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TRANSCRIPTION FACTORS AS TARGETS OF THE ANTI-INFLAMMATORY TREATMENT. A CELL CULTURE STUDY WITH EXTRACTS FROM SOME MEDITERRANEAN DIET PLANTS

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During the inflammatory response at least 2 transcription factors, NF-κB and AP-1, are involved in the altered profile of gene expression. We used human hepatoma (HepG2) and human umbilical vein endothelial cells (HUVEC) as a model system: NF-κB and AP-1 were activated by the proinflammatory cytokine IL-1 in the absence or presence of 21 selected plant extracts and the effect was evaluated by the electrophoretic mobility shift assay (EMSA). In both types of cells activation of NF-κB by IL-1 was significantly inhibited by extracts from *Scandix australis* and *Artemisia alba*, whereas extracts from *Amaranthus sp., Eryngium campestre, Thymus pulegioides* and *Reichardia picroides* elicited cell-type dependent response. The IL-1-induced AP-1 activation was diminished by extracts from *Scandix australis, Amaranthus sp.* and *Artemisia alba* more potently in HUVEC, while extracts from *Urospermum picroides* and *Scandix pecten-veneris* in HepG2 cells. Inhibitory activities of plant extracts towards cytokine activated NF-κB and AP-1 depend to some extent on the order of addition of IL-1 and plant extract to the cell culture, but the mechanism of action of extract components is not clear: although plant polyphenols may participate they are unlikely to be the only mediators, and MAP kinases were found generally not involved in down-regulation of transcription factors activity by plant extracts.

Key words: *HepG2, HUVEC, NF-κB, AP-1, STAT, plant extracts, inflammation*

INTRODUCTION

Gene expression is enhanced by specific proteins (transcription factors) which interact with matching sequences in the promoter region of a DNA molecule. Transcription factors, such as nuclear factor kappa-B (NF-κB),

activator protein-1 (AP-1), or signal transducer and activator of transcription (STAT), are involved in the induced expression of a variety of proteins, and especially cytokines controlling the inflammatory response (for references see [1]). NF-κB is present in the cytosol of many cell types, usually as a heterodimer composed of p50 and p65 subunits, held in the inactive state by IκB inhibitory subunit (2). Upon stress-induced phosphorylation, and subsequent ubiquitination and degradation of IκB the heterodimer p50/p65 is translocated to the nucleus where it binds to specific DNA sequences and stimulates transcription of the corresponding genes (3). AP-1 refers to a family of protein dimers, usually composed of c-jun/c-fos subunits, which after stress-induced phosphorylation enter the nucleus and due to binding to consensus sequences enhance the expression of the appropriate genes (4). Activation of NF-κB and/or AP-1 is often induced by the proinflammatory cytokines, such as IL-1 or TNFα (1,3). On the other hand, STAT3 belongs to the family of transcription factors activated by such cytokines as IL-6 and is involved in the up-regulation of acute phase protein synthesis in liver cells (1,5,6).

Intracellular signal transduction initiated by binding of a cytokine to the plasma membrane receptor usually involves a cascade of specific protein kinases for which the last substrate is often one of the transcription factors. The most important for the development and control of inflammation is a signaling pathway generated by interleukin-1 receptor/Toll-like receptor (7), and a cascade of mitogen activated protein kinases (MAPKs) which includes 3 distinct pathways: extracellularly-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38-mitogen activated kinase (p38 MAPK). The end point of these pathways are several transcription factors, the most important being NF-κB and AP-1 (8,9).

It has been proposed that compounds which inhibit signaling pathways leading to activation of these transcription factors may be used for the treatment of cytokine-mediated acute and chronic inflammation (for references see [10-14]). However, some authors suggest that a considerable cross-talk between those signaling pathways exists (15) and in effect inhibition of NF-κB could be counterbalanced by enhanced AP-1 activation (16). For this reason complex studies concerning the role of transcription factors as targets of anti-inflammatory treatment are required. Here we report the results of our experiments aimed at the evaluating potential anti-inflammatory activities of extracts from several species of edible plants common for the Mediterranean diet and traditionally used in localities of Spain, Italy and Crete, as described in the accompanying papers by Heinrich and co-workers (17) and Rivera and co-workers (18). Selection of extracts to be tested in the electrophoretic mobility shift assay (EMSA) was based mainly on the results of screening of 121 extracts by Bereta and co-workers (19).

MATERIALS AND METHODS

Cell culture conditions

HepG2 cells (from ATCC) were grown in culture flasks and maintained in DMEM supplemented with antibiotics and 5% FCS in a humidified incubator under 5% CO_2 at 37°C. HUVEC cells (passages 3-5) were cultured in MCDB medium containing 5% FBS and growth factors (10 ng/ml EGF, 10 ng/ml FGF). The medium (supplemented with 0.5% FBS and antibiotics) was changed 24 h before exposure to IL-1 and/or plant extracts. Cell viability assessed at the end of experiment by the LDH test was always higher than 90%.

Vacuum dried plant extracts were kindly provided by other members of Consortium "Local Food Nutraceuticals" (see: http://www.biozentrum.uni-frankfurt.de). The details of the procedure of extracts preparation are described in the accompanying paper by Loboda et al. (20). Weighted aliquots of extracts were dissolved in DMSO immediately before each experiment and added to cell cultures to obtain final concentration ranging from 5 to 50 μg/ml. The final concentration of DMSO in culture media did not exceed 0.2%. A model inflammatory response was induced in HepG2 and HUVEC cells by IL-1 (10 ng/ml) (for measuring NF-κB and AP-1), or IL-6 (10 or 25 ng/ml) (for measuring STAT3). The cells were treated with plant extracts either 10 min before IL-1 addition, or 5 min after stimulation with IL-1. After subsequent incubation for 60 min the nuclear extracts were isolated from cultured cells. For measuring STAT activation the extracts were added to the cell culture 10 min before or 5 min after IL-6 treatment, but the cells were incubated for 10 min only before cell protein isolation. Each experiment was repeated at least twice.

Preparation of nuclear or cellular extracts

Nuclear extracts were prepared by using the procedure of Suzuki et al. (21) in a modification described previously (22). In brief, 2 x 10^6 cells were washed with cold PBS, scraped in ice-cold PBS, harvested by centrifugation at 400 x g for 5 min and resuspended in buffer (10mM NaCl, 3mM $MgCl_2$, 10mM Tris pH 7.5, 1 mM DTT and 0.2 mM PMSF) followed by incubation on ice for 15 min. Nonidet NP-40 was added and samples were centrifuged for 60s at 12 000 r.p.m. Pelleted nuclei were resuspended (15 min on ice) in the buffer (10mM Hepes pH 7.5, 0.35mM NaCl, 1mM EDTA, 1mM DTT and 0.2mM PMSF) and centrifuged at 4°C for 5min at 14 000 r.p.m. The protein concentration in the supernatant was measured with bicinchoninic acid (BCA method) and the remaining supernatant was frozen (-70°C) in 10% glycerol.

For STAT activation measurement, the whole cell proteins were isolated according to Sadowski et al. (23). The cells were washed and scraped in cold PBS and centrifuged at 3000 r.p.m. for 2 min, followed by resuspension in buffer (50 mM Hepes pH 8.0, 10 mM CHAPS, 2 mM EDTA, 1 mM NaF, 1 mM DTT and 10% glycerol). After 30 min incubation on ice, the mixture was centrifuged at 14 000 r.p.m. for 5 min and supernatant proteins were measured by BCA method. The remaining supernatant was kept at -70°C until use.

For estimation of MAP kinase phosphorylation cell proteins were extracted in the lysis buffer containing 1 mM sodium vanadate to inhibit phosphatases

Electrophoretic mobility-shift assay (EMSA)

The following double-stranded oligonucleotides were used for DNA electrophoretic mobility shift assay: the oligonucleotide containing a high affinity sequence for NF-κB from the mouse kappa-light chain enhancer (5'AGC TTC AGA GAC TTT CCG AGA GG3'), the oligonucleotide specific for AP-1 from collagenase promoter (5'TCG ACT AGT ATG AGT CAG CCG3'), and oligonucleotide specific for STAT3 (the SIEm67 element - [24]) (5'AGC TCA TTT CCC GTA AAT

C3'). The described oligonucleotides were synthesized in the Laboratory of DNA Sequencing, Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw. The extracts of nuclear or cellular proteins (10 µg protein) were incubated for 30 min at room temperature in 25 µl of binding buffer (0,5% Triton X-100, 2,5% glycerol, 10mM Hepes, 4mM DTT) containing ^{32}P-end-labelled NF-κB- , AP-1- or STAT-binding oligonucleotide (c.10^5cpm) and 1µg of poly (dI-dC). To confirm specificity of binding unlabelled competitive oligonucleotides corresponding to the described probes were included in the tested samples. DNA-protein complexes were separated in a 5% non-denaturing polyacrylamide gel in Tris-boric acid-EDTA buffer pH 8.0, after the initial pre-electrophoresis (1 h at 80 V). Gels were run at 120 V for 2 h followed by drying in a gel dryer under vacuum at 80°C. The dried gels were analysed by autoradiography. The results were quantified with a scanning densitometer (Fluor S, Bio-Rad program Quantity One).

Evaluation of response of MAP kinases to plant extracts

Twenty four hours before the experiment culture medium of HepG2 cells was replaced by DMEM without FCS. The cells were treated with plant extracts suitably diluted in DMSO (final concentration 5 or 50 µg/ml of the tested extract in culture medium) for 5 min before addition of IL-1 (final concentration 10 ng/ml). After subsequent incubation for 30 min (measurement of ERK phosphorylation), or for 15 min (measurement of p38 and JNK phosphorylation) protein extracts were isolated (as described above). Samples of extracts were subjected to SDS-PAGE and electroblotted onto nitrocellulose. Phosphorylated kinases were labeled using Phospho-MAPK Family Antibody Sampler Kit from Cell Signaling Technology (www.celsignal.com) and detected using Super Signal West Pico Chemiluminescent kit (Pierce).

RESULTS

Certain plant extracts modulate the IL-1-induced NF-κB activation in HepG2 cells

The results of EMSA used for evaluation of NF-κB activation following exposure of cultured HepG2 cells to IL-1 (10 ng/ml) and to the extracts from *Scandix pecten-veneris* or *Urospermum picroides* (5 or 50 µg/ml) are shown in *Fig.1.* The NF-κB-derived band is barely visible in the control culture and cells incubated with the tested extracts alone, but as expected, it has been strongly enhanced by IL-1 treatment. Specificity of this band was confirmed by the addition of 100-fold excess of the unlabelled probe (data not shown). The tested 2 extracts almost completely blocked HepG2 cell response to the cytokine when added before IL-1 but were without effect, or even slightly enhanced NF-κB activation, when added to cell culture after IL-1. This may suggest the existence of different mechanisms of extracts activity depending on the order of addition of IL-1 and the extracts to cell culture (see the Discussion section).

The inhibitory effect of plant extracts on IL-1-induced activation of NF-κB and AP-1 in HepG2 and HUVEC cells

When the effects of several extracts on activation of two transcription factors were analyzed in two types of cultured cells, a complex picture has emerged

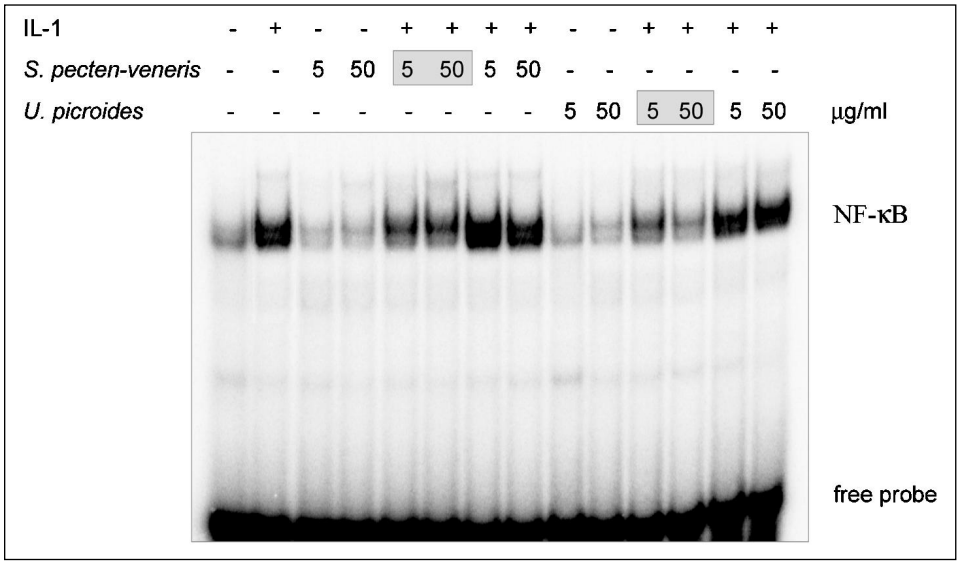

Fig. 1. The results of a typical experiment showing the effect of 2 plant extracts on IL-1-induced NF-κB activation in HepG2 cells. The cells were either pretreated (grey rectangle) with the extracts, or the addition of extracts followed IL-1 supplementation.

(*Fig. 2*). In HepG2 cells the extract of *Scandix australis* inhibited NF-κB activation more effectively when supplemented before IL-1. In HUVEC culture *Scandix australis* at the dose of 50 µg/ml totally blocked both NF-κB and AP-1 activation, irrespectively of the order of addition of the extract and IL-1. A slightly different response was observed for *Artemisia alba* extract (*Fig.2*). These results indicate that the effect of plant extracts depend also on the type of cells used in the assay and may be quite different for NF-κB and AP-1.

In order to compare quantitative effects of extracts the pictures obtained by EMSA were scanned and expressed in relative terms, assuming the value found for the IL-1-stimulated sample as 100% after subtracting values found in controls (cell cultures not exposed to IL-1 or plant extract). *Fig. 3* shows the results obtained in the experiments when plant extracts were added 5 min after IL-1 supplementation. Out of 21 plant extracts, selected for their potential anti-inflammatory properties based on the inhibition of cytokine-induced NO production in endothelial cells (see the accompanying paper by Strzelecka et al.[19]) eight extracts showed a positive response, i.e. decreased activation of NF-κB or/and AP-1.

As depicted in *Fig. 3* extracts isolated from four plant species: *Scandix australis, Artemisia alba, Thymus pulegioides and Reichardia picroides*, diminished NF-κB-specific DNA-binding activity induced by IL-1 in HepG2 cells. Two of them (*Scandix australis* and *Artemisia alba*) were active also in HUVEC cells (see *Fig.2*), while extracts from *Amaranthus sp.* and *Eryngium*

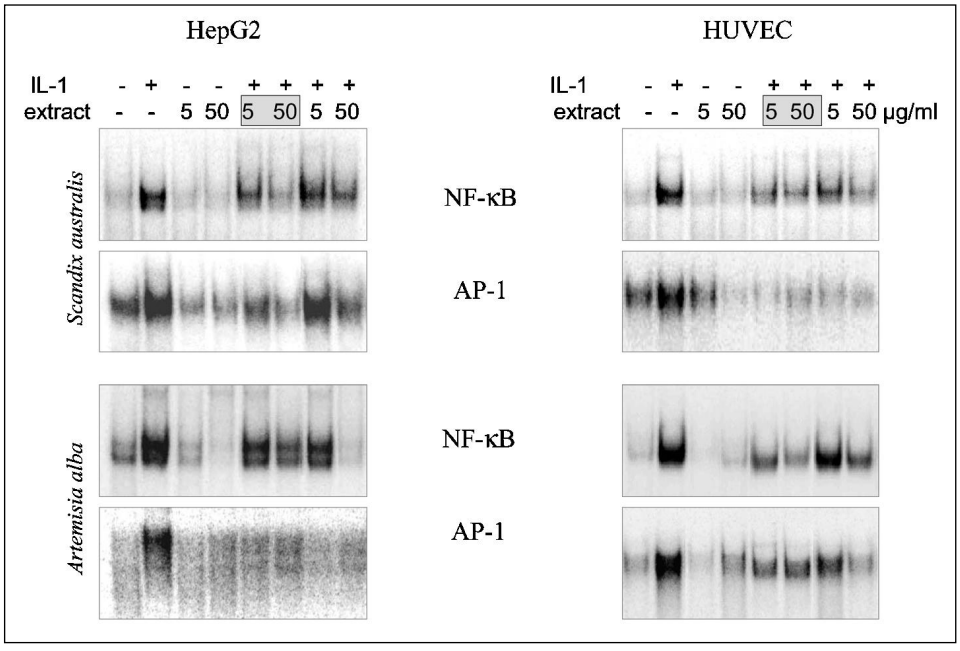

Fig. 2. Effect of extracts isolated from *Scandix australis* and *Artemisia alba* on IL-1-induced activation of NF-κB and AP-1 in HepG2 and HUVEC cells. The cells were pretreated (grey rectangle) with the extracts, or the addition of extracts followed IL-1 supplementation.

campestre inhibited NF-κB activation exclusively in HUVEC cells. As shown in *Figs 2* and *3* extracts from *Scandix australis* and *Artemisia alba* inhibit not only NF-κB but also AP-1 activation induced by IL-1 in both cell cultures, whereas other extracts (*Scandix pecten-veneris* and *Urospermum picroides*) decrease only AP-1 induction. On the other hand, the extract from *Amaranthus sp.* was active mainly in HUVEC cells (towards both NF-κB and AP-1). Interestingly, the extract from *Thymus pulegioides* down-regulated NF-κB and simultaneously enhanced AP-1 activation in HepG2 cells, while its effect in HUVEC cells was rather minor. These results suggest that down-regulation of NF-κB could be partly compensated by enhanced AP-1 activation.

Lack of the effects of tested plant extracts on the IL-6-stimulated activation of STATs in HepG2 cells

Tyrosine phosphorylation in STAT molecule induced by IL-6 enhances the expression of several genes coding for acute phase proteins (5,6). By employing EMSA with an oligonucleotide probe containing STAT3 target sequence (see Materials and Methods section) we found that IL-6 induces STAT activation in HepG2 cells but none of the tested 21 extracts was able to abolish or significantly reduce this response irrespectively whether was added before or after IL-6 (*Fig.*

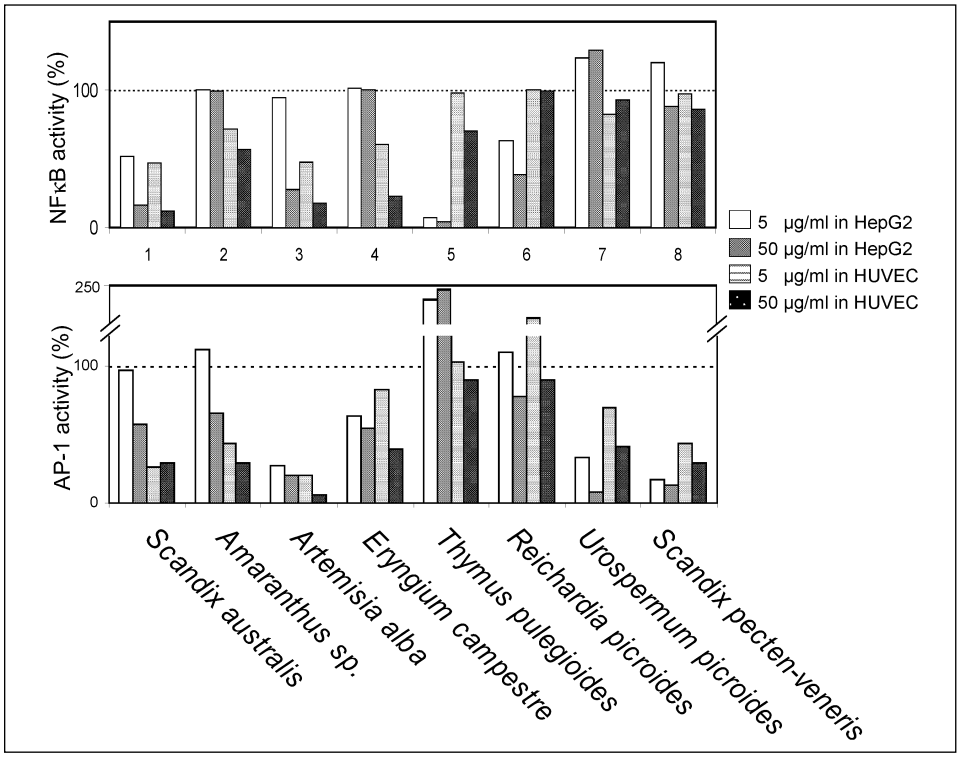

Fig. 3. Comparison of the inhibitory effects of selected plant extracts on IL-1-induced activation of NF-κB and AP-1 in HepG2 and HUVEC cells. All extracts were added 5 min after IL-1-supplementation. Transcription factors activation caused by IL-1 in the absence of plant extract = 100%. Means of 2-3 experiments.

4). This result indicates that JAK/STAT pathway is unaffected by analyzed plant extracts in HepG2 cells thus pointing to specificity of inhibition concerning other transcription factors involved in the inflammatory response: NF-κB and AP-1.

The influence of N-acetyl cysteine (NAC), quercetin, curcumin and parthenolide on NFκB and AP-1 activation in HepG2 cells

The tested plant extracts were obtained by a simple ethanol extraction (for detail see the accompanying paper by Łoboda et al [20]) and may contain several components diminishing NF-κB and AP-1 activation. Identification of these compounds by physicochemical procedures will provide a clue for the mechanism of their activity. However, the available data from the literature may be useful in defining a possible class of bioactive components. Abundant evidence indicates that some antioxidants (such as NAC) and natural products of plant origin modulate the activity of NF-κB and AP-1, as well as the expression of genes controlled by these transcription factors (26,10,14,27). In order to

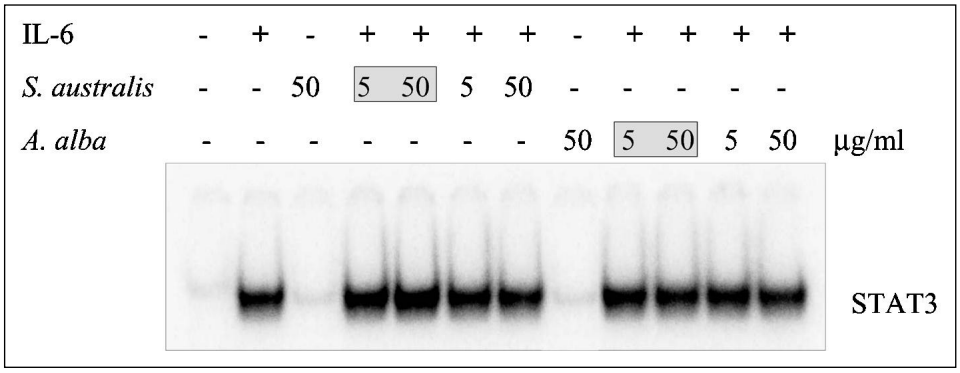

Fig. 4. Lack of the effect of analyzed plant extracts on IL-6-induced activation of STATs in HepG2 cells. The cells were either pretreated (grey rectangle) with the extracts, or the addition of extracts followed IL-6 supplementation.

validate our technique some additional experiments were carried out using cultured HepG2 cells and defined concentrations of selected compounds. Parthenolide, a sesquiterpene lactone, and quercetin, a widely distributed flavonoid, as well as curcumin (diferuloylmethane), a methylated polyphenol, are known to possess some anti-inflammatory properties and potently inhibit NF-κB and AP-1 activation (for references see [14,27,16,28]). The results of our experiments are shown in *Fig.5*. It was found that at 50 mM concentration NAC strongly inhibited activation of both NF-κB and AP-1, but only when added before IL-1. On the other hand, quercetin and curcumin were effective at micromolar concentrations towards NF-κB and AP-1 activation irrespectively of the order of addition of the tested compound and IL-1 to cultured HepG2 cells.

The effect of plant extracts on phosphorylation of MAP kinases

The down-regulated activation of transcription factors elicited by plant extracts may be due to modification of protein molecule of a given factor, or due to blocking of MAP kinase signaling pathways (9). To elucidate this problem phosphorylation of 3 MAP kinases was determined in HepG2 cells treated with some plant extracts and IL-1. Altogether, 12 plant extracts selected for their potential anti-inflammatory activity, were used in the MAP kinase phosphorylation assay. *Fig.6* shows the results of estimation of time response pattern of phosphorylation of 3 kinases and the effects of some plant extracts on p38 and JNK kinases. None of the tested extracts (final concentrations 5 or 50 µg/ml) was able to block totally phosphorylation of p38, JNK or ERK kinase. Majority of extracts showed a partial inhibition of IL-1-induced activation of one or more kinase but the results of repeated experiments were often not reproducible. The only consistent effects were: a dose-dependent inhibition of p38 phosphorylation by *Urospermum picroides*, and a slight inhibition of both

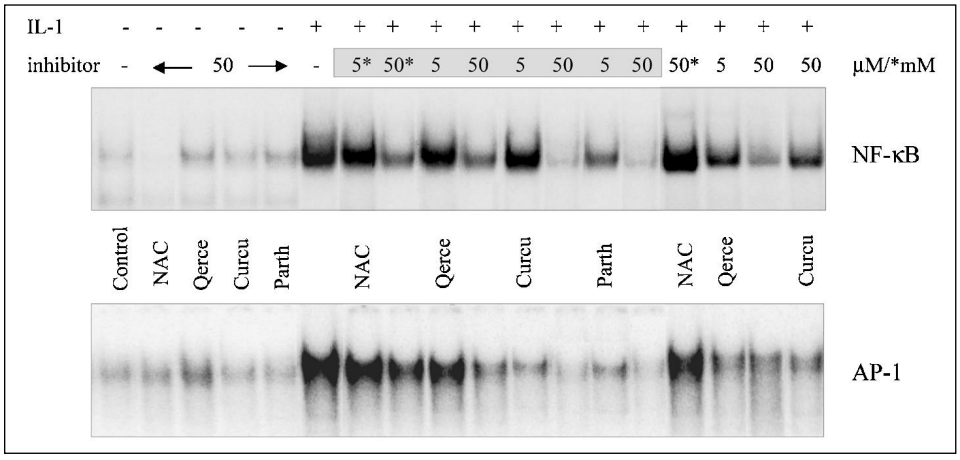

Fig. 5. The inhibitory effect of some anti-inflammatory and antioxidant compounds on NF-κB and AP-1 activation in HepG2 cells. The cells were either pretreated (grey rectangle) with the tested compound, or the compound was added to cell culture after IL-1.

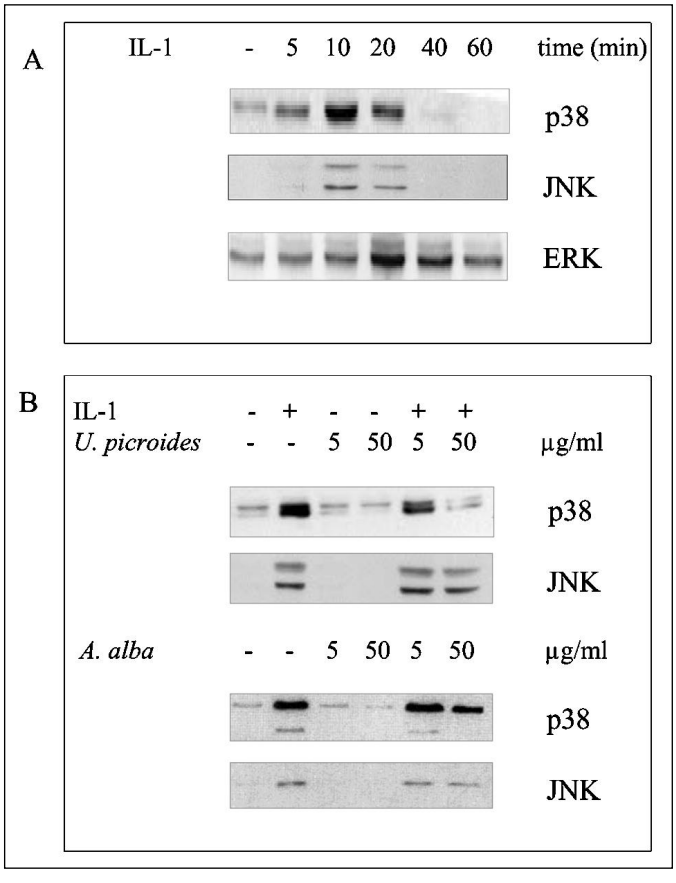

Fig. 6. Time-dependent phosphorylation of 3 MAP kinases in HepG2 cells stimulated with IL-1 (A), and the effects of extracts from *Urospermum picroides or Artemisia alba* on IL-1-induced phosphorylation of p38 and JNK kinases in cultured HepG2 cells (B). Plant extracts were added to cell culture 5 min before IL-1. For further detail see Materials and Methods section.

p38 and JNK by 50 μg/ml of Artemisia alba (*Fig.6*). On the other hand, we found that 0.1 μM quercetin totally blocked JNK phosphorylation, whereas activation of p38 and ERK kinases was partly inhibited by 10 μM quercetin (the concentration giving already some cytotoxic effects - data not shown).

DISCUSSSION

It is a widespread belief that some compounds present in edible vegetables and fruits play a beneficial role in disease prevention, or exhibit anti-inflammatory effects. The mechanism of action of such compounds, however, is in most cases not fully elucidated. The inhibition of cyclooxygenase isoenzymes involved in the synthesis of prostaglandins (28), or inhibition of pivotal transcription factors participating in the inflammatory response (11,12), may represent tissue-specific therapeutic targets. Here we describe the results of experiments aimed at finding out whether extracts of plants used in local varieties of the Mediterranean diet and showing some anti-inflammatory properties in the *in vitro* assays may act through the inhibition of NF-κB and AP-1 activation.

We have found that 8 among 21 tested extracts significantly down-regulate IL-1-induced activation of NF-κB and/or AP-1 but they all are without effect on IL-6-dependent activation of STAT3. The assayed biological activity depends to a certain extent not only on a target cell type (human hepatoma HepG2 cells and human umbilical vein endothelial cells) but also on the order of adding of IL-1 and a tested plant extract to cell culture medium (*Figs 1-3*). This may suggest that in some cases active components of the extract modify cytokine-initiated signaling cascade blocking the response to IL-1 if added earlier but are unable to stop the signal when added after IL-1 (extracts of *Scandix pecten-veneris* and *Urospermum picroides*, see *Fig.1*). It is of interest that NAC, a known antioxidant, can downregulate IL-1-induced activation of NF-κB and AP-1 only when added before the cytokine (*Fig.5*). This similarity of action may suggest that some extracts, as these two mentioned above, modulate transcription factors activation by affecting cellular redox potential. On the other hand, it seems unlikely that different responses are elicited by direct chemical interaction of IL-1 and components of the extract, although providing the experimental proof would be difficult because both tested transcription factors, NF-κB and AP-1, are very sensitive and are easily induced by change of the culture medium after addition of either IL-1 or the tested extract (data not shown).

A decreased activation of a tested transcription factor may be due to modification of its structural components, as it was reported for a sesquiterpene lactone-elicited blocking of Cys residue in p65 subunit of NF-κB (30), or due to inhibition of the corresponding signaling cascade. To confirm or exclude this possibility we measured the effect of some plant extracts on phosphorylation of MAP kinases. As shown in *Fig. 6* a very few extracts were able to reduce in a

reproducible manner the activation of one of the kinases. Moreover, comparison of the effect of extracts with their content of polyphenols listed in *Table 1* suggests that polyphenols cannot be solely responsible for the observed biological activity of plant extracts although quercetin was able to inhibit selectively the JNK kinase (data not shown).

Identification of active compounds in the tested extracts will require fractionation and employment of physico-chemical analysis but it appears that the described biological activities cannot be attributed to single-type components, such as plant polyphenols. *Table 1* has been compiled from the data on the polyphenol contents in the tested plant extracts (data provided by the Consortium) and their potency in the inhibition of NF-κB and AP-1 (see *Fig. 3*). It can be easily noted that the extract from *Thymus pulegioides*, very rich in polyphenols, inhibits only NF-κB in HepG2 cells, and even enhances AP-1 activation in these cells. On the other hand, the extract from *Amaranthus sp*. has a low polyphenol contents but efficiently inhibits activation of both NF-κB and AP-1 in HUVEC culture.

It should be mentioned here that some of the plant extracts studied by us were active in several cell types and in various assays: *Artemisia alba* (induced NO production - [19]) or *Scandix australis* (inhibition of oxidative DNA damage - [31]). We suggest that these 2 extracts will be used for further detailed analysis and for *in vivo* tests before they may be recommended as possible nutraceuticals. Moreover, before final positive conclusion is reached genetic variability of tested plant species, their growing conditions and harvesting procedures must be

Table 1. The inhibitory effect of plant extracts on NF-κB and AP-1 activation in comparison with contents of polyphenols in these extracts

The data on polyphenol contents were provided by the Consortium "Local Food Nutraceuticals" whereas values showing the effect of these plant extracts are taken from *Fig.3*. They are presented as percentage of NF-κB and AP-1 activity in cell culture supplemented with IL-1 (100%), but with only one extract concentration (the numbers indicate percentage of remaining transcription factor binding activity after treatment of cell culture with 50 μg/ml of the extract).

Extract code No.	Plant species	Family	polyphenol contents (mg/g)	NFκB % of IL-1 extract 50μg/ml		AP-1 % of IL-1 extract 50μg/ml	
				HepG2	Huvec	HepG2	Huvec
2009	*Scandix australis*	*Apiaceae*	191.55	16.6	12.3	57.3	29.1
1026	*Amaranthus sp.*	*Amaranthaceae*	25.89	99.4	57.1	65.2	29.3
4003	*Artemisia alba*	*Asteraceae*	229.90	27.7	17.4	20.2	6.3
2028	*Eryngium campestre*	*Apiaceae*	55.10	101.2	22.7	54.1	38.9
3017	*Thymus pulegioides*	*Lamiaceae*	435.10	4.5	70.7	240.7	89.5
1014	*Reichardia picroides*	*Asteraceae*	318.49	38.7	99.3	77.8	89.8
1020	*Urospermum picroides*	*Asteraceae*	245.85	129.4	93.2	8.5	41.4
1016	*Scandix pecten-veneris*	*Apiaceae*	130.65	88.5	86.3	13.0	29.2

subjected to a close scrutiny in order to fulfill the criteria of truly natural health products (32).

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