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The triterpenoid quinonemethide pristimerin inhibits induction of inducible nitric oxide synthase in murine macrophages

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Abstract

Inducible nitric oxide synthase dependent production of nitric oxide (NO) plays an important role in inflammation. We investigated whether pristimerin ((20α)-3-hydroxy-2-oxo-24-*nor*-friedela-1(10),3,5,7-tetraen-carboxylic acid-(29)-methylester), an antitumoral, antimicrobial as well as anti-inflammatory plant compound, has an effect on the inducible NO synthase system in lipopolysaccharide-activated RAW 264.7 macrophages. Pristimerin dose dependently (IC_{50}: 0.2–0.3 μM) reduces nitrite accumulation, a parameter for NO synthesis, in supernatants of lipopolysaccharide-stimulated (1 μg/ml, 20 h) macrophages. This effect correlates with a reduced inducible NO synthase enzyme activity measured by conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline and significantly lower levels of enzyme protein (Western blotting) in homogenates of cells cotreated with lipopolysaccharide and pristimerin (12 h). Northern blot analysis and polymerase chain reaction (PCR) showed decreased inducible NO synthase mRNA levels in activated macrophages exposed to pristimerin (4 h). Electrophoretic mobility shift assay (EMSA) demonstrated a markedly reduced binding activity of nuclear factor-kappa B (NFκB) in nuclear extracts of pristimerin-treated cells. These results suggest that pristimerin inhibits the induction of inducible NO synthase by a mechanism which involves inhibition of NFκB activation. This feature of pristimerin is likely to contribute to its anti-inflammatory activity. © 1997 Elsevier Science B.V.

Keywords: Inflammation; NO synthase, inducible ; Pristimerin; Triterpene; RAW 264.7 macrophages

1. Introduction

There is increasing evidence that nitric oxide (NO) plays a complex role in the modulation of the inflammatory response (for review see Clancy and Abramson, 1995; Miller and Grisham, 1995; Appleton et al., 1996). Pro-inflammatory properties of NO are attributed to an excessive production of NO by the inducible isoform of NO synthase and are influenced by factors like interaction of NO with other oxidants (e.g. peroxynitrite formation with superoxide anion), duration of NO production and substrate availability. Studies have shown that especially the chronic phase of inflammation correlates with an increase of inducible NO synthase activity (Miller and Grisham, 1995; Appleton et al., 1996). The most conclusive evidence for NO as a mediator of tissue injury has been in arthritis, based on studies in animal models, human osteoarthritis and rheumatoid arthritis (reviewed by Cochran et al., 1996).

The plant compound pristimerin ((20α)-3-hydroxy-2-oxo-24-*nor*-friedela-1(10),3,5,7-tetraen-carboxylic acid-(29)-methylester), is a representative of the triterpenoid quinonemethides, orange pigments, which in nature are found in the plant families *Celastraceae* and *Hippocrateaceae*. Pristimerin is known to exhibit antitumoral, antimicrobial and anti-inflammatory properties (Gunatilaka, 1996; Dirsch et al., 1992). Extracts containing triterpenoid quinonemethides are used widely in traditional medicine (Gunatilaka, 1996). In addition, pristimerin and related compounds had been isolated as active compounds from a phytopharmaceutical against rheumatoid arthritis (Dirsch et al., 1992). However, the mechanism of action of these compounds is still unknown.

We hypothesized that pristimerin might develop its anti-inflammatory/antirheumatic properties *via* an interference with the inducible NO system. Thus, the aim of the present study was, first, to examine the effect of pristimerin on the NO synthesis in lipopolysaccharide-activated

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macrophages and secondly, to focus on the underlying mechanism of action leading to the effect of pristimerin on the inducible NO synthase.

2. Materials and methods

2.1. Cell culture

RAW 264.7 cells obtained from the American Type Culture Collection (ATCC, TIB 71, Maryland), were cultured in Dulbecco's Modified Essential medium with 4 mM L-glutamine and 4.5 g/l glucose (DMEM, endotoxin level < 0.005 EU/ml, Bio Whittaker, Bioproducts, Heidelberg, Germany), supplemented with 10% heat-inactivated fetal calf serum (Gibco/BRL Life Technologies, Eggenstein, Germany). Cells were maintained at 37°C, 5% CO_2 and used for experiments between passage 5 and 20. In general, confluent cells were stimulated with 1 μg/ml lipopolysaccharide (*E. coli*, Serotype 055:B5 Sigma, Deisenhofen, Germany). Pristimerin (a gift from Dr. A.G. González, University of La Laguna, Tenerife, Spain). was dissolved in dimethyl sulfoxide (DMSO) and further diluted in medium. Final DMSO concentration in the cell supernatant was always < 0.1%, which had been examined not to interfere with the test systems used.

2.2. Nitrite assay

Cells were seeded in 96 well plates (8×10^4/200 μl), cultured for two days and then incubated with or without lipopolysaccharide in the absence or presence of pristimerin in various concentrations (0.1–1 μM) for 20 h. As an indicator of NO synthesis nitrite concentration was assessed in the supernatant of RAW 264.7 macrophages by the Griess reaction (Green et al., 1982). Experiments were performed 3 times in triplicates.

2.3. Cell viability

Cell respiration, an indicator of cell viability, was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan (Mosmann, 1983). Alternatively, cytotoxicity of pristimerin was examined using the sulforhodamine B colorimetric assay (Monks et al., 1991).

2.4. Inducible NO synthase enzyme activity

Macrophages, grown in cell culture flasks (75 cm^2) to confluence were incubated with or without lipopolysaccharide in the absence or presence of pristimerin (1 μM) for 12 h, washed three times with cold phosphate-buffered saline, frozen immediately at −70°C and stored until NO synthase activity was measured. NO synthase activity was determined by measuring conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in principle as described previously (Thiemermann et al., 1993). Briefly, cells were homogenized in 50 mM Tris pH 7.6 containing EDTA (0.1 mM), EGTA (0.1 mM) and the protease inhibitor phenylmethylsulfonyl fluoride (1 mM). Homogenates (100 μl) were incubated in triplicate at 37°C for 30 min with L-arginine (10 μM), NADPH (1 mM), L-valine (50 mM) and [^{3}H]L-arginine (0.2 μCi; 0.033 μM). Reactions were stopped by adding 1 ml cold sodium acetate pH 5.5 (20 mM) containing EDTA (2 mM) and L-citrulline (0.1 mM). Reaction mixtures were separated by ion exchange on Dowex 50W (Na^+ form) columns and the eluted [^{3}H]L-citrulline activity was quantified by scintillation counting (Beckmann, LS1801, Fullerton, CA, USA). For measuring specific enzyme activity, homogenates of lipopolysaccharide-stimulated macrophages were preincubated with the test substance (4°C, 10 min) and subsequently processed as described above. Protein concentration in the homogenates was determined by the Bradford protein assay (Bio-Rad, Munich, Germany).

2.5. Western blot analysis

Macrophages, grown in 6-well plates to confluence were incubated with or without lipopolysaccharide in the absence or presence of pristimerin (0.5 μM) for 12 h. Cells were washed with ice-cold phosphate-buffered saline and stored at −70°C until further analysis. Western blot analysis was performed according to Rothe et al. (1996) using a monoclonal mouse antibody against inducible NO synthase (Transduction Laboratories, Lexington, USA) and the ECL detection system (Amersham, Braunschweig, Germany). Signal intensities were evaluated by densitometric analysis (EASY plus system, Herolab, Wiesloch, Germany).

2.6. RNA extraction and Northern blot analysis

Confluent macrophages (75 cm^2 flasks) were incubated with or without lipopolysaccharide in the absence or presence of pristimerin (1 μM) or dexamethasone (10 μM) for 4 h. RNA extraction and Northern blot of mRNA was performed according to Vollmar and Schulz (1994). Membranes were hybridized to a ^{32}P-labeled murine macrophage inducible NO synthase cRNA probe (2×10^6 cpm/ml). The inducible NO synthase probe was labeled with [^{32}P]-UTP (50 μCi) using a T3 RNA polymerase transcription system (Stratagene, Heidelberg, Germany) and a HindIII linearized 558 bp inducible NO synthase cDNA subcloned in a pBluescript SK(+) vector (a gift from Dr. Kleinert, University of Mainz, Germany). The evaluation of the signal intensity was performed by densitometric analysis. To control for loading of mRNA, membranes were rehybridized with a ^{32}P-labeled β-actin probe (2×10^6 cpm/ml). To look for cyclooxygenase 2 mRNA expression, membranes were rehybridized with a ^{32}P-labeled cyclooxygenase 2 probe (2×10^6 cpm/ml). The cRNA

probe was a 2.3 kb fragment of a cDNA (TIS10) subcloned in a pGEM-7 vector (obtained from Dr. Herschman, UCLA School of Medicine, LA) and was linearized using *Eco*RI. The probe was labeled with [^{32}P]-UTP (50 μCi) using a T7 RNA polymerase transcription system (Stratagene, Heidelberg, Germany).

2.7. Reverse transcription and polymerase chain reaction (PCR)

mRNA was isolated from about 150 μg total RNA and reverse transcribed to cDNA using the PolyATtract Series 9600 system (Promega, Heidelberg, Germany). Amplification of inducible NO synthase cDNA was performed by incubating 10 ng of cDNA template in a reaction volume of 100 μl PCR buffer containing 5.0 μg bovine serum albumin, 1.5 mM $MgCl_2$, 1.0 mM dNTPs, 0.6 μg of each primer and 2.5 units of *Taq* DNA polymerase (Promega, Heidelberg, Germany) for 30 cycles of 1 min denaturation at 94°C, 1.5 min annealing at 60°C and 2 min of extension at 72°C. Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany) and had the following sequences: 5′-GATCAGGAACCTGAAGCCCC-3′ (sense) and 5′-CCTATGGGGCAAAAAAGGGC-3′ (antisense) according to Tsujino et al. (1994). To control equal template concentrations and amplification efficiency a cDNA sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using the following primers: 5′-TCACTCAAGATTGTCAGCAA-3′ (sense) and 5′-AGATCCACGACGGACACATT-3′ (antisense) (MWG-Biotech Ebersberg, Germany). PCR products were analysed on 2.5% agarose gels and stained with ethidium bromide. Evaluation of the PCR product levels was done by densitometric analysis. Experiments were performed in duplicate and repeated twice.

2.8. Electrophoretic mobiliy shift assay (EMSA)

Macrophages, grown in 6 well plates were incubated with or without lipopolysaccharide in the absence or presence of pristimerin (0.5 and 1 μM) or pyrrolidinedithiocarbamate (50 μM, Calbiochem, Bad Soden, Germany) for 3 h. Nuclear extracts were prepared as described previously (Schreiber et al., 1989). Briefly, cells were washed with phosphate-buffered saline, cell pellets resuspended in 400 μl ice cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride), kept on ice for 15 min, after which 25 μl of Nonidet NP-40 (10%) was added. Tubes were vigorously vortexed for 10 s and the homogenate centrifuged (30 s/10 000 × *g*). The pellet was resuspended in 50 μl buffer B (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM phenylmethylsulfonyl fluoride) and vigorously rocked for 15 min (4°C). The nuclear extract was centrifuged (5 min/10 000 × *g*) and after determination of protein concentration (Lowry) aliquots were either frozen at −70°C or immediately used for EMSA as described previously (Boese et al., 1996). Briefly, a double-stranded oligonucleotide containing the most common nuclear factor-kappa B (NFκB) consensus sequence (22mer, Promega, Heidelberg, Germany) was end-labeled with γ-^{32}P-ATP (300 Ci/mmol; Hartmann, Braunschweig, Germany) using the T4 polynucleotide kinase (Promega). Binding reactions were performed incubating 50 000–100 000 cpm ^{32}P-labeled DNA with nuclear protein extract (10 μg) in a final volume of 15 μl buffer (5 mM HEPES pH 7.5; 100 mM NaCl; 1 mM DTT; 5% glycerol, 1 mM EDTA; 1 μg poly dI-dC (Promega)) for 30 min (22°C). The mixture was electrophoresed on a 4.5% non-denaturing polyacrylamide gel (100 V) and the gel was exposed to an X-ray film overnight. Signal intensities were evaluated by densitometry.

2.9. Statistical analysis

Each experiment was performed at least three times. Results are expressed as the mean value ± S.D. Statistical analysis was performed using an unpaired nonparametric Mann–Whitney test or an unpaired two-tailed Student's *t*-test. P values < 0.05 were considered significant.

3. Results

In order to investigate whether NO synthesis of lipopolysaccharide-stimulated RAW 264.7 macrophages was altered by pristimerin, we determined nitrite concentration, an indicator of NO synthesis, in the cell culture supernatant. Fig. 1 shows that pristimerin (0.1–1 μM) dose dependently inhibited nitrite accumulation (20 h) in RAW 264.7 macrophages stimulated with 1 μg/ml lipopolysaccharide (IC_{50} approx. 0.2–0.3 μM).

The observed effect was not due to a potential cytotoxicity of pristimerin, since cell viability was examined after pristimerin treatment using the MTT assay or, alternatively, the protein stain sulforhodamine B. Under the conditions used for the Griess assay pristimerin showed no impairment of cell viability, except at a concentration of 1 μM (MTT: approx. 37%; sulforhodamine B: approx. 20%). Interestingly, 1 μM pristimerin showed no cytotoxicity when added to cells cultivated in cell culture flasks (75 cm^2)(data not shown).

To get a first clue whether the reduction of nitrite accumulation by pristimerin is a result from inhibition of inducible NO synthase expression or from inhibition of its enzymatic activity, time dependency of the pristimerin effect was compared with that of N^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of NO synthase enzyme activity. Pristimerin and L-NMMA were incubated simultaneously with lipopolysaccharide and at different time points after lipopolysaccharide stimulation. Fig. 2

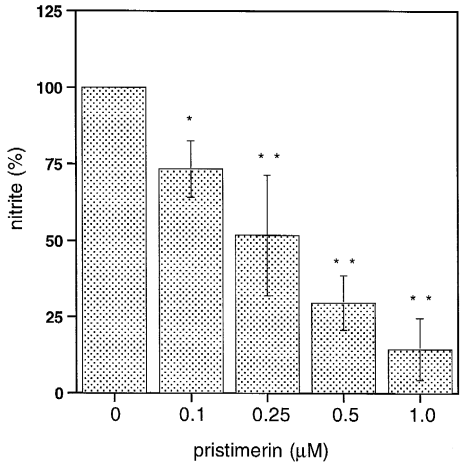

Fig. 1. Dose-dependent inhibition of nitrite accumulation in the cell culture supernatants of lipopolysaccharide-stimulated RAW 264.7 macrophages by pristimerin. RAW 264.7 macrophages were stimulated with lipopolysaccharide (1 μg/ml) for 20 h. Pristimerin was added simultaneously with lipopolysaccharide in various concentrations (0.1–1 μM). Nitrite concentration was determined by the Griess reaction as described under Section 2. Values are given in % of the nitrite concentration detected in the supernatants of stimulated cells (100%). Bars represent mean (±S.D.) of at least three independent experiments, each performed in triplicate. * $P < 0.05$, ** $P < 0.01$ (Mann–Whitney).

shows that L-NMMA inhibited nitrite accumulation virtually equally when added simultaneously with lipopolysaccharide or up to 6 h after lipopolysaccharide stimulation, a time when inducible NO synthase is already induced. In contrast, pristimerin exhibited its full inhibitory activity only when added simultaneously with lipopolysaccharide, suggesting that pristimerin does not exhibit a direct effect on the enzymatic activity of inducible NO synthase.

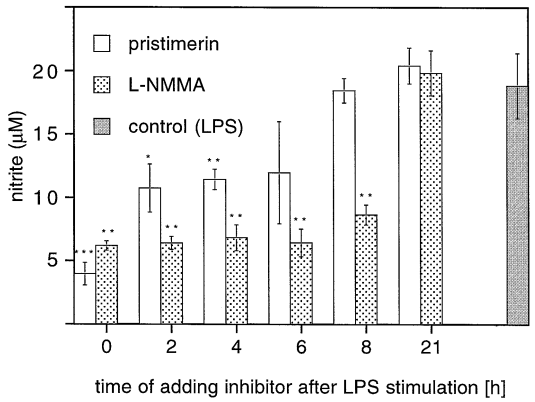

Fig. 2. Time-dependency of inhibition of nitrite accumulation by pristimerin and L-NMMA. RAW 264.7 were stimulated with lipopolysaccharide (1μg/ml) (control). Pristimerin (0.5 μM) or L-NMMA (100 μM) was added to the cell culture at different time points after lipopolysaccharide stimulation. Nitrite accumulation in the supernatants was measured 22 h after lipopolysaccharide stimulation by the Griess reaction as described under Section 2. The figure shows the result of one representative experiment out of three, performed in triplicate (mean±S.D.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test; compared to control).

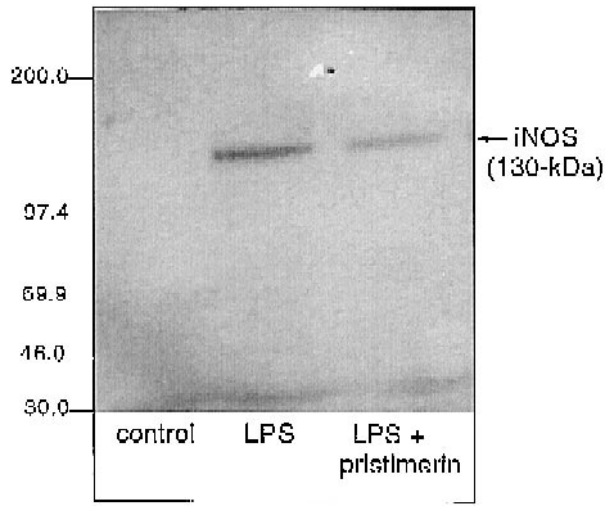

Fig. 3. Western blot analysis of inducible NO synthase in lipopolysaccharide-activated RAW 264.7 macrophages. By use of a specific antibody, the presence of the 130-kDa inducible NO synthase protein was evaluated in the cytosol of cells either untreated (control), stimulated with lipopolysaccharide (1 μg/ml) or cotreated with lipopolysaccharide (1 μg/ml) and pristimerin (0.5 μM) for 12 h. A representative blot out of three experiments with similar results is shown.

Corroboration of the above deduction that pristimerin does not affect *specific enzyme activity* was obtained by exposing homogenates of lipopolysaccharide-activated macrophages to pristimerin (1 μM) followed by measuring inducible NO synthase enzyme activity. As a control L-NMMA (1 mM), a known specific inhibitor of NO synthase enzyme activity was employed using the same protocol. L-NMMA inhibited inducible NO synthase enzyme activity more than 90%. In contrast, pristimerin showed no effect. On the other hand, when enzyme activity was measured in homogenates of macrophages stimulated with lipopolysaccharide and treated with pristimerin (1 μM) for 12 h, a significant inhibition of inducible NO synthase enzyme activity was detectable compared to the activity measured in homogenates of only lipopolysaccharide-activated cells (76.2 ± 18.8%; mean ± S.D. of three independent experiments each performed in triplicates). These findings suggest a decrease in inducible NO synthase protein concentration in cells exposed to pristimerin.

Inducible NO synthase protein levels were determined using Western blot analysis. Fig. 3 shows that lipopolysaccharide-activated macrophages exposed to pristimerin (0.5 μM, 12 h) contained significantly lower levels of the inducible NO synthase protein (130 kDa) compared to macrophages treated with lipopolysaccharide only.

Reduced mRNA expression of inducible NO synthase might be responsible for diminished inducible NO synthase concentration in pristimerin treated cells. Therefore, the level of inducible NO synthase mRNA was evaluated by reverse-transcriptase-PCR (RT-PCR) as well as Northern blot analysis. Both methods, RT-PCR (Fig. 4) and Northern blot analysis (Fig. 5) demonstrated that pris-

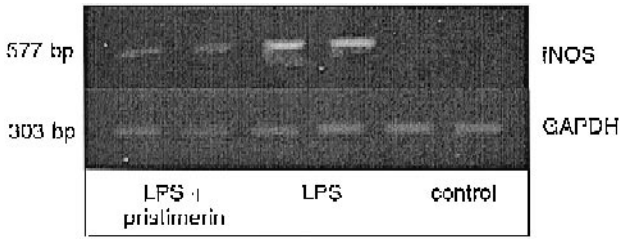

Fig. 4. A representative RT-PCR of inducible NO synthase mRNA in RAW 264.7 macrophages. cDNA (10 ng) of macrophages either not treated (control), stimulated with lipopolysaccharide (1 μg/ml) or cotreated with lipopolysaccharide (1 μg/ml) and pristimerin (0.5 μM) for 4 h were subjected to PCR in dublicate. Inducible NO synthase specific sequences (577 bp) were detected by staining the gel (2.5% agarose) with ethidium bromide. PCR of GAPDH transcripts was performed to control for similar initial cDNA content of samples. The experiment was repeated twice with similar results.

timerin reduces steady-state mRNA levels of inducible NO synthase in lipopolysaccharide-activated RAW 264.7 macrophages markedly. For control reason, levels of inducible NO synthase mRNA in lipopolysaccharide-activated macrophages treated with dexamethasone (10 μM), which is known to inhibit the induction of inducible NO synthase (Kleinert et al., 1996), were analysed. Densitometric evaluation of a representative Northern blot shown in Fig. 5 revealed a reduction of inducible NO synthase mRNA of 55% and 93% in cells exposed to pristimerin (1 μM) and dexamethasone (10 μM), respectively, compared to the amount of inducible NO synthase mRNA present in

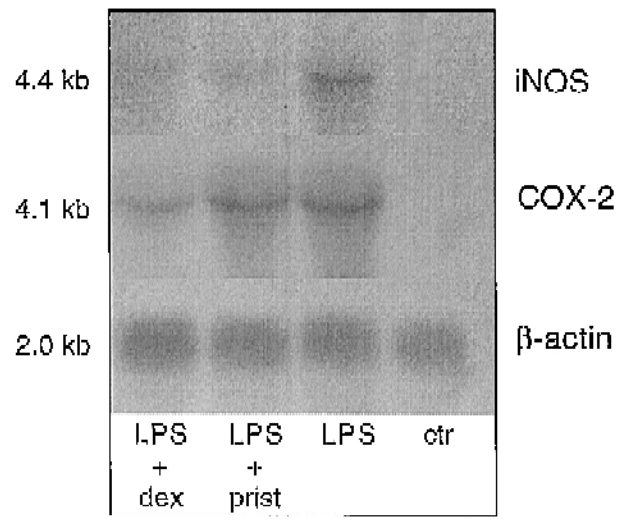

Fig. 5. Representative Northern blot analysis of inducible NO synthase and cyclooxygenase 2 mRNA. Total RNA was isolated from macrophages, which were unstimulated (control), stimulated with lipopolysaccharide (1 μg/ml), cotreated with lipopolysaccharide (1 μg/ml) and pristimerin (1 μM) or cotreated with lipopolysaccharide (1 μg/ml) and dexamethasone (10 μM) for 4 h. Blotted RNA (20 μg) was hybridized using ^{32}P-labeled cRNA probes for either inducible NO synthase, cyclooxygenase 2 or β-actin mRNA. Films were exposed for 5 (inducible NO synthase), 4 (cyclooxygenase 2) and 1 (β-actin) day(s), respectively. Similar results were obtained in two separate experiments.

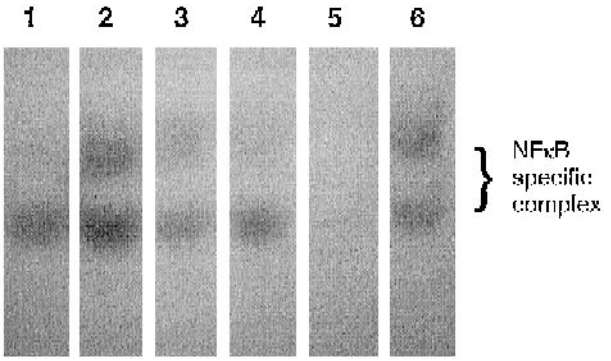

Fig. 6. Effect of pristimerin (0.5 μM) and pyrrolidinedithiocarbamate (50 μM) on the NFκB binding activity in nuclear extracts of RAW 264.7 cells activated with lipopolysaccharide (1 μg/ml) for 3 h. Equal amounts of nuclear extracts (10 μg) were subjected to EMSA as described in Section 2. Lanes: (1) untreated cells; (2) cells activated with lipopolysaccharide; (3) cells pretreated with pyrrolidinedithiocarbamate (50 μM) for 2 h and then activated with lipopolysaccharide; (4) cells treated with pristimerin (0.5 μM) and lipopolysaccharide; (5) nuclear extract of lipopolysaccharide-activated cells incubated with a 100-fold excess of unlabeled NFκB consensus oligonucleotide (unlabeled competitor); (6) nuclear extract of lipopolysaccharide-activated cells incubated with a 100-fold excess of unlabeled AP-2 consensus oligonucleotide (unlabeled noncompetitor).

lipopolysaccharide-activated cells. Inducible NO synthase signals were corrected for different RNA loading based on the signals obtained from β-actin mRNA.

To examine whether pristimerin (1 μM) selectively reduces inducible NO synthase mRNA expression or whether it also affects steady-state mRNA levels of cyclooxygenase 2 Northern blots were rehybridized with a cRNA probe for cyclooxygenase 2. Pristimerin did not diminish mRNA level of cyclooxygenase 2 in activated RAW 264.7 macrophages (Fig. 5). In contrast, dexamethasone decreased cyclooxygenase 2 mRNA levels by about 70%.

It was shown that activation of the transcription factor nuclear factor-kappa B (NFκB) is necessary to induce inducible NO synthase in murine macrophages activated with lipopolysaccharide (Xie et al., 1994). Thus, using the electrophoretic mobility shift assay (EMSA) binding activity of NFκB in nuclear extracts of lipopolysaccharide-activated and pristimerin (0.5 μM) treated cells was studied. As a control, NFκB binding activity was also investigated in activated RAW 264.7 macrophages treated with pyrrolidinedithiocarbamate (50 μM), a specific inhibitor of NFκB. Fig. 6, showing a representative EMSA, reveals that pristimerin reduces NFκB binding activity compared to the lipopolysaccharide control. The extent of reduction was in a similar range (around 60%) as those of inducible NO synthase protein and mRNA expression. For control purpose, binding activity of interferon regulatory factor-1 (IRF-1), a transcription factor required for interferon-γ-dependent transcriptional regulation of inducible NO synthase in RAW 264.7 macrophages (Martin et al., 1994)

was examined. No IRF-1/DNA complex formation could be demonstrated in lipopolysaccharide-treated RAW 264.7 cells (data not shown).

4. Discussion

The present study suggests that pristimerin is able to inhibit the induction of inducible NO synthase by reducing NFκB binding activity in lipopolysaccharide-activated murine macrophages. This notion is based on the following lines of evidence: (i) reduction of nitrite accumulation in cell supernatants was highest when pristimerin was added simultaneously with lipopolysaccharide; a delayed addition resulted in a decreased effect. (ii) Pristimerin does not show a direct effect on the enzymatic activity of inducible NO synthase. However, homogenates of cells cotreated with lipopolysaccharide and pristimerin displayed a reduced inducible NO synthase enzyme activity compared to lipopolysaccharide-stimulated control cells. (iii) Western blot analyses demonstrated markedly reduced levels of inducible NO synthase protein in lipopolysaccharide-activated cells treated with pristimerin as compared to untreated cells. (iv) mRNA analysis by Northern blot and RT-PCR displayed a lower expression of inducible NO synthase mRNA in RAW 264.7 macrophages exposed to pristimerin. (v) NFκB binding activity was shown to be reduced in nuclear extracts of pristimerin treated cells.

In contrast to the constitutively expressed NO synthases, inducible NO synthase is regulated primarily at the transcriptional level (for review see Förstermann and Kleinert, 1995). Thus, substances which interfere with the induction of inducible NO synthase are likely not to affect constitutively expressed NO synthases. This is of considerable importance since inducible NO synthase dependent over-production of NO is considered as pro-inflammatory whereas protective and anti-inflammatory properties of NO are mainly attributed to the constitutively expressed NO synthase system (Clancy and Abramson, 1995). In this respect, NO synthase inhibitors for the treatment of NO mediated inflammatory processes require high specificity for inducible NO synthase and thus, inhibitors of inducible NO synthase induction may be safe modulators of NO for various pathological conditions.

We used the murine macrophage-like cell line RAW 264.7 stimulated with lipopolysaccharide as cell model for the inducible NO synthase system, since quite detailed information about the mechanism of inducible NO synthase regulation are available for this cell model (Stuehr et al., 1991; Nathan and Xie, 1994). Xie et al. (1994) demonstrated in RAW 264.7 macrophages that activation of transcription factor NFκB is essential for the induction of inducible NO synthase after lipopolysaccharide stimulation. They showed that pyrrolidinedithiocarbamate, a specific inhibitor of NFκB activation in lipopolysaccharide-treated macrophages, blocked both the ability of lipopolysaccharide to induce binding activity for NFκB and the ability of macrophages to produce nitrite in response to lipopolysaccharide. This result was confirmed by own experiments (data not shown). Togashi et al. (1997), however, demonstrated that pyrrolidinedithiocarbamate reduces nitrite levels in cell culture supernatants via a NO scavenging effect. Thus, NO synthase activity after pyrrolidinedithiocarbamate treatment may be underestimated when determined by measuring nitrite accumulation. Importantly, the present study demonstrated that pristimerin inhibits NFκB activation as well as inducible NO synthase induction in lipopolysaccharide-activated RAW 264.7 macrophages. This results give a strong base for the suggestion that pristimerin inhibits induction of inducible NO synthase by a mechanism which involves inhibition of NFκB activation. However, effects of pristimerin on post-transcriptional, translational or posttranslational processes can not be ruled out at that point.

NFκB is a redox-sensitive transcription factor which plays a key role in the regulation of numerous genes involved in pathogen responses and cellular defence mechanisms. Genes encoding e.g. cytokines, cytokine receptors or cell adhesion molecules contain functional NFκB binding sites in their promoter regions (Schulze-Osthoff et al., 1995). Thus, besides inducible NO synthase pristimerin may affect various other gene products which are regulated by NFκB.

In addition to the inducible NO synthase system, first investigations regarding cyclooxygenase 2, an other inducible enzyme which plays an important role in the inflammatory response, were undertaken. We could demonstrate that pristimerin does not reduce the steady-state mRNA level of cyclooxygenase 2 in lipopolysaccharide-activated RAW 264.7 macrophages. The murine cyclooxygenase 2 promoter was shown to contain a functional NFκB binding site, since Yamamoto et al. (1995) demonstrated that a point mutation in an NFκB motif of the cyclooxygenase 2 promoter in the mouse osteoblastic cell line MC3T3-E1 reduced tumor necrosis factor α-induced promoter activity markedly. The different effect of pristimerin on the mRNA expression of inducible NO synthase and cyclooxygenase 2, respectively, might be the result of a different usage of the NFκB responsive element of the murine cyclooxygenase 2 promoter in different cell lines. Pristimerin might also interact with NFκB heterodimers which bind functionally to the NFκB responsive element of the inducible NO synthase but not of the cyclooxygenase 2 promoter.

We were able to demonstrate that pristimerin at low concentrations (IC_{50}: 0.2–0.3 μM) reduces the induction of inducible NO synthase, a high-output enzyme for NO production possibly via inhibiting NFκB activation. High amounts of NO and especially its derivative peroxynitrite, are recognized to be deleterious in the process of chronic inflammation. The findings presented here might therefore provide a scientific base for the use of plant extracts

containing pristimerin and related compounds against inflammatory diseases in phytomedicine.

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