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Induction of nitric oxide synthase by lipopolysaccharide and its inhibition by auranofin in RAW 264.7 cells

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Abstract

In RAW 264.7 cells, a murine macrophage cell line, treatment with lipopolysaccharide (1 to 10 ng/ml) stimulated production of nitric oxide (NO), which was inhibited by L-N^G-monomethyl-L-arginine acetate, an inhibitor of NO synthase. Auranofin, an orally active chrysotherapeutic agent, also inhibited the lipopolysaccharide-induced NO production in a concentration-dependent manner (0.3 to 3 μM). Other gold salts such as aurothioglucose and aurothiomalate had no effect. Western blot analysis demonstrated that the lipopolysaccharide (10 ng/ml)-induced expression of inducible NO synthase protein was inhibited by auranofin as well as by the protein synthesis inhibitor cycloheximide. In addition, the lipopolysaccharide-induced increase in the level of mRNA for inducible NO synthase was also lowered by auranofin. Furthermore, auranofin showed no direct effect on the conversion of [^{3}H]arginine to [^{3}H]citrulline by the cell lysate. These findings indicate that auranofin inhibits lipopolysaccharide-induced NO production by suppressing the expression of inducible NO synthase. © 1997 Elsevier Science B.V.

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1. Introduction

Nitric oxide (NO) is a free radical involved in neurotransmission, cell-mediated cytotoxicity and endothelium-dependent relaxation of vascular smooth muscles (for review, see Moncada et al., 1991). In addition, vasodilatation in acute inflammation induced by histamine, substance P or thrombin is dependent on the release of endothelium-derived relaxing factor, which is identical to NO (Palmer et al., 1987). The evidence in the literature suggests that NO also participates in inflammatory- and autoimmune-mediated tissue destruction (Mulligan et al., 1991; Kolb and Kolb-Bachofen, 1992). Farrell et al. (1992) reported elevated amounts of nitrate and nitrite, the stable end-products of NO by spontaneous oxidization, in the synovial fluid of patients with rheumatoid arthritis. Furthermore, it has been suggested that the resident mesenchymal cells of the joints and the leukocytes that have infiltrated into the synovial fluid have the capacity to produce considerable amounts of NO in vitro (Wright et al., 1989; Kirk et al., 1990; Salvemini et al., 1990). In fact, articular chondrocytes and synoviocytes respond to interleukin 1, a cytokine present in rheumatoid synovial fluid and produce high levels of NO (Stadler et al., 1991; Palmer et al., 1992; Stefanovic-Racic et al., 1994). Recently, Ueki et al. (1996) determined the concentrations of NO in serum and joint fluid in patients with rheumatoid arthritis and suggested that an increased synthesis of NO reflects abnormalities of immunoregulation in the joints of these patients.

For the treatment of rheumatoid arthritis, auranofin is used as an orally active chrysotherapeutic agent (Ward et al., 1983; Wenger et al., 1983). However, the mechanism of action of auranofin is still unclear, but may involve inhibition of lysosomal enzyme release (DiMartino and Walz, 1977; Finkelstein et al., 1977), chemotaxis (Scheinberg et al., 1982; Hafstrom et al., 1983), phagocytosis (Hafstrom et al., 1983) and superoxide generation (Davis et al., 1983) in leukocytes, inhibition of arachidonic acid metabolism (Lewis et al., 1984; Honda et al., 1987; Elmgreen et al., 1989; Peters-Golden and Shelly, 1989), and inhibition of cytokine production (Bondeson and Sundler,

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1995). Recently, inhibition of osteoclastic bone resorption (Hall et al., 1996) and inhibition of induction of cyclooxygenase-2 protein (Yamada et al., 1997; Yamashita et al., 1997) by auranofin have been reported. In the present study, we examined the effects of auranofin on NO production in RAW 264.7 cells, an Abelson leukemia virus-transformed murine macrophage cell line (Raschke et al., 1978), to obtain further insight into the mechanism of action of auranofin.

2. Materials and methods

2.1. Cell culture

RAW 264.7 cells obtained from RIKEN Gene Bank (Tsukuba, Japan) were seeded in 24-well plates at a density of 0.75×10^6 cells per well and incubated for 20 h at 37°C in 0.5 ml of Eagle's minimal essential medium (MEM), phenol red-free, (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 1% (v/v) MEM non-essential amino acid solution (Sigma, St. Louis, MO, USA) and 10% (v/v) heat-inactivated fetal bovine serum (Flow Laboratories, North Rydge, N.S.W., Australia). After three washes with medium, the cells were incubated for the indicated periods at 37°C in 0.5 ml of medium containing drugs.

2.2. Drugs

Drugs used were lipopolysaccharide (from *Escherichia coli* 0111:B4, Difco Laboratories, Detroit, MI, USA), auranofin (Sigma), aurothiomalate (Shiosol, Shionogi, Osaka, Japan), aurothioglucose (Sigma), cycloheximide (Sigma), L-N^G-monomethyl-L-arginine acetate (L-NMMA) (Wako Pure Chemical, Osaka, Japan). Lipopolysaccharide, aurothiomalate, aurothioglucose and L-NMMA were directly dissolved in medium. Other drugs were dissolved in ethanol and an aliquot of each ethanol solution was added to the medium.

The final concentration of ethanol was adjusted to 0.1% (v/v). Control medium contained the same amount of the vehicle. After treatment with drugs, the viability of the cells was examined in the Trypan blue exclusion test; no cytotoxic effects were observed.

2.3. Determination of nitrite

Nitrite concentrations in the conditioned medium were determined by using Griess reagent according to the method described by Green et al. (1982). Briefly, 100 μl aliquots of the conditioned medium were mixed with an equal volume of Griess reagent (1 part 0.1% (w/v) *N*-1-naphthylethylenediamine dihydrochloride to 1 part 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid). The absorbance at 540 nm was measured with a microplate reader (Model 450, Bio-Rad Laboratories, Hercules, CA, USA) and the nitrite concentration was determined by interpolation of a calibration curve of standard sodium nitrite concentrations against absorbance. All assays were performed on three to four replicates and repeated in at least three independent experiments.

2.4. Western blot analysis of NO synthase

Ten-milliliter aliquots of the cell suspension (1.5×10^6 cells per 1 ml medium) were poured into a plastic dish (100 mm diameter, Corning, Grand Island, NY, USA), three dishes per group and incubated for 16 h at 37°C. After three washes with medium, the cells were further incubated for 8 h at 37°C in 10 ml of medium containing the indicated concentrations of drugs. The cells were then scraped off the dishes with a rubber policeman and washed three times by centrifugation at 4°C and $800 \times g$ for 5 min. The precipitates were sonicated five times with a Handy Sonic Disrupter (UR-20P, TOMY, Tokyo, Japan) at 50% maximum power for 10 s at a time in 0.5 ml of ice-cold solubilization buffer (50 mM Tris, pH 7.4, 2% (w/v) sodium dodecyl sulfate, 1 mM phenylmethylsulphonyl fluoride, 10 μM pepstatin A and 10 μg/ml leupeptin). The sonicates were centrifuged at 4°C and $100\,000 \times g$ for 1 h and the supernatant fractions were obtained. Protein concentrations in the supernatant fractions were determined (Wang and Smith, 1975) and aliquots of 30 μg of protein were boiled for 3 min in loading buffer (50 mM Tris, pH 7.4, 4% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol and 0.05 mg/ml bromophenol blue). Aliquots of 30 μl of the solution were applied to each well of a 3% (w/v) stacking sodium dodecyl sulfate-polyacrylamide gel in 0.25 M Tris–glycine buffer (pH 6.8) and subjected to electrophoresis in 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel in 1.5 M Tris–glycine (pH 8.8) ($140 \times 140 \times 1$ mm) at 25 mA for 4 h. The fractionated proteins were transferred to nitrocellulose membranes and blotted in blocking solution (Block Ace, Dainippon Pharmaceutical, Osaka, Japan) for 1 h. The nitrocellulose membranes were then incubated at 4°C overnight with the primary antibody, a rabbit immunoglobulin G to inducible NO synthase purified from RAW 264.7 cells (Upstate Biotechnology, NY, USA), at a dilution of 1:2000. Thereafter the membranes were washed and incubated for 3 h at 4°C with rabbit anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:2000. The reaction products were incubated for 30 min at room temperature with Vectastatin ABC reagent (Vector Laboratories) and were visualized by using a chemiluminescence detection system (ECL system, Amersham, Arlington Heights, IL, USA). The membranes were exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA) at room temperature for 30 s and photographed. The levels of inducible NO synthase protein were quantified by scanning densitometry and expressed as relative densities signals.

2.5. Semiquantitation of the mRNA level of inducible NO synthase by reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells (1.5×10^7 cells) were incubated for 20 h at 37°C in 10 ml of medium, washed three times and further incubated for 8 h at 37°C in 10 ml of medium containing lipopolysaccharide (10 ng/ml) with or without auranofin.

Following incubation, cells were rinsed three times with ice-cold phosphate-buffered saline and scraped off the plate with a rubber policeman. Total RNA was then prepared by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987) and the yield of RNA extracted was determined by spectrophotometry. 1 μg of each sample was reverse transcribed at 37°C for 1 h in 20 μl of the buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) containing 5 μM random hexamer oligonucleotides (Gibco BRL, Gaithersburg, MD, USA), 200 U of the reverse transcriptase from Moloney murine leukemia virus (Gibco BRL), 500 μM 2′-deoxynucleotide 5′-triphosphate (dNTP, Pharmacia Biotech, Uppsala, Sweden) and 10 mM dithiothreitol. PCR primers for inducible NO synthase were designed according to Nadeau et al. (1995). The sequences of primers used were (former) 5′-GTGTTCCACCAGGAGATGTTG-3′ and (reverse) 5′-CTCCTGCCCACTGAGTTCGTC-3′, which amplify a 576-base pair fragment of inducible NO synthase. PCR reactions consisted of 10 μl of the reverse transcribed RNA solution and 40 μl of PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$) containing 0.2 μM of each primer, 200 μM dNTP and 1.25 U of *Taq* polymerase (Takara Shuzo, Shiga, Japan). PCR was performed for 27 cycles; 30 s denaturation at 94°C, 1 min annealing at 54°C and 1 min extension at 72°C, using a thermal cycler (GeneAmp™ PCR System 2400, Perkin Elmer Cetus, Norwalk, CT, USA). The level of mRNA for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined as an internal control. The PCR primers for rat GAPDH were (former) 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and (reverse) 5′-TCCTTGGAGGCCATGTAGGCCAT-3′, which amplify a 249-bp GAPDH fragment (Robbins and McKinney, 1992). PCR was performed for 18 cycles; 30 s denaturation at 94°C, 1 min annealing at 57°C and 1 min extension at 72°C. After PCR, 10 μl of the PCR reaction mixture was loaded onto a 1.6% agarose minigel and the PCR products were visualized by ethidium bromide staining after electrophoresis. The levels of mRNA for inducible NO synthase and GAPDH were quantified by scanning densitometry and the ratio of the density of mRNA for inducible NO synthase to GAPDH was calculated.

2.6. Determination of NO synthase activity

RAW 264.7 cells (1.5×10^7 cells) were incubated for 20 h at 37°C in 10 ml of medium, washed three times and further incubated for 8 h at 37°C in 10 ml of medium with or without lipopolysaccharide (10 ng/ml). Following incubation, cells were rinsed three times with ice-cold phosphate-buffered saline and lysed by the addition of 150 μl ice-cold homogenization buffer (0.1 mM potassium phosphate, pH 7.4, 0.25 M sucrose, 0.1 mM dithiothreitol, 1 mM EDTA). Cellular material was removed from the culture plates with a cell scraper, collected and centrifuged at $10\,000 \times g$ for 10 min at 4°C. The supernatant fraction was used as a crude preparation of NO synthase as described by Salter et al., 1991. For the assay, 30 μl of the supernatant fraction was mixed with 120 μl of assay buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 0.1 mM NADPH, 2.2×10^5 dpm L-[2,3-^{3}H]arginine (DuPont/NEN, Boston, MA, USA)) containing the indicated concentrations of auranofin or L-NMMA (300 μg/ml), and incubated for 10 min at 37°C in microcentrifuge tubes. After incubation, 100 μl of the ice-cold stop solution (100 mM Hepes, pH 5.5, 10 mM EDTA) was added to terminate the reaction (Bredt and Snyder, 1990). Then, 1 ml of a suspension of Bio-Rad AG 50W-X8 resin (resin/stop solution, 1:1, w/v) was added to each sample, mixed and centrifuged at $1700 \times g$ for 10 min at 4°C. Radioactivity in the supernatant fraction was then determined in a liquid scintillation analyzer (Packard TRI-CARB 2050CA, Packard Instrument). One set of samples was incubated for 10 min at 4°C and radioactivity in the supernatant fraction of these samples was used as background. Disintegrations per minute were converted to citrulline production and expressed in pmol per mg protein per min (Bogle and Vallance, 1996).

2.7. Determination of cell protein

Protein content was determined with the Coomassie blue protein reagent (Bradford, 1976) diluted 1:5 with distilled water before use. An appropriately diluted supernatant fraction (20 μl) was mixed with 1 ml protein reagent and incubated for 10 min at room temperature. Absorbance at 595 nm was then measured with a microplate reader (Model 450, Bio-Rad). Bovine serum albumin (essentially fatty acid-free, Sigma) was used as a standard.

2.8. Statistical analysis

The statistical significance of the results was analyzed by Dunnett's test for multiple comparison and Student's *t*-test for unpaired observation.

3. Results

3.1. Enhancement of NO production by lipopolysaccharide

Incubation of RAW 264.7 cells for 8 h in the presence of lipopolysaccharide induced NO production in a concen-

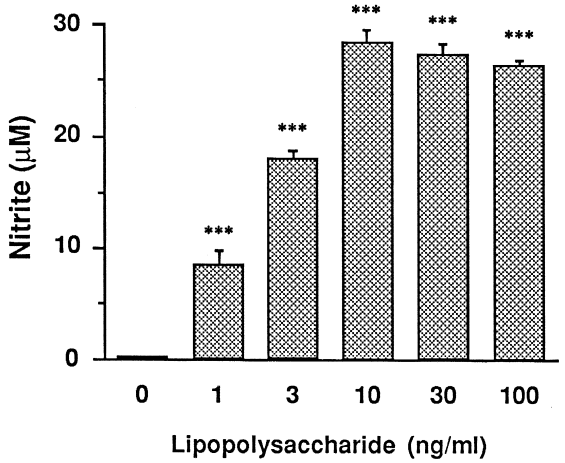

Fig. 1. NO production induced by lipopolysaccharide in RAW 264.7 cells. RAW 264.7 cells (0.75×10^6 cells) were incubated for 24 h in 0.5 ml of medium containing the indicated concentrations of lipopolysaccharide. Nitrite concentrations in the conditioned medium were determined by using Griess reagent. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: *** $P < 0.001$ versus the control.

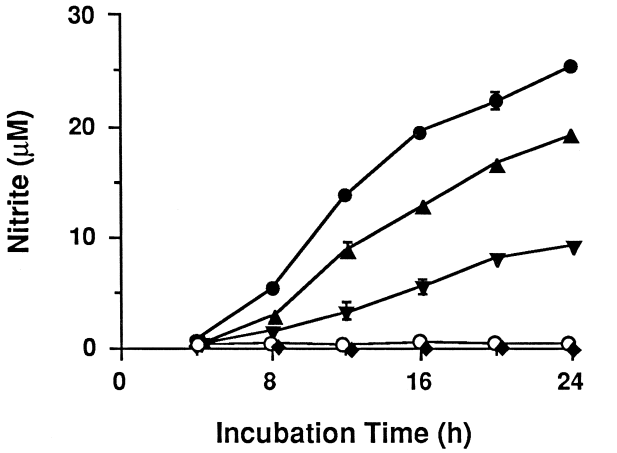

Fig. 2. Time course of the effect of auranofin on lipopolysaccharide-induced NO production. RAW 264.7 cells (0.75×10^6 cells) were incubated for the indicated periods of time in 0.5 ml of medium containing lipopolysaccharide (10 ng/ml) and various concentrations of auranofin (●, 0 μM; ▲, 0.3 μM; ▼, 1 μM; ◆, 3 μM). Nitrite concentrations in the conditioned medium were determined by using Griess reagent. Nitrite concentrations in the absence of lipopolysaccharide are shown by open circles. Values are the means from four samples.

tration-dependent manner at 1 to 10 ng/ml and the stimulatory effect of lipopolysaccharide reached a plateau at 10 ng/ml. NO production triggered by lipopolysaccharide continued to increase and almost the same concentration dependence was observed at 24 h (Fig. 1). Therefore, in the following experiments, lipopolysaccharide was used at the concentration of 10 ng/ml.

3.2. Effects of the NO synthase inhibitor L-NMMA on lipopolysaccharide-induced NO production

When RAW 264.7 cells were incubated in medium containing lipopolysaccharide (10 ng/ml) and various concentrations of L-NMMA, the lipopolysaccharide-induced NO production at 24 h was inhibited in a concentration-dependent manner. At concentrations of 100 and 300 μM, lipopolysaccharide induced NO production at 24 h was decreased to 40.0 and 94.2% of control, respectively. Almost complete inhibition was observed at 1000 μM L-NMMA.

3.3. Effects of auranofin on lipopolysaccharide-induced NO production

NO production by RAW 264.7 cells was increased time dependently by lipopolysaccharide (10 ng/ml) and was inhibited by auranofin in a concentration-dependent manner (Fig. 2). Significant inhibition was observed at concentrations of 0.3 μM and higher and almost complete inhibition was observed at 3 μM.

3.4. Effects of other gold salts on lipopolysaccharide-induced NO production

The effects of aurothiomalate and aurothioglucose on lipopolysaccharide-induced NO production at 24 h were examined. As shown in Fig. 3, aurothiomalate and aurothioglucose at concentrations of 1 to 10 μM had no effect,

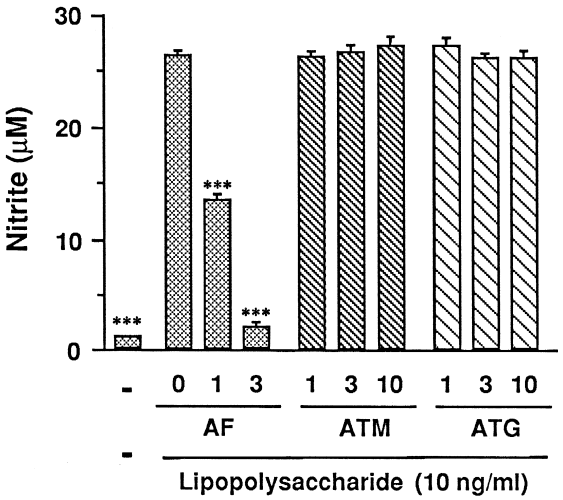

Fig. 3. Effects of aurothiomlate and aurothioglucose on lipopolysaccharide-induced NO production. RAW 264.7 cells (0.75×10^6 cells) were incubated for 24 h in 0.5 ml of medium containing lipopolysaccharide (10 ng/ml) and the indicated concentrations of aurothiomalate (ATM), aurothioglucose (ATG), or auranofin (AF). Nitrite concentrations in the conditioned medium were determined by using Griess reagent. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: *** $P < 0.001$ versus the lipopolysaccharide control.

whereas auranofin inhibited NO production at 1 μM. Among the three gold salts examined, only auranofin showed a potent inhibitory effect on NO production in the concentration range from 1 to 3 μM.

3.5. Effects of auranofin on protein levels of inducible NO synthase

As shown in Fig. 4, Western blot analysis demonstrated that treatment with lipopolysaccharide (10 ng/ml) for 8 h markedly increased the expression of inducible NO synthase protein. The expression of inducible NO synthase protein by lipopolysaccharide was suppressed by auranofin at 1 μM and almost complete suppression was observed at 3 μM auranofin. The protein synthesis inhibitor cycloheximide at 1 μM also blocked the expression of inducible NO synthase protein induced by lipopolysaccharide and inhibited the lipopolysaccharide-induced NO production. In contrast, treatment with L-NMMA (300 μM), an inhibitor of NO synthase, did not affect the lipopolysaccharide-induced expression of inducible NO synthase protein, but inhibited the lipopolysaccharide-induced NO production.

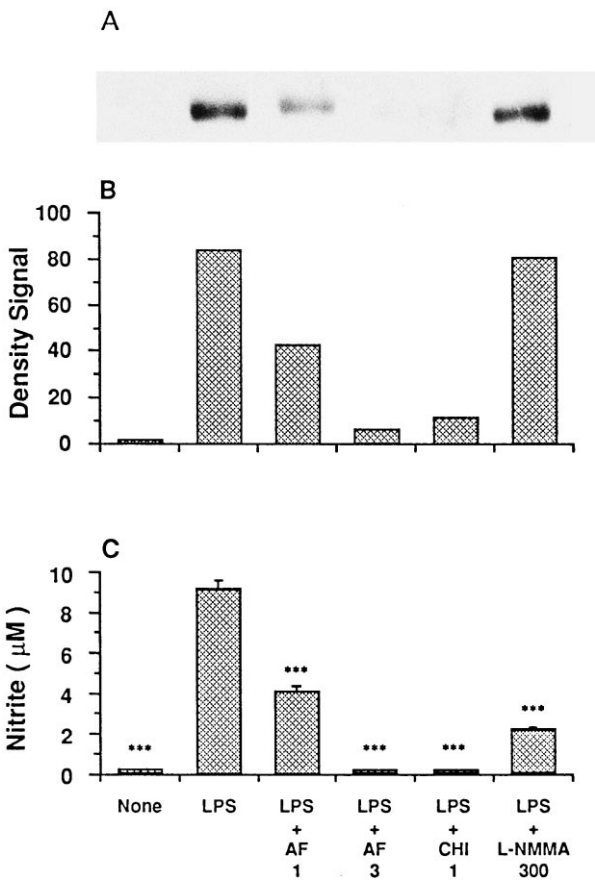

Fig. 4. Effects of auranofin, cycloheximide and L-NMMA on lipopolysaccharide-induced expression of inducible NO synthase protein. RAW 264.7 cells (1.5×10^7 cells) were incubated for 8 h in 10 ml of medium containing lipopolysaccharide (LPS, 10 ng/ml) and auranofin (AF, 1 and 3 μM), cycloheximide (CHI, 1 μM), or L-NMMA (300 μM). Western blot analysis for the expression of inducible NO synthase protein (A) was performed as described in Section 2.4. To facilitate comparison, the signal densities are shown (B). Nitrite concentrations in the conditioned medium (C) were determined by using Griess reagent. Values for the nitrite concentrations are the means from three samples with S.E.M. shown by vertical bars. Statistical significance: *** $P < 0.001$ versus the control.

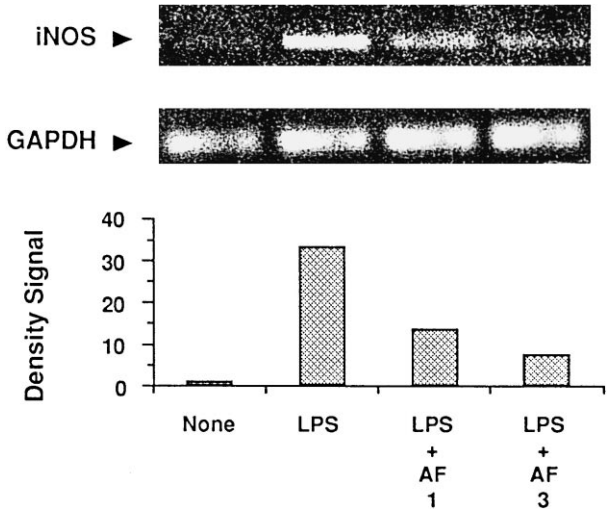

Fig. 5. Effects of auranofin on the levels of mRNA for inducible NO synthase and GAPDH. RAW 264.7 cells (1.5×10^7 cells) were incubated for 20 h at 37°C in 10 ml of medium, washed and further incubated for 8 h at 37°C in medium containing lipopolysaccharide (LPS, 10 ng/ml) with or without auranofin (AF). Total RNA was extracted and RT-PCR for inducible NO synthase mRNA and GAPDH mRNA was performed as described in Section 2. The ratio of inducible NO synthase mRNA density to GAPDH mRNA density is shown in the lower panel. The density ratio in the non-treated group (None) is expressed as 1.0. One representative out of three separate experiments is shown.

3.6. Effects of auranofin on the level of mRNA for inducible NO synthase

Treatment with lipopolysaccharide (10 ng/ml) for 8 h increased the level of mRNA for inducible NO synthase (Fig. 5). However, the lipopolysaccharide-induced increase in the level of mRNA for inducible NO synthase was decreased by auranofin in a concentration-dependent manner at 1 and 3 μM (Fig. 5). The level of mRNA for GAPDH was not affected by treatment with lipopolysaccharide (10 ng/ml) and auranofin (1 and 3 μM) (Fig. 5).

3.7. Effects of auranofin on the activity of NO synthase in cell-free systems

Incubation of the cells for 8 h in the presence of lipopolysaccharide (10 ng/ml) increased the activity of NO synthase, as determined by the conversion of [^{3}H]arginine to [^{3}H]citrulline by the supernatant fraction of the cell homogenate (Fig. 6). When auranofin (1 and 3

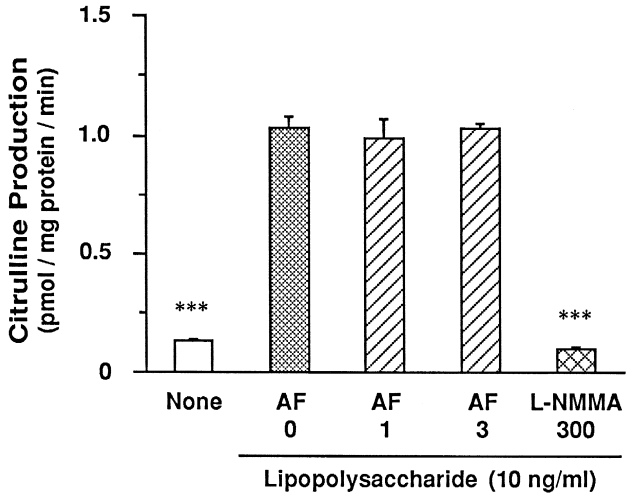

Fig. 6. Effects of auranofin on the conversion of [^{3}H]arginine to [^{3}H]citrulline by the cell lysate. RAW 264.7 cells (1.5×10^7 cells) were incubated for 8 h in 10 ml of medium with or without lipopolysaccharide (10 ng/ml). After three washes, cells were lysed with homogenization buffer and centrifuged at $10\,000\times g$ for 10 min at 4°C. The supernatant fraction was incubated for 10 min at 37°C with [^{3}H]arginine in the presence or absence of auranofin (AF) or L-NMMA. Conversion of [^{3}H]arginine to [^{3}H]citrulline was determined as described in Section 2. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: *** $P < 0.001$ versus lipopolysaccharide control.

μM) was added to the supernatant fraction obtained from the cells 8 h after incubation with lipopolysaccharide (10 ng/ml), the lipopolysaccharide-induced increase in NO synthase activity was not inhibited at all, whereas it was inhibited almost completely by L-NMMA (300 μM), an inhibitor of NO synthase (Fig. 6).

4. Discussion

Combined treatment with lipopolysaccharide and interferon γ is usually used to stimulate NO synthesis (Sheffler et al., 1995). Other agents including tumor necrosis factor α (Ding et al., 1988; Drapier et al., 1988), interleukin 2 (Cox et al., 1992; Deng et al., 1993), and picolinic acid (Melillo et al., 1993, 1994) also stimulate the expression of inducible NO synthase in combination with lipopolysaccharide in murine macrophages. In the present study, RAW 264.7 cells, a murine macrophage cell line, produced NO and showed induction of inducible NO synthase protein after stimulation with lipopolysaccharide alone.

The present study demonstrated that auranofin inhibits lipopolysaccharide-induced NO production in RAW 264.7 cells. Because conversion of [^{3}H]arginine to [^{3}H]citrulline by the lysate of RAW 264.7 cells was not affected by auranofin (Fig. 6), it seems that auranofin has no direct inhibitory effect on NO synthase. The results of Western blot analysis suggested that the inhibition by auranofin of lipopolysaccharide-induced NO production is due to the inhibition of the expression of inducible NO synthase protein (Fig. 4). In addition, RT-PCR analysis demonstrated that the level of mRNA for inducible NO synthase was increased by lipopolysaccharide treatment, and that auranofin lowered the lipopolysaccharide-induced increase in the level of mRNA for inducible NO synthase (Fig. 5). These findings suggested that auranofin acts at the transcriptional level. It has been reported that lipopolysaccharide increases inducible NO synthase transcription via its effect on the nuclear factor κB (NF-κB) in mouse macrophages (Xie et al., 1994). Recently, Nunokawa et al. (1996) reported that human inducible NO synthase gene is also transcriptionally regulated in an NF-κB-dependent manner. Therefore, it is possible that auranofin inhibits the activation of NF-κB or the binding of NF-κB to the putative NF-κB binding region. However, in an in vitro study, Yang et al. (1995) reported that the potencies of gold compounds to inhibit binding of NF-κB to DNA were as follows: aurothioglucose > aurothiomalate ≫ auranofin. In cultures of RAW 264.7 cells, lipopolysaccharide-induced NO production was inhibited by gold compounds with the following potency order: auranofin ≫ aurothiomalate = aurothioglucose (Fig. 3). Therefore, the inhibition of NO production by auranofin might not be due to inhibition of the binding of NF-κB to DNA. In mouse macrophages, the inducible NO synthase gene promoter contains consensus sequences for the binding of several transcription factors including activating protein 1, interferon γ, interferon regulatory factor and nuclear factor-interleukin 6 (Lowenstein et al., 1993; Xie et al., 1993; Kamijo et al., 1994; Martin et al., 1994), in addition to NF-κB. Therefore, it is also possible that auranofin inhibits the binding of these transcription factors to the promoter gene of inducible NO synthase.

There are several reagents that inhibit the expression of inducible NO synthase, for example, dexamethasone (Kunz et al., 1994, 1996), interleukin 4 (Liew et al., 1991; Cox et al., 1992; Bogdan et al., 1994), interleukin 10 (Ding et al., 1990; Gazzinelli et al., 1992; Oswald et al., 1992), transforming growth factor β (Ding et al., 1990; Pfeilschifter and Vosbeck, 1991; Oswald et al., 1992; Vodovotz et al., 1993), human macrophage-stimulating protein (Wang et al., 1994), trace concentrations of lipopolysaccharide added before interferon γ exposure (Vodovotz et al., 1994) and NO (Sheffler et al., 1995). Among these, transforming growth factor β has been shown to act post-transcriptionally by destabilizing the mRNA for inducible NO synthase, decreasing its translation, and accelerating the degradation of the inducible NO synthase protein (Vodovotz et al., 1993). Glucocorticoids also act at multiple levels to regulate the expression of inducible NO synthase protein. For example, dexamethasone attenuates the interleukin 1β-induced transcription of the inducible NO synthase gene, prolongs the half-life of the mRNA for inducible NO synthase, and reduces the amount of inducible NO synthase protein by reducing the translation of mRNA and increasing the degradation of the inducible NO synthase

protein in rat glomerular mesangial cells (Kunz et al., 1996). As the expression of inducible NO synthase is susceptible to control at transcriptional, post-transcriptional, translational and post-translational levels, further studies are necessary to clarify the mechanism of action of auranofin in the suppression of the expression of inducible NO synthase.

As it has been suggested that the overproduction of NO via the expression of inducible NO synthase reflects abnormalities of immunoregulation in the joints of patients with rheumatoid arthritis (Ueki et al., 1996), the therapeutic effects of auranofin in rheumatoid arthritis might be partially due to inhibition of the expression of inducible NO synthase protein and the production of NO in the synovial tissue and infiltrating leukocytes. In the present study, however, we examined the effect of auranofin in a cell culture system. Therefore, further studies are necessary to demonstrate the therapeutic effects of auranofin when administered locally at the inflammatory site.

In conclusion, auranofin inhibits the lipopolysaccharide-triggered production of nitrite, the stable end-product of NO formation, in RAW 264.7 cells. The inhibition of nitrite formation by auranofin is suggested to be due to the inhibition of the expression inducible NO synthase protein.

References

Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q.W., Nathan, C., 1994. Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. J. Leuk. Biol. 55, 227–233.

Bogle, R.G., Vallance, P., 1996. Functional effects of econazole on inducible nitric oxide synthase: production of a calmodulin-dependent enzyme. Br. J. Pharmacol. 117, 1053–1058.

Bondeson, J., Sundler, R., 1995. Auranofin inhibits the induction of interleukin 1β and tumor necrosis factor α mRNA in macrophages. Biochem. Pharmacol. 50, 1753–1759.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bredt, D.S., Snyder, S.H., 1990. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. USA 87, 682–685.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Cox, G.W., Melillo, G., Chattopadhyay, U., Mullet, D., Fertel, R.H., Varesio, L., 1992. Tumor necrosis factor-α-dependent production of reactive nitrogen intermediates mediates IFN-γ plus IL-2-induced murine macrophage tumoricidal activity. J. Immunol. 149, 3290–3296.

Davis, P., Johnston, C., Miller, C.L., Wong, K., 1983. Effects of gold compounds on the function of phagocytic cells: II. Inhibition of superoxide radical generation by tripeptide-activated polymorphonuclear leukocytes. Arthritis Rheum. 26, 82–86.

Deng, W., Thiel, B., Tannenbaum, C.S., Hamilton, T.A., Stuehr, D.J., 1993. Synergistic cooperation between T-cell lymphokines for induction of the nitric oxide synthase gene in murine peritoneal macrophages. J. Immunol. 151, 322–329.

DiMartino, M.J., Walz, D.T., 1977. Inhibition of lysosomal enzyme release from rat leukocytes by auranofin. Inflammation 2, 131–142.

Ding, A.H., Nathan, C.F., Stuehr, D.J., 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J. Immunol. 141, 2407–2412.

Ding, A., Nathan, C.F., Graycar, J., Derynck, R., Stuehr, D.J., Srimal, S., 1990. Macrophage deactivating factor and transforming growth factors-β1, -β2 and -β3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ. J. Immunol. 145, 940–944.

Drapier, J.C., Wietzerbin, J., Hibbs, J.B. Jr., 1988. Interferon-γ and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. Eur. J. Immunol. 18, 1587–1592.

Elmgreen, J., Ahnfelt-Ronne, I., Nielsen, O.H., 1989. Inhibition of human neutrophils by auranofin: chemotaxis and metabolism of arachidonate via the 5-lipoxygenase pathway. Ann. Rhem. Dis. 48, 134–138.

Farrell, A.J., Blake, D.R., Palmer, R.M., Moncada, S., 1992. Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. Ann. Rheum. Dis. 51, 1219–1222.

Finkelstein, A.E., Roisman, F.R., Walz, D.T., 1977. Effect of auranofin, a new antiarthritic agent, on immune complex-induced release of lysosomal enzymes from human leukocytes. Inflammation 2, 143–150.

Gazzinelli, R.T., Oswald, I.P., James, S.L., Sher, A., 1992. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-γ-activated macrophages. J. Immunol. 148, 1792–1796.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Hafstrom, I., Uden, A.M., Palmblad, J., 1983. Modulation of neutrophil functions by auranofin. Studies on effects in vitro and in rheumatoid arthritis patients. Scand. J. Rheumatol. 12, 97–105.

Hall, T.J., Jeker, H., Nyugen, H., Schaeublin, M., 1996. Gold salts inhibit osteoclastic bone resorption in vitro. Inflamm. Res. 45, 230–233.

Honda, Z., Iizasa, T., Morita, Y., Matsuta, K., Nishida, Y., Miyamoto, T., 1987. Differential inhibitory effects of auranofin on leukotriene B_4 and leukotriene C_4 formation by human polymorphonuclear leukocytes. Biochem. Pharmacol. 36, 1475–1481.

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S.I., Kimura, T., Green, S.J., Mak, T.W., Taniguchi, T., Vilcek, J., 1994. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science 263, 1612–1615.

Kirk, S.J., Regan, M.C., Barbul, A., 1990. Cloned murine T-lymphocytes synthesize a molecule with the biological characteristics of nitric oxide. Biochem. Biophys. Res. Commun. 173, 660–665.

Kolb, H., Kolb-Bachofen, V., 1992. Nitric oxide: a pathogenetic factor in autoimmunity. Immunol. Today 13, 157–160.

Kunz, D., Walker, G., Pfeilschifter, J., 1994. Dexamethasone differentially affects interleukin 1β- and cyclic AMP-induced nitric oxide synthase mRNA expression in renal mesangial cells. Biochem. J. 304, 337–340.

Kunz, D., Walker, G., Eberhardt, W., Pfeilschifter, J., 1996. Molecular mechanisms of dexamethasone inhibition of nitric oxide synthase expression in interleukin 1β-stimulated mesangial cells: evidence for the involvement of transcriptional and posttranscriptional regulation. Proc. Natl. Acad. Sci. USA 93, 255–259.

Lewis, A.J., Carlson, R.P., Chang, J., DeLustro, F., 1984. Effect of gold salts, D-penicillamine and benoxaprofen on type II collagen-induced arthritis in rats. Agents Actions 14, 707–714.

Liew, F.Y., Li, Y., Severn, A., Millott, S., Schmidt, J., Salter, M., Moncada, S., 1991. A possible novel pathway of regulation by murine T-helper type-2 (T_h2) cells of a T_h1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. Eur. J. Immunol. 21, 2489–2494.

Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., Murphy, W.J., 1993. Macrophage nitric oxide synthase gene: Two upstream regions mediate induction by interferon γ and lipopolysaccharide. Proc. Natl. Acad. Sci. USA 90, 9730–9734.

Martin, E., Nathan, C., Xie, Q.W., 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. J. Exp. Med. 180, 977–984.

Melillo, G., Cox, G.W., Radzioch, D., Varesio, L., 1993. Picolinic acid, a catabolite of L-tryptophan, is a costimulus for the induction of reactive nitrogen intermediate production in murine macrophages. J. Immunol. 150, 4031–4040.

Melillo, G., Cox, G.W., Biragyn, A., Sheffler, L.A., Varesio, L., 1994. Regulation of nitric-oxide synthase mRNA expression by interferon-γ and picolinic acid. J. Biol. Chem. 269, 8128–8133.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 43, 109–142.

Mulligan, M.S., Hevel, J.M., Marletta, M.A., Ward, P.A., 1991. Tissue injury caused by deposition of immune complexes is L-arginine dependent. Proc. Natl. Acad. Sci. USA 88, 6338–6342.

Nadeau, K.C., Azuma, H., Tilney, N.L., 1995. Sequential cytokine dynamics in chronic rejection of rat renal allografts: Role for cytokines RANTES and MCP-1. Proc. Natl. Acad. Sci. USA 92, 8729–8733.

Nunokawa, Y., Oikawa, S., Tanaka, S., 1996. Human inducible nitric oxide synthase gene is transcriptionally regulated by nuclear factor-κB dependent mechanism. Biochem. Biophys. Res. Commun. 223, 347–352.

Oswald, I.P., Gazzinelli, R.T., Sher, A., James, S.L., 1992. IL-10 synergizes with IL-4 and transforming growth factor-β to inhibit macrophage cytotoxic activity. J. Immunol. 148, 3578–3582.

Palmer, R.M., Ferrige, A.G., Moncada, S., 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327, 524–526.

Palmer, R.M., Andrews, T., Foxwell, N.A., Moncada, S., 1992. Glucocorticoids do not affect the induction of a novel calcium-dependent nitric oxide synthase in rabbit chondrocytes. Biochem. Biophys. Res. Commun. 188, 209–215.

Peters-Golden, M., Shelly, C., 1989. Inhibition of alveolar macrophage 5-lipoxygenase metabolism by auranofin. Biochem. Pharmacol. 38, 1589–1595.

Pfeilschifter, J., Vosbeck, K., 1991. Transforming growth factor β_2 inhibits interleukin 1β- and tumour necrosis factor α-induction of nitric oxide synthase in rat renal mesangial cells. Biochem. Biophys. Res. Commun. 175, 372–379.

Raschke, W.C., Baird, S., Ralph, P., Nakoinz, I., 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. Cell 15, 261–267.

Robbins, M., McKinney, M., 1992. Transcriptional regulation of neuromodulin (GAP-43) in mouse neuroblastoma clone NIE-115 as evaluated by the RT/PCR method. Mol. Brain Res. 13, 83–92.

Salter, M., Knowles, R.G., Moncada, S., 1991. Widespread tissue distribution, species distribution and changes in activity of calcium-dependent and calcium-independent nitric oxide synthase. FEBS Lett. 291, 145–149.

Salvemini, D., Masini, E., Anggard, E., Mannaioni, P.F., Vane, J., 1990. Synthesis of a nitric oxide-like factor from L-arginine by rat serosal mast cells: stimulation of guanylate cyclase and inhibition of platelet aggregation. Biochem. Biophys. Res. Commun. 169, 596–601.

Scheinberg, M.A., Santos, L.M., Finkelstein, A.E., 1982. The effect of auranofin and sodium aurothiomalate on peripheral blood monocytes. J. Rheumatol. 9, 366–369.

Sheffler, L.A., Wink, D.A., Melillo, G., Cox, G.W., 1995. Exogenous nitric oxide regulates IFN-γ plus lipopolysaccharide-induced nitric oxide synthase expression in mouse macrophages. J. Immunol. 155, 886–894.

Stadler, J., Stefanovic-Racic, M., Billiar, T.R., Curran, R.D., McIntyre, L.A., Georgescu, H.I., Simmons, R.L., Evans, C.H., 1991. Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. J. Immunol. 147, 3915–3920.

Stefanovic-Racic, M., Stadler, J., Georgescue, H.I., Evans, C.H., 1994. Nitric oxide synthesis and its regulation by rabbit synoviocytes. J. Rheumatol. 21, 1892–1898.

Ueki, Y., Miyake, S., Tominaga, Y., Eguchi, K., 1996. Increased nitric oxide levels in patients with rheumatoid arthritis. J. Rheumatol. 23, 230–236.

Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q.W., Nathan, C.F., 1993. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. J. Exp. Med. 178, 605–613.

Vodovotz, Y., Kwon, N.S., Pospischil, M., Manning, J., Paik, J., Nathan, C., 1994. Inactivation of nitric oxide synthase after prolonged incubation of mouse macrophages with IFN-γ and bacterial lipopolysaccharide. J. Immunol. 152, 4110–4118.

Wang, C., Smith, R.L., 1975. Lowry determination of protein in the presence of Triton X-100. Anal. Biochem. 63, 414–417.

Wang, M.H., Cox, G.W., Yoshimura, T., Sheffler, L.A., Skeel, A., Leonard, E.J., 1994. Macrophage-stimulating protein inhibits induction of nitric oxide production by endotoxin- or cytokine-stimulated mouse macrophages. J. Biol. Chem. 269, 14027–14031.

Ward, J.R., Williams, H.J., Egger, M.J., Reading, J.C., Boyce, E., Altz-Smith, M., Samuelson, C.O. Jr., Willkens, R.F., Solsky, M.A., Hayes, S.P., Blocka, K.L., Weinstein, A., Meenan, R.F., Guttadauria, M., Kaplan, S.B., Klippel, J., 1983. Comparison of auranofin, gold sodium thiomalate, and placebo in the treatment of rheumatoid arthritis: A controlled clinical trial. Arthritis Rheum. 26, 1303–1315.

Wenger, M.E., Alexander, S., Bland, J.H., Blechman, W.J., 1983. Auranofin versus placebo in the treatment of rheumatoid arthritis. Am. J. Med. 75, 123–127.

Wright, C.D., Mulsch, A., Busse, R., Osswald, H., 1989. Generation of nitric oxide by human neutrophils. Biochem. Biophys. Res. Commun. 160, 813–819.

Xie, Q.W., Whisnant, R., Nathan, C., 1993. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. J. Exp. Med. 177, 1779–1784.

Xie, Q.W., Kashiwabara, Y., Nathan, C., 1994. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J. Biol. Chem. 269, 4705–4708.

Yamada, M., Niki, H., Yamashita, M., Mue, S., Ohuchi, K., 1997. Prostaglandin E_2 production dependent upon cyclooxygenase-1 and cyclooxygenase-2 and its contradictory modulation by auranofin in rat peritoneal macrophages. J. Pharmacol. Exp. Ther. 281, 1005–1012.

Yamashita, M., Niki, H., Yamada, M., Watanabe-Kobayashi, M., Mue, S., Ohuchi, K., 1997. Dual effects of auranofin on prostaglandin E_2 production by rat peritoneal macrophages. Eur. J. Pharmacol. 325, 221–227.

Yang, J.P., Merin, J.P., Nakano, T., Kato, T., Kitade, Y., Okamoto, T., 1995. Inhibition of the DNA-binding activity of NF-κB by gold compounds in vitro. FEBS Lett. 361, 89–96.