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Synthetic cryptolepine inhibits DNA binding of NF-κB

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Abstract—The alkaloid cryptolepine is thought to mediate the anti-inflammatory effects of the climbing shrub, *Cryptolepis sanguinoleta*. The underlying mechanism of action, however, is largely unknown. In the present study, we show that the synthetic cryptolepine-hydrochloride (2.5–10 μM) dose-dependently inhibits lipopolysaccharide (LPS)-induced nitric oxide production in the murine macrophage cell line RAW 264.7. We furthermore demonstrate a strong inhibition of nuclear factor (NF)-κB, a transcription factor primarily involved in inflammatory and immune responses, by cryptolepine (2.5–10 μM) using a luciferase reporter gene assay in human HEK 293 cells. Examining the individual steps of NF-κB activation in the presence of cryptolepine we could exclude an inhibitory effect on degradation of IκB or nuclear translocation of NF-κB by the alkaloid. However, EMSA of nuclear extracts from LPS-activated RAW cells revealed reduced DNA binding activity of NF-κB by cryptolepine in vivo and in vitro. This indicates that cryptolepine may exhibit its anti-inflammatory action by blocking DNA binding of activated NF-κB and thus transcription of NF-κB-regulated proinflammatory proteins.

1. Introduction

Cryptolepine (5-methyl, 10*H*-indolo[3,2-*b*]quinoline) is an indoloquinoline alkaloid found in the West African climbing shrub *Cryptolepis sanguinoleta* (Family Asclepiadaceae). The decoction of the roots, sold locally in Guinea Bissau, is used in the treatment of hepatitis, while the leaves are used for the treatment of malaria.[1] The extracts of the roots and stems are employed clinically in the treatment of malaria, urinary and respiratory tract infections.[2] Ethnobotanical and field studies have also reported the use of the plant against fungal infections, pain and inflammation.[3]

Cryptolepine was first isolated from *C. sanguinoleta* by Gellert from root samples obtained in Nigeria.[4] The compound and its hydrochloride salt have been reported to possess a wide range of biological activities, including antimalarial,[5–9] anti-inflammatory,[10,11] anti-thrombotic,[12–15] antibacterial,[16–18] and antihyperglycaemic effects.[19,3] Furthermore, cryptolepine has been suggested to produce cytotoxic effects.[20–23]

Although cryptolepine has been shown to produce anti-inflammatory activity in vivo,[10] the underlying molecular mechanism of its action is largely unknown. One study by Noamesi and Bamgbose[11] documents a cryptolepine-mediated decrease in prostaglandin production, which tempted us to hypothesize that synthetic cryptolepine-hydrochloride (from now on referred to as cryptolepine) might target the transcription factor NF-κB, and thereby impair transcription of NF-κB-regulated genes, such as cyclooxygenase-2 (COX-2) or inducible nitric oxide synthase (iNOS). To test our hypothesis, we chose nitric oxide (NO) production from LPS-activated murine RAW 264.7 macrophages as readout, since active NF-κB was shown to be necessary for iNOS induction in this system.[24] Having observed a marked cryptolepine-mediated reduction in NO production in this model we focussed on the elucidation of the detailed mechanism of action underlying the

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anti-inflammatory action of cryptolepine. We thereby revealed that the alkaloid targets NF-κB by inhibiting its DNA binding, presumably via alkylation of cysteine residues in NF-κB subunits, without affecting activation and nuclear translocation of NF-κB.

2. Results

2.1. Cryptolepine inhibits LPS-mediated NO production in RAW 264.7 macrophages

In order to determine whether cryptolepine affects NF-κB-regulated pathways, we first evaluated its effect on nitric oxide production in LPS-stimulated RAW 264.7 macrophages.[25] RAW 264.7 cells were treated with cryptolepine, immediately followed by stimulation with 1 μg/ml LPS for 20 h. Cryptolepine (5 and 10 μM) exhibited a statistically significant inhibition of NO production (measured as the stable oxidation product nitrite). Five micromolars cryptolepine led to a 30% reduction in NO production whereas 10 μM of the compound reduced NO even by 50% compared to vehicle-treated LPS-stimulated control cells (Fig. 1A). A concomitant cell toxicity assay (Crystal Violet staining of attached cells) revealed a significant, but minor (in relation to NO-inhibition), cytotoxic effect of cryptolepine in the concentrations employed (Fig. 1B).

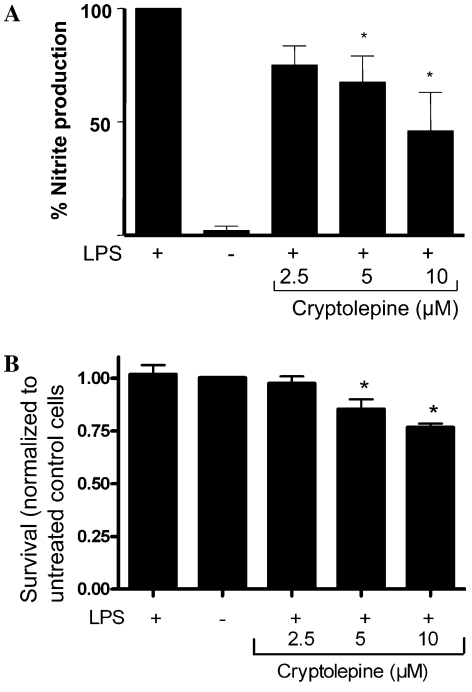

Figure 1. Cryptolepine inhibits LPS-induced NO release from RAW 264.7 macrophages. (A) RAW 264.7 cells were activated with LPS (1 μg/ml) in absence or presence of the indicated concentration of cryptolepine. Nitrite accumulation was quantified 20 h later in cell culture supernatants by the Griess reaction as described under Section 5. Results are means ± SD of six independent experiments. * significantly different from control, $P < 0.05$ (ANOVA/Dunnett). (B) Cell numbers were assessed by Crystal Violet staining as described in Section 5.

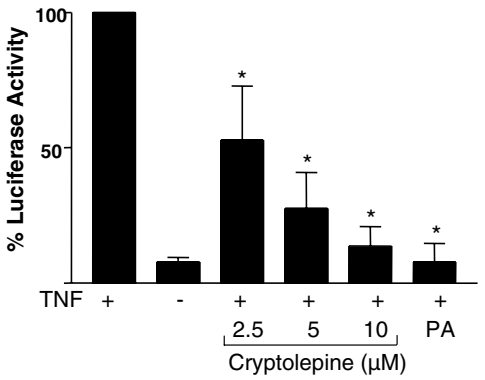

Figure 2. Cryptolepine inhibits NF-κB-mediated gene expression. HEK 293 transiently transfected with a firefly luciferase gene driven by a NF-κB-regulated promotor were either left untreated or were pretreated for 30 min with the indicated concentrations of cryptolepine before activation with TNFα (1 ng/ml) for 6 h. The cells were then lysed and extracts analyzed for luciferase activity. Parthenolide (PA, 10 μM) was used as a positive control. The data are means ± SD of at least four experiments performed in triplicate, * significantly different from control, $P < 0.05$ (ANOVA, Dunnett).

2.2. Cryptolepine inhibits NF-κB-mediated gene transcription

Activation of NF-κB leads to its translocation into the nucleus where it contributes to the expression of multiple pro-inflammatory genes, including iNOS. Prompted by the observation that cryptolepine inhibits nitric oxide production in LPS-activated RAW 264.7 macrophages, we decided to quantify the suppressive effect of the alkaloid on NF-κB-dependent gene expression in general. For this, a plasmid construct bearing a luciferase reporter gene under the control of NF-κB was transiently transfected into HEK 293 cells. Treatment of transfected cells with TNF-α caused activation of the NF-κB-driven luciferase expression which was significantly ($P < 0.05$) inhibited by cryptolepine in a concentration-dependent manner (2.5–10 μM), as shown in Figure 2. As a positive control for inhibition of NF-κB activity we included parthenolide, a sesquiterpene lactone from *Tanacetum parthenium* whose mode of action has been extensively studied,[26–29] in this and the following experiments.

2.3. Cryptolepine does not interfere with phosphorylation and degradation of IκB

Encouraged by the dose-dependent inhibition of NF-κB activity by cryptolepine, we next focussed on the individual steps in the activation cascade of NF-κB and their susceptibility to cryptolepine action. One of the first responses of macrophages to a pro-inflammatory stimulus such as LPS or TNF-α is phosphorylation and degradation of IκB, the inhibitor of NF-κB, that sequesters the NF-κB in the cytosol under resting conditions.[30] Using Western blot analysis, we could demonstrate that cryptolepine does not interfere with IκB phosphorylation (Fig. 3A) or with IκB degradation (Fig. 3B). In both untreated and cryptolepine-treated cells IκB is phosphorylated 1–5 min upon LPS exposure and degraded after 15 min. Whereas untreated cells

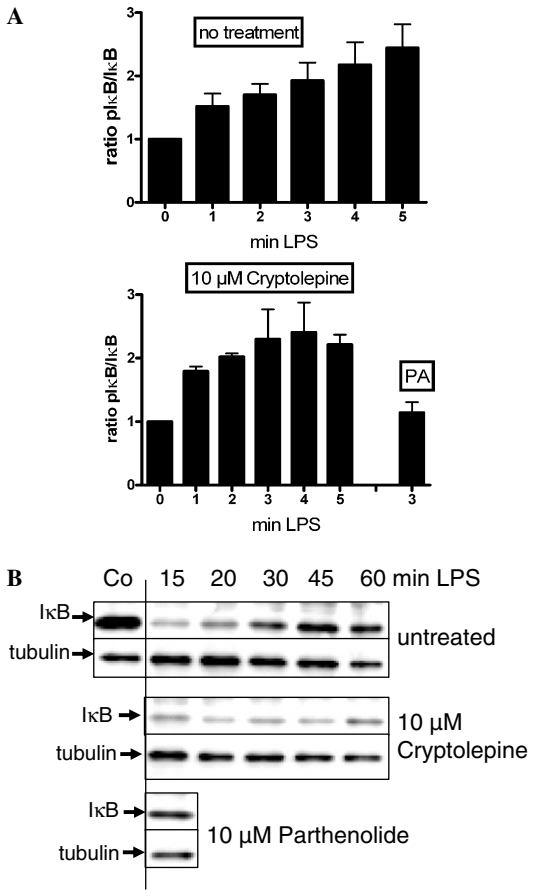

Figure 3. Cryptolepine does not interfere with IκB phosphorylation and degradation. (A) RAW 264.7 macrophages were grown in 6-well plates and pretreated with cryptolepine (10 μM) or parthenolide (PA; 10 μM) for 30 min before they were stimulated with LPS (1 μg/ml) for the indicated periods of time. Cell lysates were subjected to a Western blot analysis for phospho-IκB and subsequently for IκB after stripping of the membranes. Densitometric analyses of the membranes were performed with AIDA software. Ratios p-IκB/IκB as fold to unstimulated control cells are plotted for each time point (3 min for parthenolide) for untreated (*left panel*) and cryptolepine-treated (*right panel*) cells. The shown data present a compilation of three independent experiments and are depicted as means ± SD. (B) RAW macrophages were seeded into 6-well plates, left untreated (Control) or preincubated with 10 μM cryptolepine or parthenolide for 30 min, respectively, and then stimulated with LPS (1 μg/ml) for the indicated periods of time. Cells were lysed and lysates probed for IκB and tubulin for protein normalization. The depicted blots are representative of three independent experiments with consistent results.

almost fully resynthesize IκB within 45 min after LPS stimulus, cryptolepine-treated cells are impaired in IκB regeneration (Fig. 3B). This again stresses the inhibitory effect of cryptolepine on NF-κB action as the resynthesis of IκB, as part of a negative feedback loop, is NF-κB-dependent.[30] Parthenolide inhibited IκB phosphorylation and degradation as reported previously.[26]

2.4. Cryptolepine does not interfere with nuclear translocation of p65/NF-κB

Once freed from its inhibitor, NF-κB migrates into the nucleus in order to initiate transcription of its downstream effectors. To rule out a negative effect of cryptolepine on nuclear translocation of NF-κB, we employed confocal laser scanning microscopy and examined the nuclear translocation of the p65 subunit of NF-κB in activated RAW macrophages in the presence and absence of the alkaloid (Fig. 4). Unstimulated RAW macrophages displayed predominantly cytosolic localization of the p65 subunit of NF-κB, whereas LPS stimulation led to an increase of nuclear p65. As expected, parthenolide treatment interfered with nuclear translocation of p65, whereas cryptolepine, even at a concentration of 10 μM, did not show any inhibitory effect on the nuclear entry of p65. Therefore, cryptolepine does not affect nuclear translocation of p65/NF-κB, or most probably other members of the NF-κB family of transcription factors.

2.5. Crytolepine interferes with the DNA binding capacity of NF-κB

Next we aimed to investigate the last step of NF-κB activation, namely binding to its DNA consensus sequence. For this reason we performed EMSAs. These revealed that cryptolepine inhibits DNA binding of NF-κB in a dose-dependent manner. As shown in Figure 5A, nuclear extracts of resting RAW 264.7 cells did not contain substantial amounts of active NF-κB. Stimulation with LPS led to a massive increase of active NF-κB in the nuclear extracts and to binding to its labelled consensus sequence. NF-κB activation was markedly reduced by treatment of cells with 5 and 10 μM cryptolepine. Parthenolide (10 μM), again used as a positive control for NF-κB inhibition, also interfered with NF-κB binding activity.

In an alternative experimental setup we wanted to explore whether cryptolepine inhibited DNA binding of NF-κB via direct chemical modification, for example, alkylation of NF-κB subunits. For this, we employed isolated active NF-κB (from nuclei of LPS-stimulated macrophages) and incubated it with different concentrations of cryptolepine for 30 min before adding the radioactively labelled NF-κB consensus sequence. The gel shift revealed that cryptolepine was able to inhibit DNA binding of NF-κB in vitro at a concentration of 250 μM. An addition of an excess of DTT to the mixture of NF-κB and cryptolepine attenuated the observed in vitro inhibition (Fig. 5B). This finding is in line with a cryptolepine-mediated alkylation of cysteine residues in NF-κB which is abrogated in the case of an excess of competing thiol groups of DTT.

3. Discussion

The alkaloid cryptolepine is thought to mediate the anti-inflammatory effects of the climbing shrub, *C. sanguinoleta*, but the underlying mechanisms were not elucidated so far. In a commonly accepted in vitro model of inflammation, namely LPS-stimulated RAW 264.7 macrophages, cryptolepine displayed inhibitory activity on NO production in a dose-dependent manner with half maximal inhibitory activity at a concentration of 10 μM (Fig. 1). Since NF-κB is the main mediator of

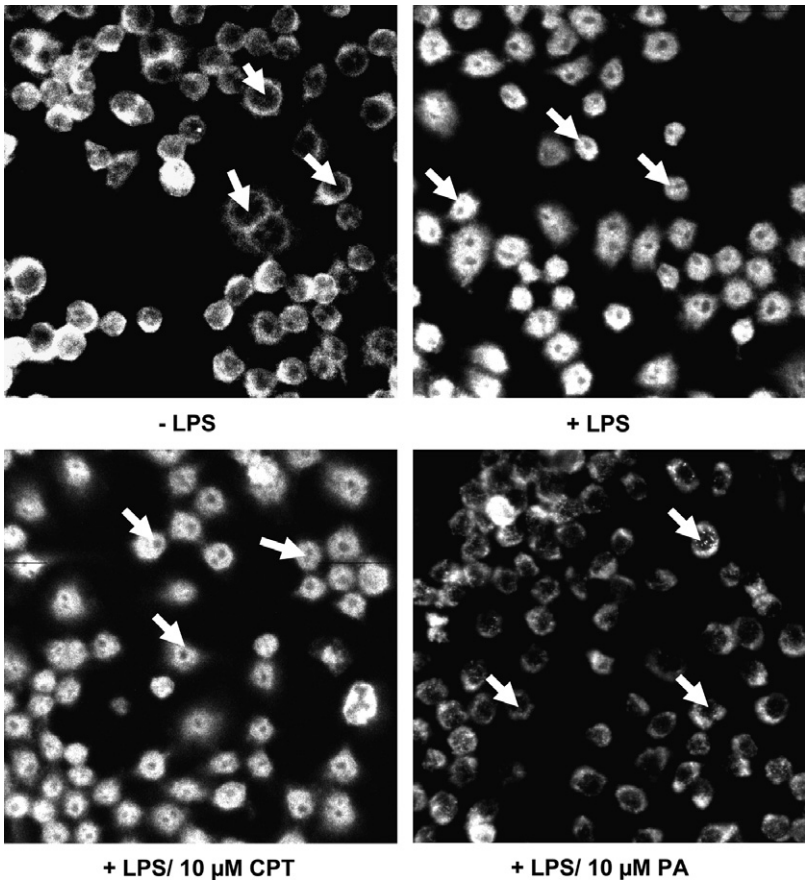

Figure 4. Cryptolepine does not affect nuclear entry of p65/NF-κB. RAW 264.7 cells were grown on coverslips, treated as indicated and described in detail in Section 5, FITC-immunostained for NF-κB/p65 and viewed under a confocal laser scanning fluorescence microscope (excitation at 488 nm). White arrows indicate the localization of the nucleus and CPT or PA stand for cryptolepine or parthenolide, respectively.

LPS-triggered iNOS, we tested the effect of cryptolepine on NF-κB-mediated gene expression. Again, cryptolepine was effectively able to block NF-κB-mediated luciferase transcription (Fig. 2). While further tracking down the immediate target of cryptolepine in the NF-κB activation pathway we could demonstrate that the alkaloid does not influence phosphorylation and degradation of IκB (Fig. 3) or the nuclear entry of NF-κB (Fig. 4), steps known to be upstream of NF-κB-mediated gene expression. Rather, cryptolepine directly acted on the DNA binding step of activated NF-κB (Fig. 5) and thus impaired the initiation of transcription of not only proinflammatory proteins, but also regulators of the NF-κB pathway such as IκB (Fig. 3). As reported for other molecules, for example, the sesquiterpene lactone helenalin,[31] cryptolepine might alkylate one of the crucial cysteine residues of p65/p50 and thereby impair DNA binding of NF-κB. One point arguing for such a direct interaction of cryptolepine with NF-κB is the observation that cryptolepine inhibited NF-κB DNA binding in a pure in vitro binding assay of isolated active NF-κB (Fig. 5B), and that the inhibition could be overcome by an excess of DTT. The thiol groups of DTT are assumed to compete with NF-κB for alkylation by cryptolepine. It has to be noted that the in vitro inhibition of DNA binding of NF-κB (Fig. 5B) required much higher concentrations than the inhibitory effect in in vivo experiments. One explanation could be that cryptolepine accumulates in the cell during the up to 24 h long incubations of in vivo experiments and thereby reaches intracellular concentrations much higher than the initially applied concentrations in the low μM range. Another issue to take into account is a potential metabolism of cryptolepine in the cell into a more potent anti-inflammatory agent. Regarding the cytotoxicity profile of crypolepine, the postulated alkylating activity seems to be rather specific for NF-κB and maybe few additional proteins. A crude unselective alkylans of proteins or even DNA would be expected to exhibit stronger cytotoxic effects that are missing in our experimental setup.

4. Conclusion

Overall, we here demonstrate that cryptolepine inhibits NF-κB-mediated gene expression by interfering with DNA binding of the transcription factor NF-κB. The possible underlying mechanism is at least partly based on the alkylating nature of cryptolepine. Our data provide novel insights into the mode of action of cryptolepine and present a molecular explanation for its known, but so far unexplained, anti-inflammatory effect. Interestingly, also the anti-malarial activity of cryptolepine may be based on its inhibitory effect on NF-κB, since a recent report stresses the importance of the NF-κB pathway for the pathogenesis of malaria[32] and

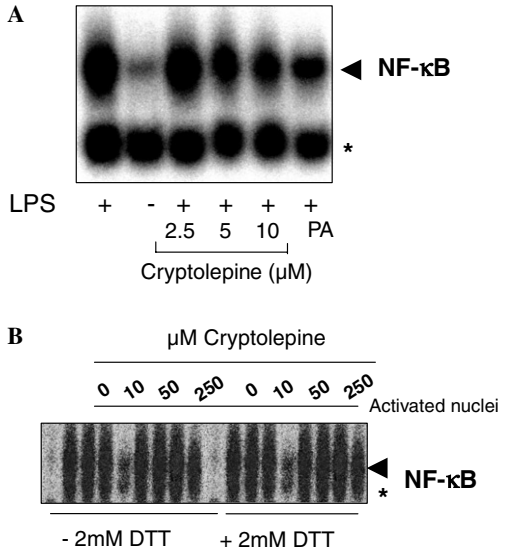

Figure 5. Cryptopepine impairs DNA binding activity of NF-κB in vivo and in vitro. RAW 264.7 cells were treated with LPS (1 μg/ml) for 1 h after a 30 min pre-treatment with 2.5, 5, and 10 μM cryptolepine, as well as parthenolide (10 μM), which was used as positive control. Nuclear extracts were subjected to EMSA as described under Section 5 (A). Nuclei were isolated from LPS-stimulated macrophages and served as a source of active NF-κB. Equal amounts of nuclear protein were incubated with the indicated concentrations of cryptolepine for 30 min in the absence or presence of 2 mM DTT, before the mixtures were subjected to an EMSA as described above. Lane 1 depicts DNA binding of nuclear extracts from unstimulated Raw macrophages (negative control) (B). Data shown are representative of at least two separate experiments with consistent results and ∗ indicates unspecific binding.

also the activity of artemisinin, used as alternative antimalaria therapy, could be partly explained by its targeting NF-κB.[33]

5. Experimental

5.1. Synthesis of cryptolepine

Cryptolepine (in the hydrochloride salt form) was synthesized by methylation of quindoline as described earlier[8] and based on the methodology of Holt and Petrow.[34] The alkaloid was prepared in sterile water for biological studies and kept at −20 °C.

5.2. Cell culture

RAW 264.7 cells obtained from American Type Culture Collection (ATCC TIB 71) were cultured in Dulbecco's Modified Essential Medium (DMEM) with 4 mM L-glutamine and 4.5 g/L glucose (endotoxin level <0.005 endotoxin U/ml, BioWhittaker, Bioproducts Heidelberg, Germany) supplemented with 10% heat-inactivated FBS (Gibco-BRL Life Technologies). Cells were maintained at 37 °C, 5% CO_2 and used for experiments between passages 5 and 20. The human embryonic kidney cell line 293 (HEK293; DSMZ-German collection of microorganisms and cell cultures, ACC 305) was grown in DMEM (BioWhittaker, Bioproducts, Heidelberg, Germany) supplemented with 10% FCS (Biochrom KG, Berlin, Germany) and 2 mM glutamine (Merck, Munich, Germany). Cells were split 1:10 when they reached ≈85–90% confluence using 0.05% trypsin/0.02% EDTA in PBS.

5.3. Assessment of LPS-mediated NO production

Quantification of nitrite accumulation was carried out as described earlier by Dirsch et al.[35] Cells were seeded in 96-well plates (2×10^5/200 μl/well), cultured for 2 days, and then incubated with or without lipopolysaccharide (1 μg/ml LPS) in the absence or presence of cryptolepine for 20 h. As a parameter of NO synthesis nitrite concentration was assessed in the supernatant of RAW 264.7 macrophages by the Griess reaction.[36] Briefly, 100 μl of cell culture supernatant was removed and combined with (i) 90 μl 1% of sulfanilamide in 5% H_3PO_4 and (ii) 90 μl 0.1% of *N*-(1-naphthyl) ethylenediamine dihydrochloride in H_2O in a 96-well plate, followed by spectrophotometric measurement at 550 nm (reference wavelength 620 nm) using a SPECTRA microplate reader (SLT-Lab instruments). Nitrite concentrations in the supernatants were determined by comparison with a sodium nitrite standard curve. Experiments were performed at least three times and performed in triplicate.

5.4. Cytotoxicity assay

RAW 264.7 cells first probed for NO production as described above were stained with Crystal Violet (0.5% in 20% methanol). Bound dye, a measure for attached cells, was solubilized with 50% 1 M sodium citrate/50% ethanol and quantified in TECAN plate reader (wavelength 540 nm).

5.5. Transient transfection and reporter gene assay

In order to determine the effect of cryptolepine on the transactivation of NF-κB, a luciferase reporter gene assay was carried out as described earlier by Keiss et al.[37] HEK293 cells were seeded at a concentration of 5×10^5 cells/60-mm dish. The next day, cells were transfected with the pNF-κB-luc plasmids using the Ca^{2+}-phosphate method. Transfected cells were seeded in 24-well plates at a concentration of 15,000 cells/well and grown for an additional 16 h. Then cells were either preincubated with cryptolepine for 30 min and subsequently stimulated with 1 ng/ml TNF-α for 6 h. NF-κB-mediated gene expression was measured with a commercial luciferase kit (Promega, Heidelberg, Germany) according to the manufacturer's instructions using a AutoLumat plus luminometer (Berthold, Bad Wildbad, Germany).

5.6. Western blot

RAW 264.7 cells in 6-well plates were pretreated with 10 μM cryptolepine for 30 min and then stimulated with LPS (1 μg/ml) for the indicated periods of time. Cells were then washed with ice-cold phosphate buffer saline (PBS) and lysed using the lysis buffer (50 mM Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS),

2% mercaptoethanol, 10% glycerol and 0.004% bromophenol blue). Equal amounts of protein were separated by PAGE on 10–12% gels and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (50 mM Tris, pH 8.0, 150 mM NaCl) with 0.2% Tween 20 (TBS-T pH 8.0) for 2 h and then incubated with the indicated primary antibody (1:500–1:1000 in TBS-T/5%BSA, Santa Cruz Biotechnology, CA, USA (IκB, Tubulin) or Cell Signalling Technology, Inc. (phospho-IκB)) overnight at 4 °C. After three washes with TBS-T, pH 8.0, membranes were incubated with the appropriate horseradish peroxidase coupled secondary antibodies for 1 h at room temperature. Proteins were visualized with ECL Plus (Amersham Biosciences, Freiburg, Germany) and subsequent development in a CCD camera (LAS, 3000, Fujifilm). Densitometric analysis was performed using AIDA software (Fujifilm).

5.7. Immunocytochemistry

RAW 264.7 macrophages were grown on gelatine-covered coverslips and preincubated with 10 μM cryptolepine or 10 μM parthenolide for 30 min as indicated. Then, LPS (1 μg/ml) was added and after another 30 min cells were fixed with 2% formalin and permeabilized with 0.2% Triton X-100/PBS. Unspecific binding sites were blocked with 5% BSA/PBS for 20 min. After incubation with anti-p65 antibody (1:50 in BSA/PBS, Santa Cruz) for 2 h at RT, cells were washed with BSA/PBS and a FITC-conjugated anti-rabbit antibody (SIGMA) was applied for 1 h in the dark. Cells were mounted, analyzed using a confocal laser scanning microscope (ZEISS; FITC excitation/488 nm) and photographed.

5.8. Electrophoretic mobility shift assay (EMSA)

RAW 264.7 cells were grown in 6-well plates and stimulated with LPS (100 ng/ml) in the presence or absence of cryptolepine for 60 min. Nuclear extracts were prepared as earlier described.[37] Briefly, cells were washed with PBS, resuspended in 400 μl hypotonic buffer A 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9; 10 mM KCl; 0.1 mM ethylenediaminetetraacetic acid (EDTA); 0.1 mM ethyleneglycoltetraacetic acid (EGTA); 1 mM dithiothreitol (DTT); 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and were allowed to swell on ice for 15 min. Nonidet P-40 (10%, 25 μl) was added followed by 10 s of vigorous vortexing and centrifugation at 12,000*g* for 30 s. The supernatant was removed and the nuclear pellet was extracted with 50 μl of hypertonic buffer B (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) by shaking at 4 °C for 15 min. The extract was centrifuged at 12,000*g* and the supernatant was frozen at −85 °C for further use. Double-stranded oligonucleotide probes containing a consensus binding-sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) (Promega, Heidelberg, Germany) was 5′ end-labelled with (γ^{32}P)-ATP (3000 Ci/mmol, Amersham, Braunschweig, Germany) using T4 polynucleotide kinase (Promega, Heidelberg, Germany). EMSA experiments were performed as described previously.[38] Briefly, equal amounts of nuclear protein were incubated with the labelled probe for 30 min at room temperature and resolved in a 4.5% non-denaturing polyacrylamide gel. Visualization of bands was performed by phosphorimaging (Packard, Meriden, USA).

5.9. Statistical analysis

All data are expressed as means ± SD. One-way ANOVA with Dunnett's multiple comparison test was used to check on significant differences between control and treatment groups. Differences with $P < 0.05$ were considered significant. Analyses of the data were performed using the software GraphPad PRISM, Version 4.0 (GraphPad Software, San Diego, CA).

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