In vitro investigations on the effect of peat humic substances on inflammation

Renate Klöcking[1], Ralf Junek[2], Carola Kleiner[3], Roland Schubert[4] and Juergen I. Schoenherr[5]

Research Institute for Peat and Natural Products, Hochschule Zittau/Görlitz (FH) – University of Applied Sciences, Friedrich-Schneider-Str. 26, D-02763 Zittau, Germany,
[1] Phone: +49 3583 61 2303, Fax: +49 3583 61 2300, e-mail: rkloeck@hs-zigr.de
[2] Phone: +49 3583 61 2331, Fax: +49 3583 61 2300, e-mail: Ralf.Junek@gmx.net
[3] Phone: +49 3583 61 2362, Fax: +49 3583 61 2300, e-mail : ckleiner@hs-zigr.de
[4] Phone: +49 3583 61 1845, Fax: +49 3583 61 1740, e-mail: RSchubert@hs-zigr.de
[5] Phone: +49 3583 61 2304, Fax: +49 3583 61 2300, e-mail: j.schoenherr@hs-zigr.de

Summary

With the objective of understanding the role of humic substances in inflammation, the influence of humic and fulvic acids on the release of the inflammation marker TNF-α in both lipopolysaccharide (LPS) stimulated and non-stimulated U937 cells was investigated. Humic substances were isolated by conventional alkaline extraction and acid precipitation methods from a peatland in the Lower Lusatia region (Germany). Using a sandwich ELISA technique for TNF-α determination the results show that neither humic nor fulvic acids develop a pro-inflammatory effect in non-stimulated cells. In LPS-stimulated cells, however, low humic acid concentrations cause a significant increase of TNF-α release which is reversed at higher humic acid concentrations (anti-inflammatory effect). Fulvic acids did not activate LPS-induced cellular defence reactions. Basing on the results the necessity of pre-examining the TNF-α releasing potential of humic substances intended for cosmetic, body care and medical use should be considered.

Key index words: peat, humic substances, humic acids, fulvic acids

Introduction

The anti-inflammatory effect of peat and peat humic substances (HS) is one of the oldest and most popular healing promises made for curative peat application. The anti-inflammatory effect was experimentally 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 cascade by naturally occurring humic acids (HA) as well as of synthetic HA-like polymers provided an explanation for the anti-inflammatory effect of HA (Schewe *et al.*, 1991). In the last decades, however, pro-inflammatory properties of HS were also reported (Inglot *et al.*, 1993) and 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). With the objective of better understanding the role of HS in inflammation and cellular defense, the present study addresses the influence of different HS preparations on the release of the inflammation marker TNF-α in differentiated LPS-stimulated and non-stimulated human U937 cells.

Materials and methods

Humic substances

Black peat humic acids (BLP-HA) were extracted from peat of the Altteich Peatland (Saxonian Lower Lusatia, Germany) using 0.2 mol/l sodium hydroxide. After removing undissolved particles by centrifugation and membrane filtration, HA were precipitated with 1 mol/l hydrochloric acid at pH 1.5–2.0, separated from the solution by centrifugation, rinsed with demineralised water and lyophilised. The molecular mass distribution of BLP-HA determined by high performance size exclusion chromatography (HPSEC) is characterised by peaks at 27.6 kDa and 2.0 kDa (Kinne, 2005).

Brown water humic substances (BRW-HS) consisting mainly of water- and acid-soluble fulvic acids (FA), 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 polyethersulfone membrane followed by lyophilisation of the retentate. The molecular mass distribution of BRW-HS showed only one peak in the range of 1.3-1.4 kDa (Kinne, 2005).

Determination of cytotoxicity

Human promonocytic U937 cells (ATCC CRL 1593) were cultivated in RPMI 1640 medium containing 10% foetal bovine serum. The cytotoxicity of HS was determined in 96-well flat-bottom microtitre plates using the XTT tetrazolium reduction assay EZ4U (Biozol, Eching, Germany). The test volume was 200 µl per well and contained $2*10^5$ cells and serially diluted HS in serum-free, colorless RPMI 1640 medium. Cell controls receiving the same medium were kept HS-free. The plates were incubated at 37° C/5% CO_2 for 1 and 24 h, respectively, before the XTT reagent was added. To assess the cytotoxicity of the test compounds, half-maximum cytotoxic concentrations (CC_{50}) were calculated. Details of the method have been published elsewhere (Klöcking *et al.*, 1995).

TNF-α assay

In-vitro experiments on TNF-α release were performed in differentiated U937 cells. The basic level of TNF-α release was induced with the bacterial lipopolysaccharide (LPS). Before starting the experiment, 50 µl of serially diluted HS in serum-free, colorless 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, several cell controls with and without LPS and HS, respectively, were included in each experiment. After four hours incubation at 37° C the plates were centrifuged at 300 g and TNF-α was determined in the supernatant using the BioLegend Human TNF-α ELISA MAX™ Kit (Biozol, Eching, Germany). The absorbance of the final reaction product of this multistep procedure, tetramethylbenzidine, is measured at 450 nm (reference wave length: 570 nm). The TNF-α concentration in the samples is calculated based on the regression equation obtained from the standard row absorption data.

Results

Cytotoxicity

The cytotoxicity of HS was primarily determined to exclude toxic effects of the test substances on the experimental results of the TNF-α release test. Towards this end U937 cells were exposed to graduated HS concentrations from 1 µg/ml to 1000 µg/ml for 1 and 24 h. The results show that the fulvic acid-like BRW-HS are well tolerated by the cells and that this behavior is largely independent of the duration of exposure (Fig. 1). A weak tendency of increasing cytotoxicity was observed for BLP-HA concentrations >500 µg/ml, but in no case a level of 20 % has been exceeded. This means that significant cytotoxic effects of the test substances can be widely excluded for the concentration range used in the TNF-α release experiments.

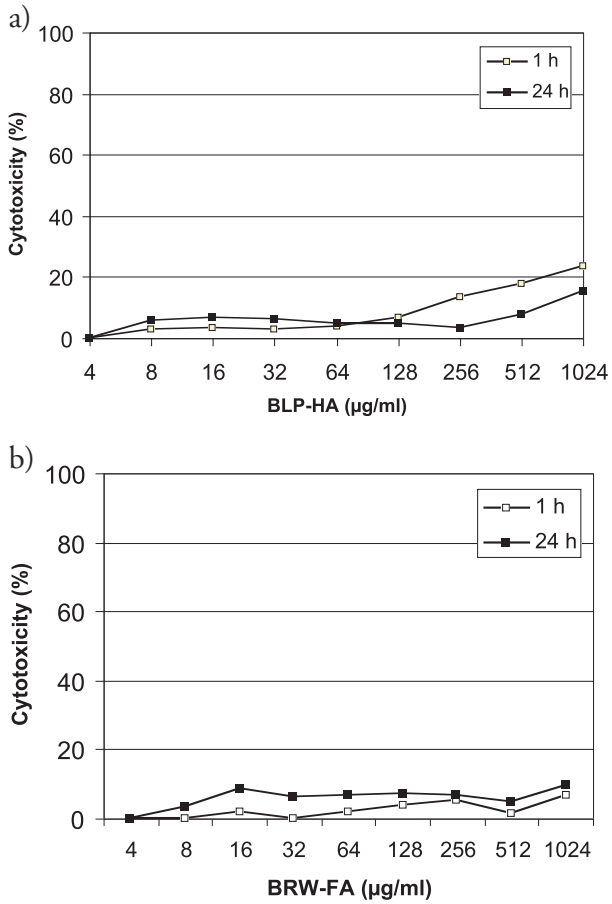

Figure 1. Cytotoxicity of (A) Altteich black peat humic acids (BLP-HA) and (B) Altteich brown water fulvic acids (BRW-FA). Cytotoxicity is represented by mean values of at least three independent experiments with triplicate.

TNF-α release

In a first step of investigations, HS were examined for inducing TNF-α release in non-stimulated U937 cells. The results revealed that neither HA from the Altteich peat (peak molecular masses 27.6 and 2.0 kDa) nor FA from the Brown Water humic substances(peak molecular mass (1 kDa) exert a pro-inflammatory LPS-like effect on differentiated non-stimulated U937 cells (data not shown). However, in LPS-stimulated cells, low humic acid concentrations cause a significant increase of TNF-α release which is reversed at higher humic acid concentrations (Fig. 2). Fulvic acids failed to enhance TNF-α release, and showed only a negligible anti-inflammatory effect at higher substance concentrations. Therefore it may be suggested that the pro-inflammatory effect of HA is limited to the high molecular fraction as it was not observed with the low-molecular FA.

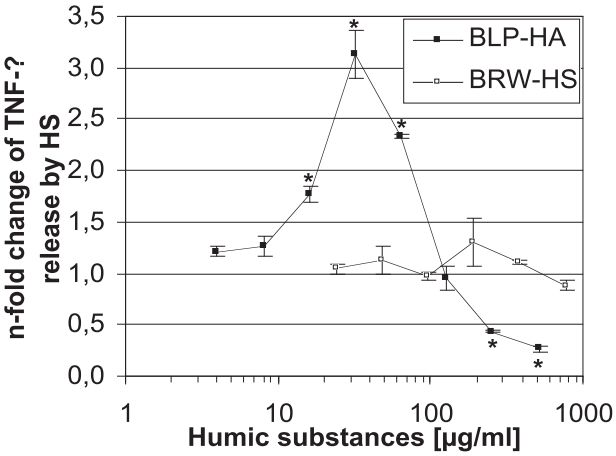

Figure 2. Influence of Altteich black peat humic acids (BLP-HA) and Altteich brown water humic substances (BRW-HS) on the TNF-α release from differentiated, LPS-stimulated U937 cells (mean ± standard deviation, n = 3). The results are expressed as fold TNF-α release of LPS-stimulated, but not HS-treated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

Conclusion

HA in the presence of LPS have significant effects on the TNF-α release of U937 cells. The effects depend on substance concentration and the molecular mass distribution of the isolated HS. The increase of TNF-α release

induced by low HA concentrations may suggest an activation of LPS-induced cellular defence reactions the escalation of which is prevented by increasing the HA concentration. Investigations on the mechanism by which HA act either as activators or as inhibitors of the TNF-α release are in progress.

Acknowledgements

This study is part of a research project financed by the German Federal Ministry for Education and Research, FKZ 1746X06.

References

Inglot, A.D., Zielińska-Jenczylik, J. and Piasecki, E. (1993). Tołpa peat preparation (TPP) induces interferon and tumor necrosis factor production in human peripheral blood leukocytes. *Arch. Immunol. Ther. Exp.* (Warsaw) **41**, 73-80.

Kinne, M. (2005). *Isolation and characterization of humic substances*. Master's Thesis, Coventry University (UK).

Klöcking, R., Hofmann, R. and Mücke, D. (1968). Experimental studies in animals on the antiphlogistic activity of humates. *Arzneimittelforschung* (Drug Research) **18**, 941-942.

Klöcking, R., Schacke, M. and Wutzler, P. (1995). Primary screening of antiherpetic compounds with EZ4U. *Chemotherapie J.* **4**, 141-147.

Riede, U.N. (2000). Pro-inflammatory effect of humic substances on epidermis cells. 53th Congress of DGGG – Deutsche Gesellschaft für Gynäkologie und Geburtshilfe, München, Germany (http://www.thieme.de/abstracts/gebfra/abstracts2000/fr_inhalt.html).

Schewe, Ch., Klöcking, R., Helbig, B. and Schewe, T. (1991). Lipoxygenase-inhibitory action of antiviral polymeric oxidation products of polyphenols. *Biomed. Biochim. Acta* **50**, 299-305.

Taugner, B. (1963). Experimental studies in animals on a sodium humate salicylic acid bath. *Arzneimittelforschung* (Drug Research) **13**, 329-333.