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Bimodal effect of humic acids on the LPS-induced TNF-α release from differentiated U937 cells

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Abstract

Humic substances (HS) have been reported to possess anti-inflammatory as well as pro-inflammatory properties. The anti-inflammatory activity was demonstrated in the rat paw edema model and we found a preliminary explanation in the 5-lipoxygenase inhibitory effect of humic acids (HA). The pro-inflammatory activity is reflected by the production and release of pro-inflammatory cytokines in HA-treated neutrophilic granulocytes. With regard to the potential use of HA as antiviral and UV-protective agents it appears advisable to investigate the role of HS in the inflammation process in more detail. Hence we tested four different HS preparations – two naturally occurring HA from the Altteich peatland in Germany, one fulvic acid (FA) preparation from a Finnish spruce forest and a synthetic HA-like polymer (caffeic acid oxidation product, KOP) for their influence on the lipopolysaccharide (LPS)-induced TNF-α release in human U937 cells. In addition, the cytotoxicity of HS was determined.

The results demonstrate a concentration-dependent bimodal effect of HA on the TNF-α release of differentiated LPS-stimulated U937 cells for both the natural black peat HA from the Altteich peatland and the HA-like polymer KOP. Low HA concentrations (10–80 µg/ml) enhanced the TNF-α release by up to threefold (pro-inflammatory activity), while HA concentrations >100 µg/ml reduced it about 10-fold (anti-inflammatory activity). FA failed to enhance TNF-α release, but reduced it at higher concentrations (>200 µg/ml) by the half. Brown water HA did not exert any significant effect on TNF-α release. No HS-stimulated TNF-α release was also observed in the absence of exogenously supplied LPS. This means that HS, unlike endotoxin, are no inflammation-causing agents for LPS-untreated cells. Differences in the effect of individual HS on TNF-α release are discussed in connection with the polyanionic character of HS, their molecular mass distribution and the hitherto imperfectly known chemical structure.
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Introduction

Natural humic substances (HS) represent the whole ensemble of yellow to dark brown coloured organic molecules that originate preferably from decaying plant materials during humification. According to their

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solubility in alkaline and/or acid media three major HS fractions are distinguished: the practically insoluble humin, the alkali-soluble humic acids (HA) and the fulvic acid (FA) fraction, which is soluble in alkaline, neutral and acid media. In contrast to the insoluble humin, HA and a large percentage of FA have polyanionic properties with phenolic hydroxyl and carboxyl groups as the most common functional groups (Perdue, 1985). Although considerable effort has been made to elucidate the chemical structure of HA, a generally valid principle of their basic structure has not yet been found due to the highly complex composition of natural HS. The analysis of simple synthetic HA is therefore considered a promising attempt to address the problem in future investigations (Klöcking et al., 2008).

The main natural resource of HS is peat containing more than 30% organic substances (mainly HS) in the dry mass. HS considerably contribute to the extraordinary heat capacity of peat (Young and LeBoeuf, 2000), which forms the physical basis of traditional peat applications to gynecological and rheumatic disorders (Baatz, 1988; Kleinschmidt, 1988).

Associated with the increasing knowledge of the antiviral and UV-B protective effect of HA (Klöcking et al., 2000, 2004; Klöcking and Helbig, 2001), the potential use of HA to prevent UV-B-induced skin diseases has become a novel focus of interest. Contrary to peat application as peat bath or peat packs that contain HA to a large extent undissolved in the peat mass, isolated HA (mostly in the form of soluble sodium or ammonium salts) have direct contact to the body. For this reason the question of skin compatibility of HA and their influence on inflammatory processes is of particular importance.

HS have been reported to possess anti-inflammatory as well as pro-inflammatory properties. The anti-inflammatory activity was demonstrated in the paw oedema model and in the granuloma pouch model in rats (Taugner, 1963; Klöcking et al., 1968). On the biochemical level, the inhibition of the 5-lipoxygenase pathway of the arachidonic acid (AA) cascade by naturally occurring HA as well as synthetic HA-like polymers provided an explanation for the anti-inflammatory effect of HA (Schewe et al., 1991). Moreover, the suppression by 65–90% of the heat-induced AA release by sodium humate and the HA-like caffeic acid oxidation product (KOP) supported the characterization of HA as anti-inflammatory substances (Dunkelberg et al., 1997; Klöcking et al., 1997). On the other hand, HA have been shown to stimulate the release of the pro-inflammatory tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) in neutrophilic granulocytes (Riede, 2000; Zeck-Kapp et al., 1991). Inglot et al. (1993) already reported on a low-molecular HA-containing fraction of a peat extract (Tołpa peat preparation, TPP) that induces TNF-α and interferon in human peripheral blood leukocytes.

The pro-inflammatory cytokine TNF-α is produced by different cell types, particularly macrophages and monocytes, in response to inflammatory stimuli such as endotoxin lipopolysaccharide (LPS) of gram-negative bacteria and peptidoglycan, lipoteichoic acid, and lipoproteins from gram-positive bacteria (Fan et al., 2005). LPS stimulates the TNF-α release also in differentiated, macrophage-like U937 cells. These cells have been applied as an in vitro model to investigate the TNF-α response to several bacterial infections (Caron et al., 1994; Roberts et al., 1997) and were chosen for the investigations in this study.

Four different HS including two HS from Altteich peatland in Germany, fulvic acids (FAs) from a Finish spruce forest and the synthetic HA-like caffeic acid oxidation product KOP were tested for their influence on the LPS-induced TNF-α production in human U937 cells. To avoid cross-reactions of HS with components of the TNF-α sandwich ELISA, the test protocol was adapted according to special requirements. In addition, the cytotoxicity of the test substances to U937 cells was determined using the XTT tetrazolium reduction assay EZ4U.

The results demonstrate a concentration-dependent bimodal effect of HA on the LPS-stimulated TNF-α release in human U937 cells, which is not detectable in LPS-unstimulated and in FA-treated cells. These findings are discussed mainly with respect to the polyanionic character of HA, their molecular mass distribution and possible differences in the hitherto imperfectly understood chemical structure of HA and FA.

Materials and methods

Chemicals

Lipopolysaccharide (LPS) from E. coli, Serotype 055:B5, all-trans retinoic acid (vitamin A acid) and cholecalciferol (vitamin D_3) were purchased from Sigma-Aldrich, Deisenhofen, Germany. RPMI 1640 medium (with and without phenol red and L-glutamine) was obtained from C-C-Pro, Oberdorla, Germany. Fetal bovine serum (FBS) and Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+} were from Biochrom, Berlin, Germany. All other chemicals and biochemicals were purchased from commercial sources and were of the highest quality available.

Cell culture and differentiation

The promonocytic human cell line U937 (ATTC CRL 1593) was cultivated in 10% FBS-containing RPMI

1640 medium at 37 °C and 5% CO_2 in a humidified atmosphere. The differentiation towards macrophage-like cells necessary for U937 cells to produce TNF-α was performed on the basis of Caron et al. (1994). Instead of 10^{-7} mol/l all-trans retinoic acid and 10^{-7} mol/l 1,25 dihydroxyvitamin D_3, a concentration of 10^{-6} mol/l all-trans retinoic acid and vitamin D_3 at a concentration of 10^{-6} mol/l were applied to the cell medium for 3 days of cultivation.

Test substances

Black peat humic acids (*BLP-HA*) were extracted from peat of the Altteich Peatland, situated in the Lower Lusatia region, Germany. For this, 50 g of peat was suspended under agitation (300 rpm) in 300 ml deionised water and extracted with 0.2 mol/l sodium hydroxide for 1 h. Undissolved particles were removed by centrifugation (3500 g, 20 min) followed by membrane filtration (cellulose acetate filter 0.45 μm, Sartorius AG, Göttingen, Germany). The dissolved BLP-HA was precipitated with 1.0 mol/l HCl at pH 1.5–2.0, separated from the solution by centrifugation (3500 g, 20 min), rinsed twice with deionised water and lyophilised. The molecular mass distribution of BLP-HA determined by high-performance size exclusion chromatography (HPSEC) is characterized by two peaks, one minor at about 27.6 kDa and a major second one at about 2.0 kDa (Kinne et al., 2005).

Brown water humic substances (*BRW-HS*) were obtained from the drainage channel that pervades the Altteich Peatland. The brown water was concentrated by means of ultrafiltration through a 1.0 kDa OMEGA polyethersulfon membrane (PALL GmbH, Dreieich, Germany) followed by lyophilisation of the retentate. The molecular mass distribution shows only one single peak at about 1.3–1.4 kDa (Kinne et al., 2005).

Fulvic acid (*FA*) was kindly supplied by Prof. Dr. Martin Hofrichter (Unit of Environmental Biotechnology, International Graduate School Zittau, Germany). It was prepared from a Finish spruce forest according to the IHSS method (Aiken, 1985). The maximum of the molecular mass distribution curve was found at 1.09 kDa (Kinne et al., 2005).

Na-KOP, a synthetic HA-like polymer, has been synthesized from caffeic acid by periodate oxidation as described earlier (Helbig et al., 1997). The dark brown product is well soluble in water and weak alkaline media. The molecular mass distribution shows a minor peak at about 22 kDa and a major second one at about 1.6 kDa (Kinne et al., 2005). Na-KOP has been characterized as an HA-like polymer by FT-IR spectroscopy and classical wet chemical methods (Helbig et al., 1997), by ^{13}C and ^1H NMR spectra (Hänninen et al., 1987) and by TMAH thermochemolysis in combination

with pyrolysis gas chromatography/mass spectroscopy (Poerschmann et al., 2003).

Determination of cytotoxicity

The cytotoxicity of HS was determined in 96-well flat-bottom microtitre plates using the XTT tetrazolium reduction assay EZ4U (Biozol, Eching, Germany). The assay has been applied for U937 cells by Klöcking et al. (1998) and was slightly modified concerning cell number and incubation time. Briefly, 100 μl of serum-free RPMI 1640 containing 10^6 cells/ml was used per well and 100 μl of serially diluted HS in colourless RPMI medium was added. A set of at least six wells was reserved for cell controls receiving medium without HS. Cells were incubated at 37 °C for 1 and 24 h, respectively, before the XTT reagent was added. After further incubation at 37 °C for 3 h, the optical density (OD) of the produced formazan was measured at 492 nm (reference wave length: 620 nm) in a microplate reader (Synergy^{TM}, BIO-TEK$^®$, Inc., Winooski, VT, USA). The percentage of cytotoxicity was calculated from the measured OD values using the following equation: $[OD_{xo} - OD_{xi}/OD_{xo}] \times 100\%$, where x_i and x_o stand for the arithmetic mean of sample values and cell controls, respectively.

Determination of TNF-α release

Differentiated U937 cells were centrifuged at 200 g, washed twice with PBS for 5 min and were resuspended at a density of 10^6 cells/ml in 10% FBS containing RPMI 1640.

Before starting the experiment, 50 μl of serially diluted HS in serum-free, colourless RPMI 1640 followed by 50 μl of LPS (2 μg/ml) in FBS-containing RPMI were filled into the test wells of a 96-well plate. One hour later, 100 μl of the cell suspension was added. Besides these samples provided for the dose–response curve, cell controls w/o LPS and HS, cells with LPS but w/o HS for determining the basal TNF-α release, and cells with HS but w/o LPS to detect possible cross-reactivity of HS with components of the ELISA Kit, were included in each experiment. After 4 h incubation at 37 °C the plates were centrifuged at 300 g for 5 min and 60 μl of each supernatant was transferred into a micro-test tube and stored at −80 °C until the TNF-α determination was carried out.

The TNF-α concentration was determined using the BioLegend Human TNF-α ELISA MAX^{TM} Kit (Biozol, Eching, Germany) according to the manufacturer's instructions. Because of the high cross-reactivity of HA with components of the sandwich ELISA Kit and to achieve a high sensitivity for TNF-α in the concentration range of interest (4–125 pg/ml), the method was slightly modified. Instead of the recommended BioLegend assay

diluent, a solution of 0.5% BSA in PBS was used as diluent (for the detection antibody and the avidin–horseradish peroxidase) and blocking reagent. To achieve the same final concentration of BSA in each well, the samples and the TNF-α standard row were diluted with FBS-containing RPMI 1640 medium supplemented with 1.0% and 0.5% BSA, respectively. The test plate was kept sealed for 2 h and slightly shaken at about 140 rpm. The remaining steps (addition of detection antibody, avidin–horseradish peroxidase and tetramethylbenzidine, TMB) were carried out as described in the manufacturer's instructions. After acidifying with $1 \, mol/l \, H_2SO_4$ the absorption of the final TMB reaction product was measured at 450 nm (reference wave length: 570 nm). The TNF-α concentration in the samples was calculated on the basis of the regression equation obtained from the standard row absorption data.

Data analysis

Each experiment was processed at least three times; XTT and TNF-α assay were performed in triplicate. In the figures, the results of the cytotoxicity are given as the arithmetic mean ± standard error of the mean (SEM). The results of the TNF-α release correspond to the arithmetic mean ± standard deviation (SD) of single values. The calculation of the statistical significance included all the obtained experimental data. Student's t-test for independent samples was used. If the results did not comply with the requirements for the t-test, the non-parametric U-test according to Wilcoxon, Mann and Whitney was applied. Values for $p \leqslant 0.05$ were considered significant.

Results and discussion

In a first step of investigations, the cytotoxicity of HS was determined by exposing U937 cells to graduated HS concentrations from 1 to 1000 μg/ml for 1 and 24 h. The results show that three (BRW-HS, FA and Na-KOP) of the four investigated HS are well tolerated by the cells and that this behavior is largely independent of the duration of exposure (Fig. 1A–D). Although a weak tendency of increasing cytotoxicity could be observed for BLP-HA concentrations $> 500 \, \mu g/ml$, a level of 20% cytotoxicity has not been exceeded in any case. Thus, significant cytotoxic effects of the test substances can be widely excluded for the concentration range used in the TNF-α release experiments.

The results of the TNF-α release experiments in the presence of BLP-HA and BRW-HS are shown in Fig. 2. BLP-HA, when used at low concentrations (10–80 μg/ml), exerts a significant increase in the TNF-α release from LPS-stimulated, differentiated U937 cells. In comparison to untreated cells, cells treated with 32 μg/ml BLP-HA cause a nearly threefold TNF-α release after 4-h LPS stimulation. At concentrations greater than 100 μg/ml, the curve turns downward and at 512 μg/ml BLP-HA TNF-α release is reduced by about 80%. In the case of BRW-HS, no significant effect on TNF-α release could be observed (Fig. 2).

The dose–response curve of the synthetic HA-like polymer Na-KOP takes a similar course as that of BLP-HA (Fig. 3). Na-KOP at concentrations between 10 and 100 μg/ml causes a twofold increase of TNF-α release, while concentrations greater than 100 μg/ml reduce the TNF-α release about 10-fold. Unlike HA and Na-KOP, FA at low concentrations do not enhance the
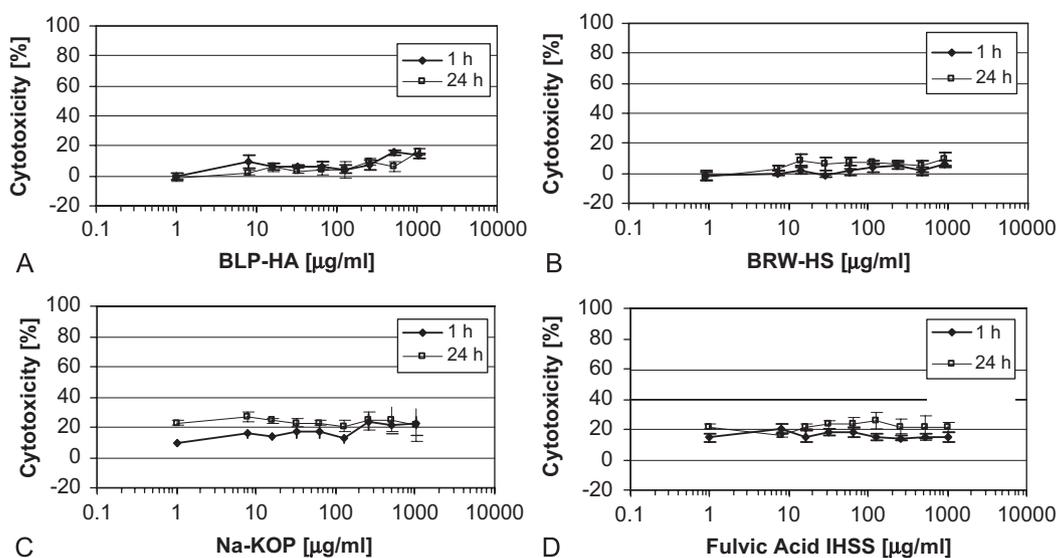

Fig. 1. Cytotoxicity of Altteich black peat humic acids (BLP-HA), Altteich brown water humic substances (BRW-HS), HA-like caffeic acid oxidation product (Na-KOP) and a Finish fulvic acid preparation after 1- and 24-h treatment of U937 cells. Cytotoxicity is represented by mean values ± SEM of at least three independent experiments with triplicate.

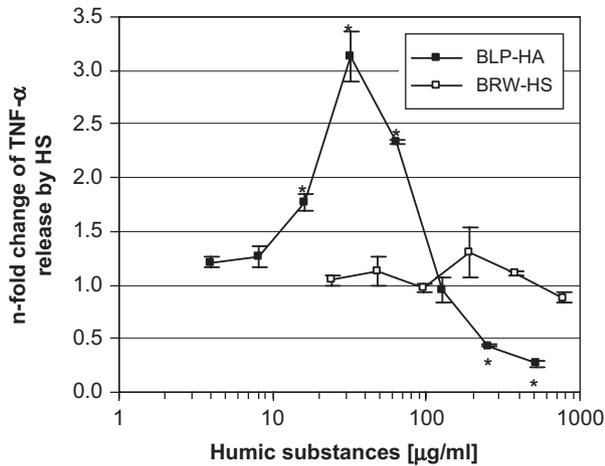**Fig. 2.** Influence of Altteich black peat humic acids (BLP-HA) and Altteich brown water humic substances (BRW-HS) on the TNF-α release from differentiated, LPS-stimulated U937 cells (mean ± standard deviation, $n = 3$). The results are expressed as fold TNF-α release of LPS-stimulated, but not HS-treated cells, with $*p \leqslant 0.05$. The data of one experiment, representative of three (BRW-HS) or four (BLP-HA) performed, are shown.
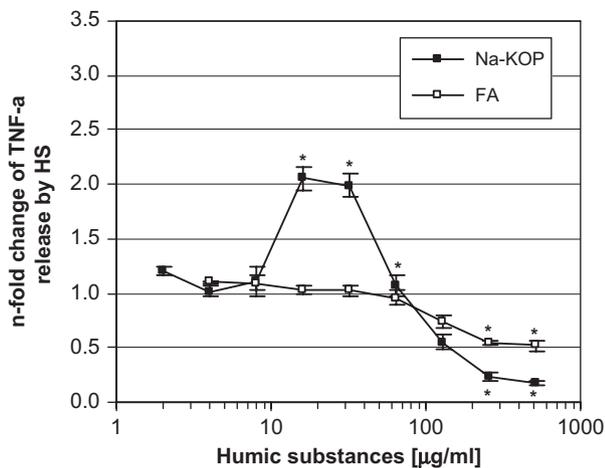

Fig. 3. Influence of caffeic acid oxidation product (Na-KOP) and a Finish fulvic acid preparation (FA) on the TNF-α release of differentiated, LPS-stimulated U937 cells (mean ± SD, $n = 3$). The results are expressed as fold TNF-α release of LPS-stimulated, but not HS-treated cells, with $*p \leqslant 0.05$. The data of one experiment, representative of three (FA) or four (Na-KOP) performed, are shown.

TNF-α release, but reduce it at higher concentrations ($> 200 \, \mu g/ml$) by the half (Fig. 3). No inflammation-inducing effect of HS could be detected in LPS-untreated cells (data not shown).

Considering the results of this study, it is obvious that HS influence LPS-stimulated human U937 cells differently. For the first time it is shown that naturally occurring as well as synthetic HA can exert a

concentration-dependent opposite influence on TNF-α release. Effects of this type are well-known in pharmacology and usually referred to as bimodal (Desai et al., 1973). This means that low doses of the active agent, e.g. an immunomodulator, show an activating effect on target cells (lymphocytes, monocytes, macrophages), whereas high doses exhibit in general a suppressing effect (Matar et al., 2002; López et al., 2006).

Our observations are consistent with experimental data of Klingner (2002) who studied the interaction of a panel of synthetic HA-like polymers with phospholipase A_2 (PLA$_2$), the key enzyme of the AA cascade. The enzyme was found to be strongly stimulated by low ($0.1–1 \, \mu g/ml$) and normalized or slightly inhibited by higher ($> 10 \, \mu g/ml$) HA concentrations, suggesting a modulating potency of HA in phospholipid metabolism (Klöcking and Helbig, 2001).

The bimodal effect of HA on TNF-α release is of particular interest, as this cytokine plays a critical role in initiating immune response reactions against microbial infections. Excessive TNF-α levels, however, are associated with many painful inflammatory diseases such as rheumatoid arthritis (El Bahri et al., 2007), osteoarthritis (Iannone and Lapadula, 2003), Morbus Crohn (Sartor, 2006), pelvic adhesions (Cheong et al., 2002) and chronic airway inflammation (Kim and Remick, 2007). The results of the present study suggest that unlike other TNF-α antagonists (e.g. the therapeutically utilized infliximab) BLP-HA and KOP are able to support both the unspecific immune response (pro-inflammatory effect) and the protection of cells from uncontrolled high TNF-α production (anti-inflammatory effect). The indifferent behavior of BLW-HA and the rather low anti-inflammatory effect of FA may be due to their low molecular mass and to differences in activity-relevant chemical structures (Klöcking et al., 2006) among HS. The elucidation of these relationships requires a larger panel of natural and synthetic HS and remains the subject of further investigations.

Other reasons for changes in TNF-α release such as the different origin of test substances or the preparation methods used have obviously less influence on the results. BLP-HA of the Altteich peat and the synthetic HA KOP, for example, have similar effects on TNF-α release, although they are quite different in origin and preparation. The same is true for BRW-HA and FA.

The mechanism of how HA affect the TNF-α release of U937 cells is so far unclear. One could speculate that the stimulating and/or inhibiting effect of HA may be related to the negative charged functional groups of HA because other polyanionic compounds also induce changes in cytokine production. Chang et al. (1994) comparing the effect of different polyanionic compounds on TNF-α production in LPS-activated THP-1 cells found synthetic polysulfated hyaluronic acid with a

sulfate hexosamine molar ratio of 3.9 to be a strong inhibitor of TNF-α production. In contrast, naturally polysulfated glycosaminoglycans, such as chondroitin-4-sulfates, keratin sulfate, heparan sulfate and heparin, failed to inhibit TNF-α production or, as observed for chondroitin-6-sulfate, even increased it significantly. An explanation for the enhanced inflammatory response of LPS-stimulated monocytes to polyanionic compounds was suggested by Heinzelmann and Bosshart (2005). The authors found that sulfated polysaccharides like heparin or dextrane sulfate associate with the LPS-binding serum protein (LBP), displace LPS and accelerate the transfer of LPS to the membrane receptor CD14, thus enhancing the inflammatory response. A different target that provides a possible explanation for the anti-inflammatory activity of HA was found by Gau et al. (2000). Investigations in human umbilical vein endothelial cells (HUVEC) revealed that HA suppress the LPS-stimulated activation of NF-kappaB, a key factor in the innate immune response, in a dose- and time-dependent manner.

In conclusion, the results demonstrate for the first time a concentration-dependent bimodal effect of HA on the LPS-induced TNF-α release in U937 cells, which was not found for FA. Regarding the biocompatibility of HS, the presented results give no indication for an inflammation-inducing effect of HS in normal, LPS-untreated human cells. However, the TNF-α-enhancing effect of low HA concentrations to LPS-stimulated cells requires further detailed investigations of a larger panel of natural and synthetic HS in LPS-dependent as well as LPS-independent inflammation models.

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