein were separated by 7.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinilidene difluoride filter (ATTO, Tokyo, Japan). Non-specific binding of the blot was then blocked with phosphate-buffered saline (PBS) containing 5% skim milk (Difco, Detroit, MI) and 0.2% Tween 20 for 2 h, and the filter was incubated with 1:250 of mouse anti-cyclooxygenase-2 monoclonal antibody or 1:1000 of mouse anti-cyclooxygenase-1 antibody in the blocking buffer at 4°C overnight. The blot was subsequently incubated with horseradish peroxidase-linked goat anti-mouse IgG (1:2000 dilution) and analyzed using the ECL system (Crofford et al., 1994). Hyperfilm with cassette closure times of 15 to 60 s resulted in adequate exposure to visualize the bands.

2.6. Extraction of RNA and Northern blot analysis

Synoviocytes were incubated alone or in the presence of auranofin (0.1, 1, 10 μM) or 1 μM of dexamethasone for 4 h. Dexamethasone was used as a cyclooxygenase-2 inhibitor. Then the synoviocytes were stimulated with or without interleukin-1β (1 ng/ml) and incubated for an additional 6 h. Then the cells were trypsinized and total RNA was extracted using a modified version of the technique previously described (Chomczynski and Sacchi, 1987). Twenty micrograms of total RNA was subjected to electrophoresis through a 1.5% agarose/4% formaldehyde gel and transferred onto a nylon membrane (Hybond N+). Human cyclooxygenase-1 and -2 cDNA (Crofford et al., 1994) and β-actin cDNA (Clontech, Palo Alto, CA) were radiolabeled with ^{32}P-dCTP (NEN Lifescience Products, Boston, MA) using a Megaprime labeling kit and the blot was hybridized in Hybrisol I (Oncor, Gaithersburg, MD) at 42°C for 24 h with the probes. Washing was performed twice for 20 min in 2× saline–sodium citrate (SSC), 0.1% SDS at 55°C for cyclooxygenase-1 and -2, and four times for 20 min in 2× SSC, 1% SDS at 65°C and twice for 20 min in 0.1× SSC, 0.5% SDS at 55°C for β-actin. The membrane was exposed to Fuji imaging plate (Fuji Photofilm, Kanagawa, Japan) for 1 h at room temperature with cassette. Densitometry was carried out using a Fuji Bas 2000 (Fuji photofilm) scanner. Cyclooxygenase-1 and -2 were normalized to β-actin detected on the same Northern blot for quantitation.

2.7. Immunostaining

Synoviocytes were preincubated in LabTek chamber slides (Nunc) and treated or not with auranofin (0.1, 1 μM) for 4h, then stimulated with interleukin-1β (1 ng/ml) for 1 h. Then the cells were fixed with acetone for 30 s and immunoperoxidase staining was performed at room temperature with Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's suggested protocol (Sano et al., 1992). The primary antibody against nuclear factor-κ B p65 (Santa Cruz Biotechnology, Santa Cruz, CA) or 50 μg/ml of purified rabbit IgG (Vector Laboratories) was reacted for 30 min in a humid chamber. The color of the cells was developed by immersion in a solution of 0.05% w/v 3,3′-diaminobenzidine tetrahydrochloride (Sigma), 0.01% of hydrogen peroxide in 0.05 M Tris (pH 7.4) for 6 min. Then the cells were counterstained with Mayer's hematoxylin (Wako Pure Chemical) for 2 min. The developing reagent gave a brown color deposit for positive signals.

2.8. In vitro cytotoxicity assay

The cytotoxic effect of auranofin on synoviocytes was determined in a standard ^{51}Cr release assay (Hashiramoto et al., 1999). In the assay, the cells were radiolabeled and ^{51}Cr was released only from injured cells and ^{51}Cr activity in the supernatants was measured with a liquid scintillation counter. In brief, synoviocytes were labeled with ^{51}Cr sodium chlomate (NEN) and incubated with or without various concentrations of auranofin (0.1, 1, 3, 10 μM) on 96-well multititer plates for 16 h. After incubation, 10 μl of Triton X-100 was added to untreated wells and pipetted vigorously in order to measure maximum dissociation. Then, the plates were centrifuged and 100-μl aliquots of supernatants containing the released ^{51}Cr were measured

by liquid scintillation counting. Spontaneous release was determined by performing control incubations in the absence of reagents and was subtracted from the total counts of each sample. Each sample was assayed in triplicate and the result was expressed as the ratio to maximum dissociation.

2.9. Statistical analysis

All results are presented as the means ± S.E.M. Comparison of the means was done with Fisher's exact test and differences were considered significant when the *P*-value was below 0.05.

3. Results

3.1. Effects of auranofin on prostaglandin E_2 production in cultured synoviocytes

To study whether auranofin affects prostaglandin E_2 production of synoviocytes stimulated by interleukin-1β, the concentration of prostaglandin E_2 in the cultured supernatants was measured by ELISA. Under basal conditions, the cells secreted prostaglandin E_2 at the concentration of 86–130 pg/ml (Fig. 1A). Stimulation with interleukin-1β for 6 h markedly increased prostaglandin E_2 production of the cells 6- to 13-fold. The inhibitory effect of auranofin (1 μM) was observed from 2 h after administration and its maximal inhibitory effect was found at 8 h (Fig. 1A).

To study the dose-dependent effect of the agent on prostaglandin E_2 production of the cells stimulated by interleukin-1β, the cells were treated with the concentrations of auranofin as shown or with 1 μM of dexamethasone for 4 h. Auranofin significantly reduced the interleukin-1β-induced prostaglandin E_2 production of the cells in a dose-dependent manner (Fig. 1B). As anticipated, dexamethasone markedly attenuated prostaglandin E_2 production of the cells (Fig. 1B).

In addition, the effects of auranofin on the catalyzed prostaglandin E_2 production from exogenous arachidonic

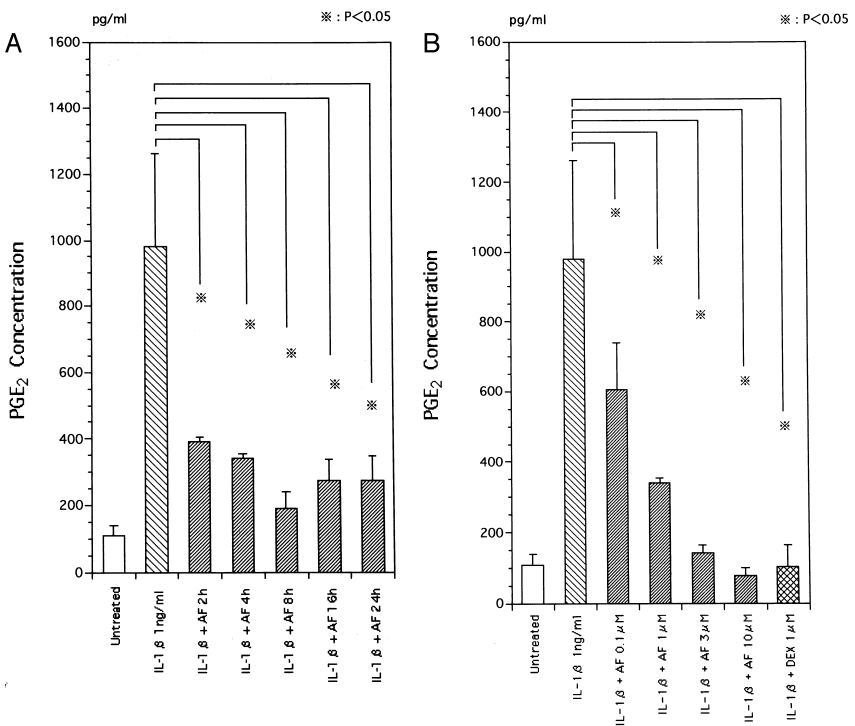

Fig. 1. Effects of auranofin on the prostaglandin E_2 production in cultured synoviocytes. In order to determine the effects of auranofin on prostaglandin E_2 production in cultured synoviocytes, ELISA were performed. (A) Synoviocytes (4×10^4 cells/well) were incubated in 1 ml of conditioned media with or without 1 μM of auranofin (AF) for the time periods shown followed by stimulation with interleukin (IL)-1β (1 ng/ml) for 6 h and incubation with fresh media for 4 h. Under basal conditions, the cells secreted prostaglandin E_2 at the concentration of 86–130 pg/ml. Stimulation with interleukin-1β markedly increased prostaglandin E_2 production of the cells 6- to 13-fold. Auranofin attenuated interleukin-1β-induced prostaglandin E_2 production and its maximal effect was found at 8 h. (B) Synoviocytes were incubated with or without the concentrations of auranofin (AF) shown or 1 μM of dexamethasone (DEX) as a cyclooxygenase-2 inhibitor for 4 h followed by stimulation with interleukin-1β for 6 h and incubation with fresh media for 4 h. Auranofin significantly diminished interleukin-1β-induced prostaglandin E_2 production in a dose-dependent manner. Dexamethasone also attenuated prostaglandin E_2 production by the cells. (C) Synoviocytes were incubated with or without the concentrations of auranofin (AF) shown or with 5 μM of indomethacin (IM) as a cyclooxygenase-1 inhibitor for 4 h followed by incubation with 30 μM of arachidonic acid (AA) for 20 min. Auranofin augmented spontaneous prostaglandin E_2 production at a concentration of 10 μM and enhanced prostaglandin E_2 production synthesized from exogenous arachidonic acid at concentrations of 3 and 10 μM. Indomethacin reduced prostaglandin E_2 production irrespective of the presence or absence of exogenous arachidonic acid. Values are expressed as means ± S.E.M. of three different experiments ($n = 3$) with triplicate samples. Statistical significance: (A) and (B) ※$P < 0.05$ compared to corresponding control interleukin-1β. (C) ※$P < 0.05$ compared to corresponding control untreated.

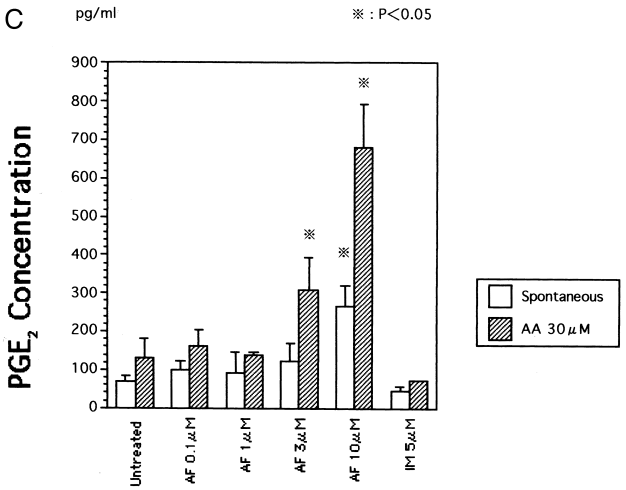

Fig. 1 (*continued*).

acid under unstimulated conditions were studied. Auranofin augmented the spontaneous prostaglandin E_2 production at a concentration of 10 μM and enhanced prostaglandin E_2 production synthesized from exogenous arachidonic acid at concentrations of 3 and 10 μM. (Fig. 1C). As was expected, indomethacin reduced prostaglandin E_2 production irrespective of the presence or absence of exogenous arachidonic acid. Cell viability under these conditions was measured by trypan blue dye exclusion and did not decrease significantly under any of the treatment conditions (data not shown).

3.2. Effects of auranofin on the expression of cyclooxygenase-1 and -2 polypeptides in cultured synoviocytes

To investigate whether auranofin inhibited cyclooxygenase protein expression of synoviocytes, Western blotting was performed. Synoviocytes constitutively expressed cyclooxygenase-1 protein (70 kDa) and minimally expressed cyclooxygenase-2 protein (70 kDa) under basal conditions (Fig. 2: lane 1). Cyclooxygenase-2 protein expression was dramatically increased after 8-h stimulation with interleukin-1β without interference with cyclooxygenase-1 protein expression (Fig. 2: lane 2). The interleukin-1β-induced cyclooxygenase-2 protein expression of the cells was inhibited by auranofin treatment at 4 h in a dose-dependent fashion (Fig. 2: lanes 3–5). In contrast, auranofin did not attenuate cyclooxygenase-1 protein expression of the cells (Fig. 2: lanes 3–5). As was expected, dexamethasone suppressed cyclooxygenase-2 protein expression without inhibition of cyclooxygenase-1 protein expression (Fig. 2: lane 6).

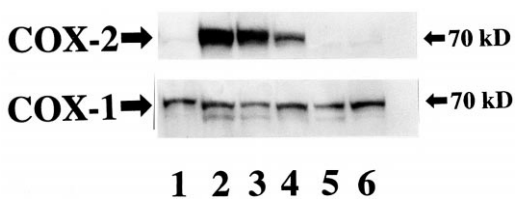

Fig. 2. Cyclooxygenase-1 and -2 protein expression in auranofin-treated synoviocytes, by Western blot analysis. To determine the effects of auranofin on cyclooxygenase protein expression, Western blot analysis was performed. Synoviocytes were incubated with the concentrations of auranofin or dexamethasone shown for 4 h followed by stimulation with interleukin-1β (1 ng/ml) for 8 h. Then the cells were lysed and proteins were extracted. Aliquots of protein were separated by 7.5% SDS–PAGE and the filter was incubated with 1:1000 of anti-cyclooxygenase-1 antibody or 1:250 of anti-cyclooxygenase-2 antibody and analyzed by Western blotting as described in Materials and methods. Synoviocytes constitutively expressed cyclooxygenase-1 protein and minimally expressed cyclooxygenase-2 protein under basal conditions (lane 1). Cyclooxygenase-2 protein expression was dramatically increased after 8-h stimulation with interleukin-1β without interference with cyclooxygenase-1 protein expression (lane 2). Auranofin inhibited interleukin-1β-induced cyclooxygenase-2 protein expression by the cells in a dose-dependent fashion, whereas no effect was found on cyclooxygenase-1 expression (lanes 3–5). Dexamethasone also selectively suppressed cyclooxygenase-2 protein expression (lane 6). Lane 1: untreated, lane 2: interleukin-1β 1 ng/ml, lane 3: interleukin-1β + auranofin 0.1 μM, lane 4: interleukin-1β + auranofin 1 μM, lane 5: interleukin-1β + auranofin 10 μM, lane 6: interleukin-1β + dexamethasone 1 μM. Similar results were obtained in three separate experiments.

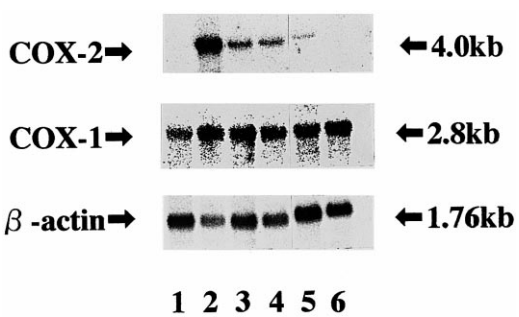

Fig. 3. Cyclooxygenase-1 and -2 mRNA expressions in auranofin-treated synoviocytes by Northern blot analysis. To determine the effects of auranofin on cyclooxygenase mRNA expression, Northern blot analysis was carried out. Synoviocytes were incubated with the concentrations of auranofin or dexamethasone shown for 4 h followed by stimulation with interleukin-1β (1 ng/ml) for 6 h. Then total RNA was extracted and subjected to electrophoresis and transferred onto a nylon membrane. The blot was hybridized with radiolabeled cyclooxygenase-1, -2, and β-actin cDNA and analyzed by Northern blotting as described in Materials and methods. Synoviocytes constitutively expressed cyclooxygenase-1 mRNA and no expression of cyclooxygenase-2 mRNA was observed under basal culture conditions (lane 1). Cyclooxygenase-2 mRNA expression was markedly increased after 6-h stimulation with interleukin-1β (lane 2). The interleukin-1β-induced cyclooxygenase-2 mRNA expression of the cells was completely suppressed on auranofin treatment in a dose-dependent manner, whereas cyclooxygenase-1 mRNA expression of the cells was neither enhanced by the stimulus, nor did auranofin attenuate cyclooxygenase-1 mRNA expression of the cells (lanes 3–5). Dexamethasone also diminished cyclooxygenase-2 mRNA expression without suppression of cyclooxygenase-1 mRNA (lane 6). Lane 1: untreated, lane 2: interleukin-1β 1 ng/ml, lane 3: interleukin-1β + auranofin 0.1 μM, lane 4: interleukin-1β + auranofin 1 μM, lane 5: interleukin-1β + auranofin 10 μM, lane 6: interleukin-1β + dexamethasone 1 μM. Representative result obtained from three separate experiments is shown.

3.3. Effects of auranofin on cyclooxygenase-1 and -2 mRNA expression in synoviocytes with or without interleukin-1β stimulation

To determine whether auranofin suppressed cyclooxygenase-2 mRNA expression, Northern blot analysis was carried out. Synoviocytes constitutively expressed cyclooxygenase-1 mRNA (2.8 kb) and no expression of cyclooxygenase-2 mRNA (4.0 kb) was observed under basal culture conditions (Fig. 3: lane 1). Cyclooxygenase-2 mRNA expression was markedly increased after 6-h stimulation with interleukin-1β (Fig. 3: lane 2). The interleukin-1β-induced cyclooxygenase-2 mRNA expression of the cells was completely suppressed by auranofin treatment at 4 h in a dose-dependent manner (Fig. 3: lanes 3–5). As in Western blot analysis, cyclooxygenase-1 mRNA expression of the cells was not enhanced by the stimulus, and auranofin did not attenuate cyclooxygenase-1 mRNA expression of the cells (Fig. 3: lanes 3–5). Dexamethasone also diminished cyclooxygenase-2 mRNA expression without suppression of cyclooxygenase-1 mRNA (Fig. 3: lane 6).

3.4. Effects of auranofin on the nuclear factor-κ B activity in interleukin-1β-stimulated synoviocytes

To examine whether auranofin interfered with the activity of nuclear factor-κ B, the interleukin-1β-induced translocation of nuclear factor-κ B p65 protein was detected with immunostaining using the antibody against nuclear factor-κ B p65. Under basal culture conditions, nuclear factor-κ B p65 was expressed predominantly in the cytoplasm of synoviocytes (Fig. 4A). Nuclear factor-κ B p65 immediately translocated from cytoplasm to nucleus

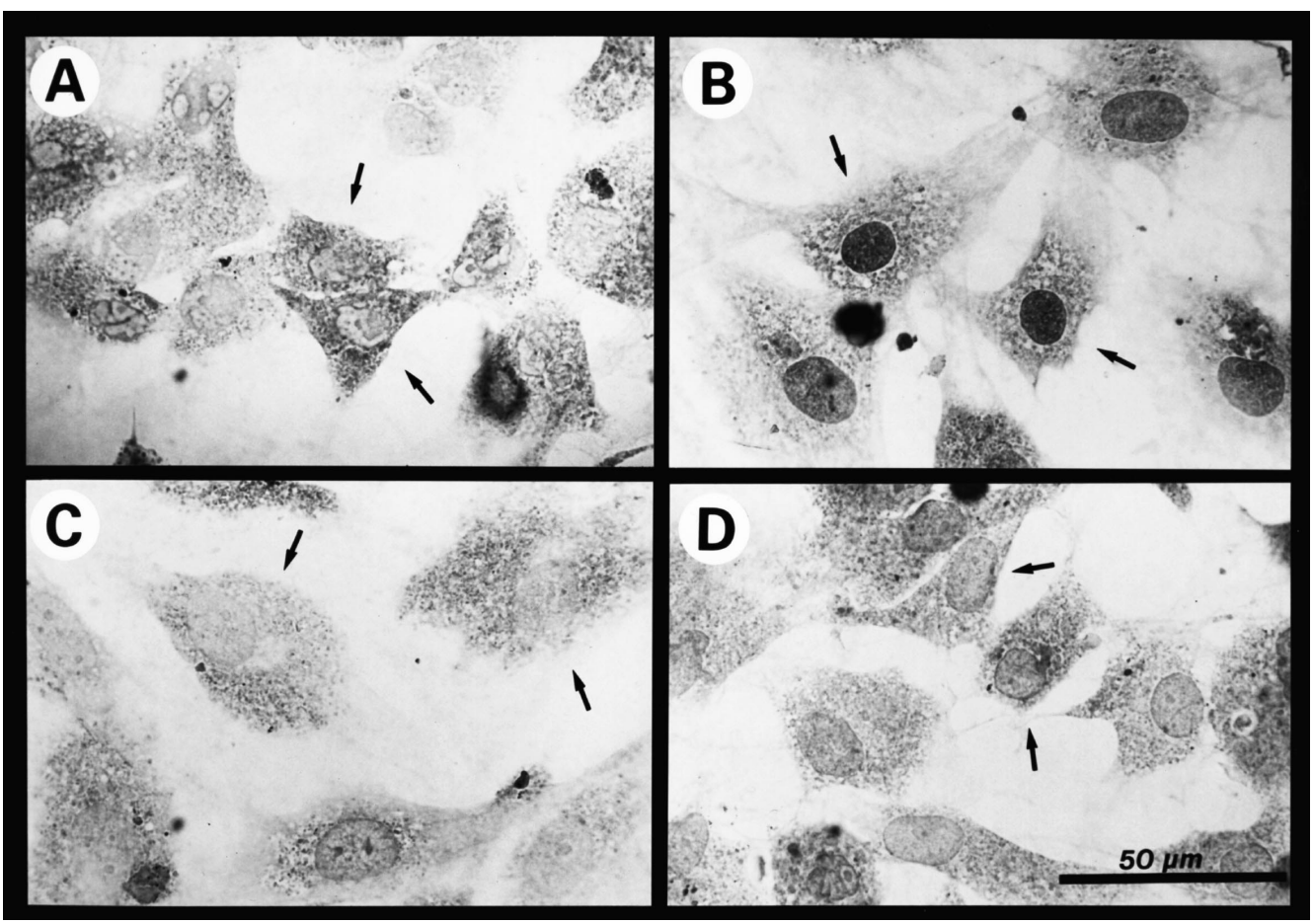

Fig. 4. Nuclear factor-κ B immunostaining in cultured synoviocytes. To examine whether auranofin interfered with the activity of nuclear factor-κ B, the interleukin-1β-induced translocation of nuclear factor-κ B p65 protein was detected by immunostaining. Synoviocytes were incubated with or without the concentration of auranofin shown for 4 h followed by stimulation with interleukin-1β (1 ng/ml) for 1 h. Then the cells were fixed and stained with anti-nuclear factor-κ B p65 IgG (50 μg/ml) as described in Materials and methods. Counterstaining was done with hematoxylin. Positive staining is indicated by brown deposits. Control staining with rabbit IgG (50 μg/ml) and anti-nuclear factor-κ B p65 IgG absorbed with synthetic nuclear factor-κ B p65 was completely negative (data not shown). (A) Under basal culture conditions, nuclear factor-κ B p65 was expressed predominantly in the cytoplasm of synoviocytes. (B) Rapid translocation of nuclear factor-κ B from cytoplasm to nucleus was found after stimulation by interleukin-1β. (C) and (D) Auranofin interfered strongly with the nuclear import of nuclear factor-κ B p65. (A) Untreated, (B) interleukin-1β (1 ng/ml), (C) interleukin-1β + auranofin 0.1 μM, (D) interleukin-1β + auranofin 1 μM. Similar results were obtained in three separate experiments.

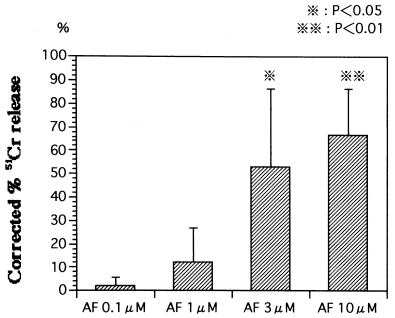

Fig. 5. Cytotoxic effects of auranofin on synoviocytes. To determine whether or not auranofin-induced cytotoxicity in synoviocytes, a ^{51}Cr release assay was carried out. Synoviocytes were incubated for 16 h with or without the concentrations of auranofin (AF) shown and the ^{51}Cr released in the culture supernatants was counted in a liquid scintillation counter as described in Materials and methods. As shown, 0.1 to 1 μM of auranofin treatments showed no significant effect whereas a significant increase in ^{51}Cr release occurred with 3 to 10 μM of the compound, indicative of cytotoxicity. Values are expressed as means ± S.E.M. of five experiments ($n = 5$) with triplicate samples. Statistical significance: ※ $P < 0.05$ compared to corresponding control. ※※ $P < 0.01$ compared to corresponding control.

on stimulation with interleukin-1β (Fig. 4B), and auranofin interfered strongly with the nuclear import of nuclear factor-κ B p65 as shown in Fig. 4C and D. Control staining with normal rabbit IgG and anti-nuclear factor-κ B p65 absorbed with synthetic nuclear factor-κ B p65 was completely negative (data not shown).

3.5. Cytotoxic effects of auranofin on synoviocytes

To study the cytotoxic effect of auranofin on synoviocytes, the ^{51}Cr release assay was carried out. Auranofin, at concentrations of 0.1 to 1 μM, did not increase significantly the ^{51}Cr release from the cells. However, treatment with 3 to 10 μM of the compound significantly augmented ^{51}Cr release, indicative of cytotoxicity on the cells (Fig. 5).

4. Discussion

Recent studies have demonstrated that some disease-modified anti-rheumatic drugs inhibit cyclooxygenase-2 activity and prostaglandin E_2 production (Tao et al., 1998; Vergne et al., 1998). We have now shown that auranofin diminishes interleukin-1β-induced prostaglandin E_2 synthesis on synoviocytes. Contradictory results obtained with effects of gold compounds on arachidonate metabolism and prostaglandin E_2 production have been attributed to differences in cell types, but Yamada et al. (1997) have recently demonstrated that there is an effect of the cell stimulus. However, the mechanism of auranofin suppression of stimulus-induced cyclooxygenase-2 expression is unclear. Recent studies have indicated that the cyclooxygenase-2 gene has transcriptional regulatory sequences of two nuclear factor-κ B sites (Appleby et al., 1994; Crofford, 1997), interleukin-1 activates nuclear factor-κ B through the phosphorylation of I κ B (Beg et al., 1993), and interleukin-1β-induced cyclooxygenase-2 expression and prostaglandin E_2 formation of synoviocytes are regulated by nuclear factor-κ B, especially the p65 subunit (Roshak et al., 1996; Crofford et al., 1997). Although there are recent reports that gold compounds inhibit the function of nuclear factor-κ B (William et al., 1992; Daniel et al., 1995), Yang et al. (1995) have reported that auranofin does not efficiently inhibit the DNA-binding activity of nuclear factor-κ B. It is known that transcriptional activation of nuclear factor-κ B requires two central events, namely, nuclear import and DNA binding (Baeuerle, 1991). Our data demonstrated that auranofin interferes with the interleukin-1β-induced nuclear import of nuclear factor-κ B from the cytoplasm on synoviocytes. Nuclear import of the protein has been shown to be required for the activation of protein kinase C (Baeuerle, 1991) and auranofin was found to be a specific inhibitor of protein kinase C (Froscio et al., 1989; Mahoney et al., 1989; Parente et al., 1989; Wong et al., 1990). While auranofin unexpectedly fails to inhibit protein kinase C in some studies (Hashimoto et al., 1992; Bondeson and Sundler, 1993), this may be attributed to the difference in sensitivities of protein kinase C from different cell types (Hashimoto et al., 1992) such as T cells and macrophages. Thus, we suggest that auranofin interfered with interleukin-1β-induced translocation of nuclear factor-κ B by inhibition of I κ B phosphorylation via suppression of protein kinase C activity on synoviocytes.

In contrast, similar to some reports (Peters-Golden and Shelly, 1988; Yamashita et al., 1997), higher concentrations of auranofin augmented spontaneous prostaglandin E_2 synthesis and that catalyzed from exogenous arachidonic acid on synoviocytes. This enhancement of prostaglandin E_2 synthesis by auranofin may be due to the activation of cyclooxygenase-1 and/or phospholipase enzymes (Yamada et al., 1997), these being the main enzymes that catalyze arachidonate under unstimulated conditions. However, further studies are required to explore the exact mechanisms of auranofin action. It is not proved that auranofin acts clinically as an anti-inflammatory agent because its onset of action is slow. However, this may be due to the pharmacokinetics of auranofin, in that administration of standard doses of the compound leads to a wide range of concentrations and it takes approximately 12 weeks to reach therapeutic concentrations (1.5–5.1 μM) (Gottlieb, 1982; Tett, 1993).

We have also examined the cytotoxic effect of auranofin on cultured synoviocytes. Auranofin did not induce significant cytotoxicity at concentrations below 1 μM, that is close to its therapeutic concentration. In addition, its inhibitory effect on prostaglandin E_2 synthesis was found starting from a concentration of 0.1 μM. All these findings make it unlikely that cytotoxicity and cyclooxygenase-2 inhibition by the compound occur simultaneously in vivo

because the two needed quite different concentrations. Further studies to clarify the mechanisms of its cytotoxicity are underway.

Synoviocytes and macrophages appear to play major roles in the destructive aspects and cytokine networks of rheumatoid arthritis (Firestein, 1996). Previous reports have indicated that auranofin inhibits cytokine production by macrophages (Yanni et al., 1994; Bondeson and Sundler, 1995). Also, our data demonstrate that the compound inhibits prostaglandin E_2 production in synoviocytes. Furthermore, it has been reported that auranofin inhibits osteoclastic bone resorption (Hall et al., 1996). Although the effects of intact auranofin in vitro are not completely identical to its in vivo effects because it dissociates in the plasma membrane of the digestive tract (Kean et al., 1991), these findings suggest a possible mechanism in which auranofin would directly and indirectly inhibit prostaglandin E_2 biosynthesis of synoviocytes in vivo, leading to amelioration of joint destruction and the inflammatory process of rheumatoid arthritis. The beneficial and adverse effects of gold compounds are clinically well-known, and they still play pivotal roles in the management of rheumatoid arthritis. Our study may lead to a better understanding of the exact action of auranofin in the treatment of rheumatoid arthritis.

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