Plant extracts from stinging nettle (*Urtica dioica*), an antirheumatic remedy, inhibit the proinflammatory transcription factor NF-κB

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Abstract Activation of transcription factor NF-κB is elevated in several chronic inflammatory diseases and is responsible for the enhanced expression of many proinflammatory gene products. Extracts from leaves of stinging nettle (*Urtica dioica*) are used as antiinflammatory remedies in rheumatoid arthritis. Standardized preparations of these extracts (IDS23) suppress cytokine production, but their mode of action remains unclear. Here we demonstrate that treatment of different cells with IDS23 potently inhibits NF-κB activation. An inhibitory effect was observed in response to several stimuli, suggesting that IDS23 suppressed a common NF-κB pathway. Inhibition of NF-κB activation by IDS23 was not mediated by a direct modification of DNA binding, but rather by preventing degradation of its inhibitory subunit IκB-α. Our results suggests that part of the antiinflammatory effect of *Urtica* extract may be ascribed to its inhibitory effect on NF-κB activation.

Key words: Antiinflammatory; Nuclear factor κB; Rheumatoid arthritis; Stinging nettle extract; Transcription factor; *Urtica dioica* L.

1. Introduction

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation, hyperproliferation of the synovial lining and cartilage destruction. Cytokines, in particular tumor necrosis factor (TNF), are elevated in the synovial fluid and presumably involved in the disease process by upregulation of a multitude of inflammatory mediators [1,2]. The success of anti-TNF antibodies in clinical trials underlines that TNF is a major pathogenic factor in rheumatoid diseases [3]. The definition of TNF as a therapeutic target led to a search for signaling pathways that control TNF production in rheumatoid arthritis.

Transcription factors of the NF-κB family are critical for the inducible expression of many genes involved in inflammatory responses [4,5]. NF-κB is ubiquitously found as an inactive complex in the cytoplasm bound to its inhibitory subunit IκB. In the active form NF-κB is a heterodimer which is frequently composed of a p50 and p65 protein, but also other subunits such as p52, c-Rel and RelB may participate in complex formation. A large variety of inflammatory conditions including bacterial and viral infections, lipopolysaccharide, cytokines and various forms of stress induce NF-κB activity. Several lines of evidence indicate that reactive oxygen intermediates (ROIs) serve as second messengers in the activation pathway of NF-κB [6]. A key event for the activation of NF-κB is the inducible phosphorylation of the inhibitory subunit IκB-α on serines 32 and 36. This covalent modification by IκB kinases induces the conjunction of IκB to ubiquitin, followed by a rapid degradation of the inhibitor at the proteasome [7–9]. The active form of NF-κB then translocates to the nucleus and binds to regulatory sequences of target genes. Among the numerous target genes of NF-κB are those encoding inflammatory and chemotactic cytokines such as interleukin-1 (IL-1), IL-2, IL-6, IL-8 and TNF, cell adhesion molecules, major histocompatibility complex class I molecules, cytokine receptors and proinflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2 [4,10]. In many inflammatory diseases NF-κB activation is increased leading to the overexpression of proinflammatory gene products. In rheumatoid arthritis, elevated activation of NF-κB has been detected in synoviocytes and endothelial cells [11–14]. Furthermore, using adenoviral expression of IκB-α it has been demonstrated that TNF production by macrophages is indeed NF-κB dependent [15].

Another transcription factor which has been implicated in the pathogenesis of rheumatoid arthritis is activator protein-1 (AP-1). The factor is a dimer composed of protooncogene products of the Fos and Jun family and plays a major role in cellular differentiation and proliferation processes [16]. It has been suggested that increased AP-1 activation may be responsible for synovial hyperplasia in rheumatoid arthritis [17,18]. Target genes of AP-1 include the matrix metalloproteinases MMP-1 and MMP-3, which are involved in degrading connective tissues. AP-1 can physically interact with NF-κB and cooperatively induce cytokine gene expression [19]. It has also been demonstrated that the activity of both factors is inhibited by their interaction with the glucocorticoid receptor [20–22]. Interestingly, the antiinflammatory action of several antirheumatic drugs has been attributed to their ability to modulate NF-κB activation [10].

In the present study, we investigated the effect of leaf extracts from stinging nettle (*Urtica dioica* L.) on the activation of NF-κB. *Urtica* extracts are used as an adjuvant remedy in rheumatoid arthritis with a proven therapeutic efficacy. Previously, it has been shown that *Urtica* extracts inhibit the expression of several cytokines as well as eicosanoid formation in stimulated peripheral blood cells [23,24]. Here we demonstrate that IDS23 (Rheuma-Hek, Strathmann AG), a standardized extract of stinging nettle leaves, potently suppresses

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Abbreviations: AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PMA, phorbol myristyl acetate; ROI, reactive oxygen intermediate; tk, thymidine kinase; TNF, tumor necrosis factor

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the activation of NF-κB in response to several stimuli by the inhibiting the proteolytic degradation of its inhibitor IκB-α. IDS23 furthermore exerted an inhibitory effect on the activation of AP-1, suggesting that at least part of the antirheumatic effect of *Urtica* extract may be attributed to its ability to inhibit proinflammatory transcription factors.

2. Materials and methods

2.1. Cell culture and reagents

The human T-cell line Jurkat, the macrophage cell line MonoMac6, the epithelial cell line HeLa and mouse L929 fibrosarcoma cells were maintained in RPMI 1640 medium containing 10% FCS, 2 mM glutamine and antibiotics. Phorbol myristyl acetate (PMA) and lipopolysaccharide (LPS) were obtained from Sigma (Deisenhofen, Germany). Recombinant TNF was from Knoll AG (Ludwigshafen, Germany). Extracts of stinging nettle leaves were kindly provided by Strathmann AG (Hamburg, Germany) as a commercial drug preparation (IDS23, Rheuma-Hek) and a water-soluble fraction (IDS23/1) [23,25]. For the preparation of IDS23, one part dried and chopped *Urticae dioicae folia* drug was originally extracted with 10 parts of ethanol at 50°C for 4 h. For the preparation of the water-soluble fraction IDS23/1, lyophilized IDS23 was solubilized in culture medium (12.6 mg/ml) by sonication. After ultracentrifugation at $33\,000\times g$ for 30 min and sterile filtration, the supernatant was used for further experiments. According to gravimetric analysis, 375 μg of the dry mass of the original extract IDS23 was solubilized in 1 ml IDS23/1 [23,25].

2.2. Electrophoretic mobility shift assay

Cells were routinely plated at $1–2\times10^6$/well in six-well plates and pretreated for 1 h with stinging nettle extracts, followed by the addition of the appropriate stimulus. After 1 h total cell extracts were prepared and used for electrophoretic mobility shift assays (EMSAs) and Western blot analysis. Extracts were prepared in a high-salt buffer, incubated with the ^{32}P-endlabeled NF-κB-, Sp1- or AP-1-specific oligonucleotides (Promega, Heidelberg, Germany) and run on a 4% non-denaturing polyacrylamide gel as described [7]. To characterize the NF-κB-DNA complex, supershift analyses were performed using antibodies against NF-κB subunits (Santa Cruz, Heidelberg, Germany).

2.3. Transfections and luciferase assays

In order to measure the transactivating activity of NF-κB, cells were transfected by calcium phosphate coprecipitation with a luciferase reporter gene construct which was controlled by six κB binding sites fused to the minimal thymidine kinase (tk) promoter. After 16 h cells were stimulated with the indicated agents for 5 h. Cells were then lysed in 25 mM glycylglycine, pH 7.8, 1% Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, and centrifuged at $13\,000\times g$ at 4°C for 5 min. 50 μl of the supernatant was assayed in 100 μl buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KPi, pH 7.8, 1 mM DTT, 1 mM ATP) using a luminometer. Following injection of 100 μl luciferin (0.3 mg/ml) light emission was recorded as relative light units.

2.4. Western blotting

To measure the degradation of IκB-α cellular proteins from 2×10^6 cells were separated on a 12.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% non-fat dry milk powder in PBS and incubated for 1 h with an IκB-α-specific rabbit antiserum (Santa Cruz). Membranes were washed three times with TBS/0.05% Tween-20 and incubated with peroxidase-conjugated rabbit anti-mouse IgG for 1 h. Following extensive washing, the reaction was developed by enhanced chemoluminescent staining.

3. Results

3.1. Urtica extracts are potent inhibitors of NF-κB activation

To investigate the effect of *Urtica* extracts on NF-κB activation, we incubated HeLa cells with various concentrations of a stinging nettle preparation, termed IDS23 (Rheuma-Hek). Cells were then stimulated with TNF, after which cell extracts were prepared and analyzed for DNA binding to a

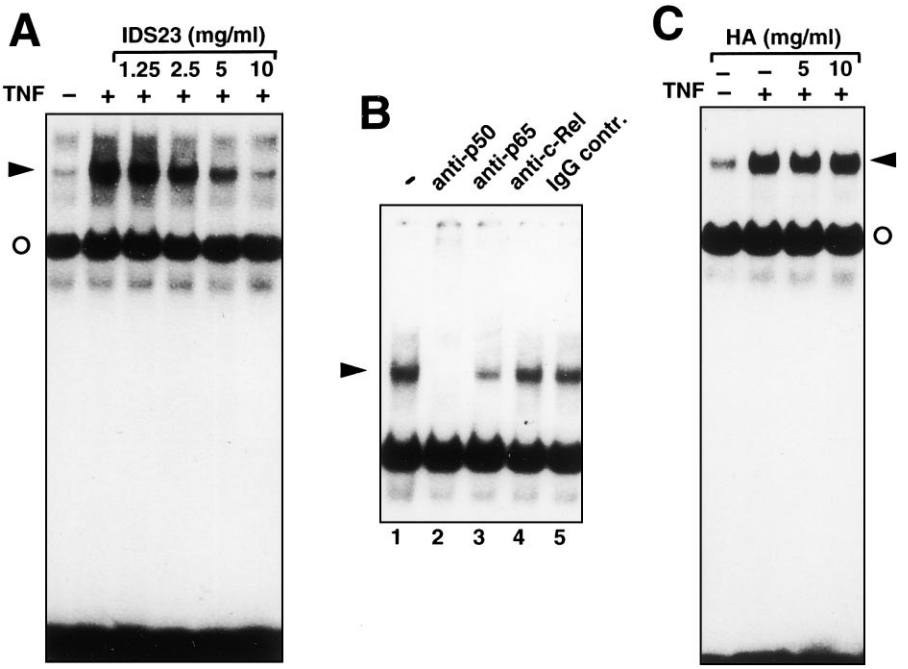

Fig. 1. *Urtica* extract inhibits NF-κB DNA binding. A: The effect of IDS23. HeLa cells were treated for 1 h with the indicated concentrations of IDS23 and then stimulated with 200 U/ml TNF. After 1 h total cell extracts were prepared and analyzed by EMSA. The NF-κB DNA complex is indicated by a filled arrowhead. A faster migrating non-specific complex is marked by a circle. B: Specificity and subunit composition of the NF-κB DNA complex. Total cell extracts from TNF-treated HeLa cells were either left untreated or incubated with antibodies against the NF-κB subunits p50, p65, c-Rel or with control IgG. Only a section of the autoradiogram is shown. C: The effect of a *Harpagophytum* control extract (HE) on NF-κB activity. An extract of *Harpagophytum procumbens*, which was prepared similarly as IDS23, was used to investigate the specificity of *Urtica* extract for the inhibition of NF-κB activity.

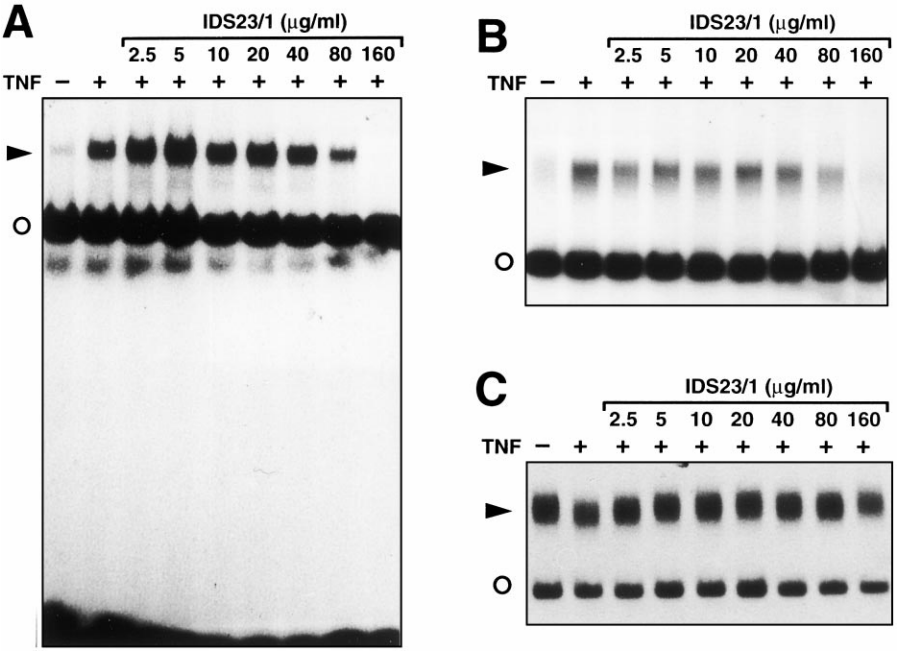

Fig. 2. The effect of the water-soluble extract IDS23/1 on the DNA binding activity of NF-κB, AP-1 and Sp1. HeLa cells were treated with IDS23/1 at different concentrations and analyzed for the DNA binding activity to an oligonucleotide containing a consensus motif for NF-κB (A), AP-1 (B) and SP-1 (C). In B and C only sections of the autoradiograms are shown. The arrowheads indicate the positions of the specific DNA complexes. The indicated concentrations of IDS23/1 refer to the solubilized plant material which was determined by gravimetric analysis as described in Section 2.

^{32}P-labeled κB-specific oligonucleotide. As determined by EMSAs, treatment with TNF caused the appearance of a novel NF-κB protein-DNA complex (Fig. 1A). A faster migrating, non-specific DNA complex was not affected by the treatments and provided an internal control for the amount and integrity of the cell extracts. Pretreatment of cells with IDS23 dose-dependently inhibited the DNA binding activity of NF-κB.

Because NF-κB complexes may constitute different homo- and heterodimers, we analyzed the composition of the DNA complex. Antibodies against the NF-κB subunits p50, p65, and c-Rel were added to an extract of cells stimulated with TNF. The NF-κB-DNA complex was retarded completely by anti-p50 (Fig. 1B), whereas the non-specific DNA complex was not affected. Formation of the upper DNA complex was also reduced by anti-p65, while anti-c-Rel or control IgG had no effect. This indicates that the TNF-induced and IDS23-inhibited NF-κB complex consisted of a p50/p65 heterodimer. We further analyzed the effects of an extract from devil's claw (*Harpagophytum procumbens*), which was prepared in a similar manner as IDS23 and taken as a control extract. At concentrations which effectively inhibited NF-κB DNA binding in the case of IDS23, *Harpagophytum* extract did not interfere with NF-κB (Fig. 1C). This indicates that the effect on NF-κB activity is specific to *Urtica* extracts and not mediated by a ubiquitous plant product.

Previously, it has been demonstrated that the cytokine-suppressing activity of stinging nettle extracts is enriched in a water-soluble fraction, called IDS23/1 [23–25]. Pretreatment of HeLa cells with IDS23/1 indeed more strongly inhibited the formation of the NF-κB-specific DNA complex than IDS23 (Fig. 2A). To determine whether *Urtica* extracts specifically inhibit NF-κB or whether the DNA binding of other transcription factors was suppressed, we analyzed the extracts used in Fig. 2A in EMSAs with DNA probes specific for AP-1 and Sp1. As shown in Fig. 2B, AP-1, another transcription factor implicated in rheumatoid arthritis, was partially inhibited by IDS23/1. In contrast, the activity of Sp1 remained virtually unchanged (Fig. 2C).

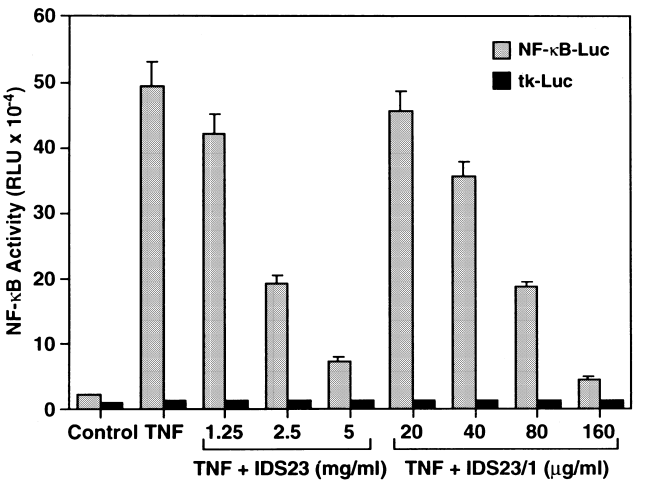

Fig. 3. Inhibition of NF-κB-controlled gene expression by *Urtica* extracts. HeLa cells were transiently transfected with a luciferase reporter gene driven by either the minimal tk promoter (tk-Luc; black bars) or by the tk promoter fused to six NF-κB binding sites (NF-κB-Luc; gray bars). 16 h after transfection, cells were pretreated with the indicated concentrations of IDS23 or IDS23/1, stimulated with TNF and harvested after 5 h of further incubation. Mean values ± S.D. of NF-κB activity given as relative light units (RLU) from triplicate experiments are shown.

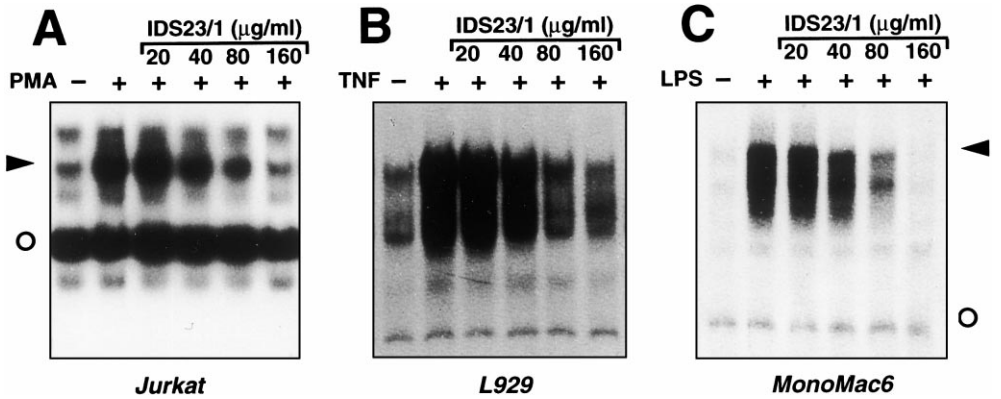

Fig. 4. The effect of IDS23/1 on NF-κB activation in response to distinct stimuli. Different cell lines were incubated for 1 h with the indicated concentrations of IDS23/1 followed by treatment with the appropriate stimuli for another 1 h. Jurkat T-cells (A) were stimulated with PMA (50 ng/ml) and L929 fibrosarcoma cells (B) with TNF (200 U/ml). Macrophage-like MonoMac6 cells (C) were stimulated with LPS (1 μg/ml). Total extracts of the different cell lines were analyzed by EMSAs as described in Fig. 1A.

3.2. *Urtica extracts inhibit NF-κB-dependent gene expression*

The previous experiments demonstrated that *Urtica* extracts potently inhibits NF-κB DNA binding. Therefore, we investigated whether this effect resulted in the functional inhibition of NF-κB-driven gene expression. To this end, HeLa cells were transfected with reporter gene constructs which were controlled either by the minimal tk promoter alone or by a tk promoter fused to six NF-κB binding sites. Cells were then pretreated with *Urtica* extracts and stimulated with TNF. Treatment of cells with TNF increased NF-κB reporter gene activity approximately 20-fold (Fig. 3). This activation was almost completely and dose-dependently prevented by IDS23 and even more strongly by the water-soluble extract IDS23/1.

3.3. *Urtica extract interferes with a common step of NF-κB activation*

NF-κB can be activated by various stimuli. We asked whether the effect of *Urtica* extract was restricted to TNF-activated HeLa cells or whether the extract was also capable of inhibiting NF-κB in response to other stimuli in different cell types. As shown in Fig. 4A, incubation of Jurkat T-cells with IDS23/1 dose-dependently inhibited NF-κB induced by the phorbol ester PMA. An inhibitory effect on NF-κB activity was also observed in TNF-stimulated L929 fibrosarcoma cells (Fig. 4B). Because macrophage-derived cytokines play a pathogenic role in rheumatoid arthritis, we investigated the effect of IDS23/1 in MonoMac6 cells which express several characteristics of mature monocytes. In these cells, LPS strongly induced NF-κB activation which was likewise impaired by *Urtica* extract (Fig. 4C). It was noteworthy that, at concentrations used in the EMSAs, IDS23/1 did not markedly compromise the viability of the different cells (data not shown). Thus, the results demonstrate that *Urtica* extracts inhibit NF-κB in response to diverse stimuli in different cell types.

3.4. *Urtica extract does not abolish NF-κB DNA binding activity but inhibits IκB degradation*

An explanation for the inhibitory effect of *Urtica* extracts could be a direct interference with the DNA binding activity of NF-κB. It has been shown that the p50 NF-κB subunit contains redox-active cysteine residues within the DNA binding domain which are highly susceptible to chemical modification [26]. To analyze the effect of *Urtica* extracts on the DNA binding activity, IDS23/1 was directly added to an extract of TNF-stimulated HeLa cells. Even high concentrations of IDS23/1 did not prevent DNA binding of active NF-κB in vitro (Fig. 5A).

A key step of NF-κB activation is the dissociation of its inhibitor IκB which is mediated through the phosphorylation and subsequent proteolytic degradation. We therefore incubated Jurkat cells for different times with PMA in the presence and absence of IDS23/1 and subsequently analyzed the degradation of IκB-α by Western blot analysis. Treatment of cells with PMA led to the rapid degradation of IκB-α, whereas a protein that reacted non-specifically with the anti-IκB-α antibody was not affected (Fig. 5B). The degradation of IκB-α was strongly inhibited by *Urtica* extract in PMA-stimulated Jurkat cells (Fig. 5B) as well as in TNF-stimulated HeLa cells (data not shown). These results suggests that *Urtica* extract inhibits NF-κB activation not by directly interfering with its

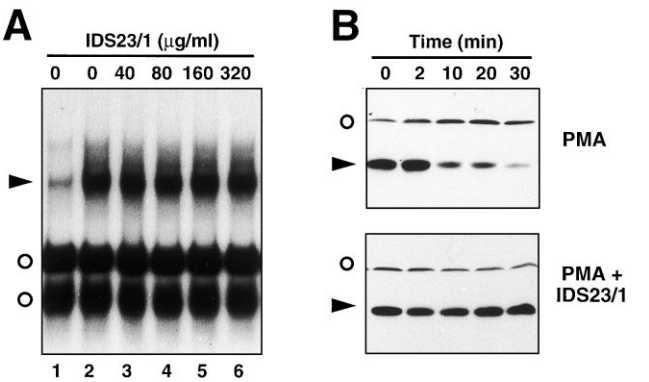

Fig. 5. *Urtica* extract does not directly modify NF-κB DNA binding (A), but prevents the proteolytic degradation of IκB-α (B). A: An extract of PMA-stimulated Jurkat cells was treated in vitro with the indicated concentrations of IDS23/1 and analyzed for NF-κB DNA binding after 2 h by EMSA. B: Jurkat cells were either left untreated (upper panel) or pretreated with IDS23/1 (160 μg/ml; lower panel). After 1 h cells were stimulated with PMA for the indicated times. Cell extracts were analyzed by Western blotting with an IκB-α-specific antibody. The position of IκB-α is indicated by an arrowhead. Noteworthy, a slowly migrating protein, which reacted non-specifically with the antibody, was not affected by the treatments.

DNA binding, but by preventing the degradation of IκB-α or upstream signaling events.

4. Discussion

Plant remedies have become increasingly popular and are often preferred to synthetically derived pharmaceuticals. It is therefore of interest to determine their active components and to elucidate their molecular mechanisms of action. In the present study we show that leaf extracts from stinging nettle, which are used in the treatment of rheumatoid arthritis, potently inhibit transcription factor NF-κB. An inhibitory effect is demonstrated in different cell types including T-cells, macrophages, fibrosarcoma and epithelial cells, as well as in response to several stimuli, suggesting that *Urtica* extracts interfered with a common target in the NF-κB pathway. In this respect, we show that *Urtica* extracts stabilize the inhibitor IκB-α by preventing its proteolytic degradation. Since inflammatory processes are strictly dependent on NF-κB activity, our results suggest a molecular basis for the antiinflammatory effects of *Urtica* extracts.

Recent studies have shown that NF-κB activity is elevated in rheumatoid arthritis, particularly in the synovial lining and endothelial cells [11–14]. NF-κB controls the expression of numerous proinflammatory gene products including cytokines, molecules involved in endothelial cell adhesion and antigen presentation as well as inflammatory enzymes such as cyclooxygenase II and inducible nitric oxide synthase [4,10]. It has been demonstrated that TNF expression in macrophages is NF-κB-dependent, because adenoviral infection with IκB-α strongly inhibited TNF production in rheumatoid joint cultures [15]. Thus, by decreasing transcription of these various proinflammatory genes in concert NF-κB inhibition should modulate several aspects of inflammation.

Besides NF-κB, we show that transcription factor AP-1 may be an additional target of *Urtica* extracts. AP-1 is a complex of the Fos and Jun protooncogene products and has been implicated in the hyperplasia of synovial tissues [17]. Target genes of AP-1 include the matrix metalloproteinases MMP-1 and MMP-3 which play a major role in the degradation of cartilage matrix molecules. Recently, it could be demonstrated that inhibition of AP-1 activity by antisense approaches prevented collagen-induced arthritis in an animal model [18].

Activation of NF-κB requires the phosphorylation and subsequent proteolytic degradation of IκB by the proteasome pathway [7]. The precise molecular target of *Urtica* extracts remains to be investigated. We provide evidence that *Urtica* extracts do not interfere with NF-κB DNA binding, but inhibit the proteolytic degradation of IκB. This inhibition may be the result of a direct prevention of IκB degradation or the inhibition of IκB kinases or further upstream signaling molecules. The inhibition of NF-κB may be ascribed to an antioxidant property of *Urtica* extract, as intracellular ROI formation is essential for NF-κB activation in response to various stimuli [6]. It has been reported that certain flavonoids and phenol carbon acids such as quercetin and curcumin inhibit NF-κB activation presumably by an antioxidant mechanism [27,28]. The main phenolic ingredient in *Urtica* extracts is caffeic malic acid, which suppresses eicosanoid formation but, compared with *Urtica* extract, is rather ineffective in preventing cytokine production in peripheral blood cells [23,25]. Thus, whether flavonoids or unrelated substances in *Urtica* extracts mediate the NF-κB inhibitory effects remains to be shown. Other naturally occurring compounds which potently inhibit NF-κB activation are plant sesquiterpene lactones or gliotoxin [29–31]. It is interesting to note that several drugs traditionally used in antirheumatic therapy, including sulfosalazine, salicylates, glucocorticoids and gold compounds, exert at least part of their antiinflammatory action by interfering with the NF-κB pathway [20,21,32–34]. The inhibitory effect on NF-κB activation may therefore provide a simple means to identify the active antirheumatic compound in *Urtica* extracts.

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