Extended abstract No. 428

PRECLINICAL STUDIES ON HUMIC SUBSTANCES OF DIFFERENT ORIGIN

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SUMMARY

With the aim of developing humic preparations for medical use we investigated two naturally occurring and three synthetic humic substances (HS) for their effects on inflammation, spontaneous contractile activity of smooth muscles, and the reaction time of blood coagulation. The results indicate that low concentrations (1-10 µg/ml) of HS enhance the spontaneous contractile activity of smooth muscle strips from guinea pigs by stimulating alpha(2)-adrenoceptors and dopamine D2 receptors, intermediate HS concentrations (10-250 µg/ml) reduce the release of tumor necrosis factor alpha (TNF-α), an inflammation biomarker, and high HS concentrations (250-1000 µg/ml) act as anticoagulants. Interestingly, a few humic samples were found to exhibit bimodal dose-response curves the reason of which still needs to be clarified.

KEYWORDS: Natural and synthetic humic substances, LPS-stimulated TNF-α release, spontaneous contractile activity of smooth muscles, blood coagulation, bimodal effect

INTRODUCTION

Humic substances (HS) are known to exert multiple pharmacological effects, for example, antiviral activity against a wide range of pathogenic viruses (KlöckingR *et al*., 2006), remarkable influence on inflammation processes (KlöckingR. *et al*., 1968; Riede, 2000), UV-B protective activity in bacterial and human cell cultures (Muela *et al*., 2000; KlöckingR *et al*., 2004) and significant interactions with blood coagulation factors (KlöckingHP *et al*., 2004). For many years now, the alkali-soluble fraction of HS (humic acids, HA) have been practically applied in veterinary medicine for prevention and therapy of digestive disorders of farm, zoo and pet animals (Kühnert *et al.,* 1989). In addition, HA have been identified as remedies for mitigation of environmentally related fish diseases (Heidrich, 2004; Meinelt *et al.*, 2008). On the other hand, because of their insufficient chemical and toxicological

characterization, the application of HA in human medicine is still limited to some few nutraceuticals and medical products.

With the aim to develop HS for medical use we employed appropriate biological models for testing HS in vitro and ex vivo. Especially, we examined two naturally occurring and three synthetic HS for their effects on inflammation, on spontaneous contractile activity of smooth muscles and on blood coagulation. Here we present the results with regard to the underlying dose-effect relationships, a critical point for medical applications of HA.

MATERIAL AND METHODS

Test substances

Water-soluble sodium and ammonium humates were prepared from the brown water of an ombrotrophic mire near Dierhagen-Neuhaus (Dierhäger Moor, Mecklenburg-Western Pomerania, Germany) according to Klöcking *et al.* (1977). HA from a black peat layer of the Altteich peatland near Weißwasser (Altteicher Moor, Lower Lusatia, Saxony, Germany) were extracted with sodium hydroxide at pH 9 and precipitated either with hydrochloric acid according to standard procedures (→Black peat HA, BLP-HA 2005) or with halogen-free organic acids (Klöcking *et al.*, 2010). In addition, we prepared three synthetic HA by periodate oxidation of caffeic, chlorogenic and hydrocaffeic acid resulting in the corresponding HA-like oxidation products KOP, CHOP and HYKOP (Helbig *et al.*, 1997).

Reference humic substances

IHSS Waskish Peat HA (IR107H) from a Sphagnum peat of Pine Island Bog in Koochiching County, Minnesota (USA) was used as reference HA.

In-vitro inflammation model to investigate LPS-induced TNF-α release

The human macrophage-like cell line U937 was chosen for the investigations in this study. The experiments were carried out in 96-well cell culture plates. TNF-α concentration was determined using the BioLegend Human TNF-α ELISA MAX™ Kit (Biozol, Eching, Germany). 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 original method was slightly modified (Junek *et al.*, 2009).

Ex-vivo method to measure the spontaneous contractile activity (SCA) of smooth muscles

The SCA of smooth muscles were measured under isometric conditions according to the standardized method of Golenhofen (1976). The preparation of the smooth muscle fibers and the experimental design have been described in detail by Grozeva *et al.* (2005). To avoid differences in the SCA of the individual smooth muscle preparations, the changes in SCA were evaluated as a percentage of the maximum contraction of preparations induced by 10^{-5} mol/l acetylcholine.

Thrombelastographic measurements

The reaction time (r) is representative of early fibrin formation. It was determined by thrombelastographic measurements following the method of Wilson *et al.* (2009). The concentration-dependent influence of HS on the reaction time is expressed as the percentage deviation of untreated controls.

RESULTS

TNF-α release.

The influence of HS on TNF-α release in U937 cells is presented in Figure 1. It is clearly visible from the dose-response curves that HS at concentrations between 10 and 250 µg/ml inhibit the LPS-induced TNF-α release in a concentration-dependent manner (anti-inflammatory effect). Sodium KOP and BLP-HA proved to be most effective. At lower dosages, the test substances were either inactive or even produced an increase of TNF-α release (pro-inflammatory effect) as shown, for example, with sodium humate HD80. The real cause of this so-called bimodal effect has still to be investigated.

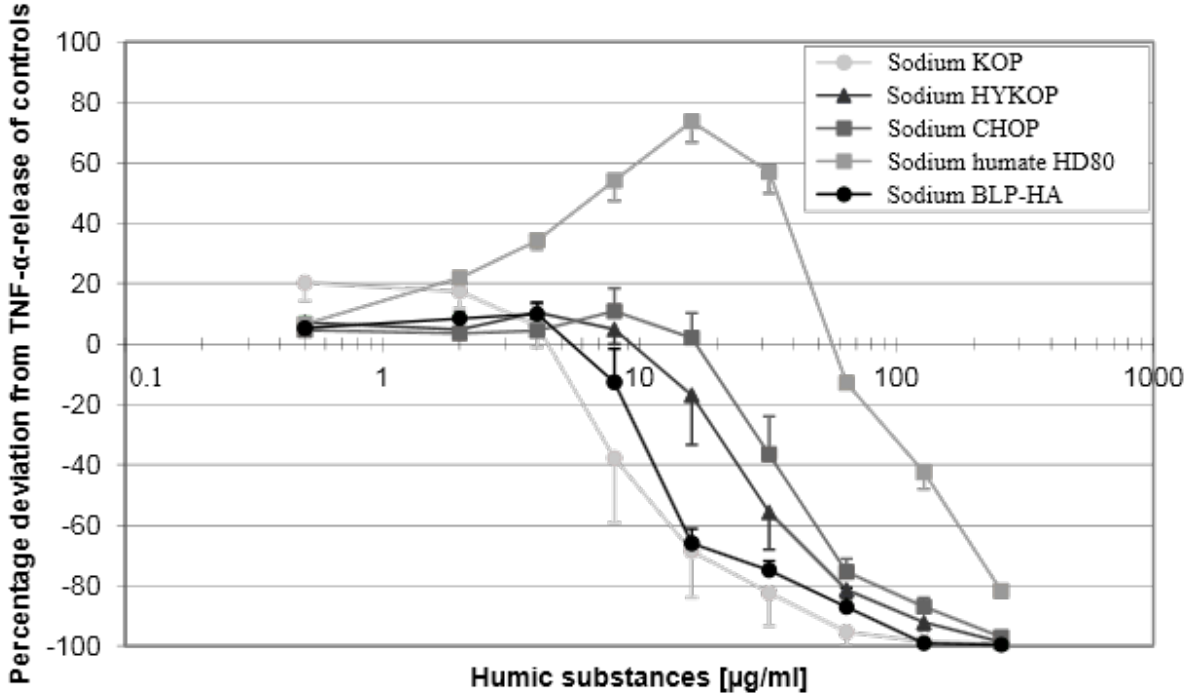

Fig. 1. Influence of humic substances of different origins on the LPS-induced TNF-α release of U937 cells. Results are expressed as a percentage deviation from TNF-α release of LPS-induced untreated cells; n = 3.

Spontaneous contractile activity (SCA) of smooth muscles

The influence of HS on the SCA of smooth muscle strips of the guinea pig stomach has been studied extensively by Beer et al. (2000). The method is very sensitive and permits the analysis of agonistic and antagonistic effects of HS on α_2-adrenoceptors and Dopamin-D2 receptors in the nanogram range. The results are shown in Figure 2 for sodium humate, ammonium humate and the synthetic HA, sodium KOP.

Differing in intensity and kinetics, HS concentrations between 0.3 and 10 µg/ml develop an agonistic effect on the SCA of guinea pig smooth muscle strips ranging between 12 and 38 % of 10^{-5} mol/l Ach. Experiments with specific inhibitors of α_2-adrenoceptors and dopamine-D2 receptors confirmed the specificity of this effect (data not shown). Further fractionation of sodium KOP, the strongest agonist of the tested HS, into hymatomelanic (HyA) and fulvic acids (FA) underlined the high activity of HyA.

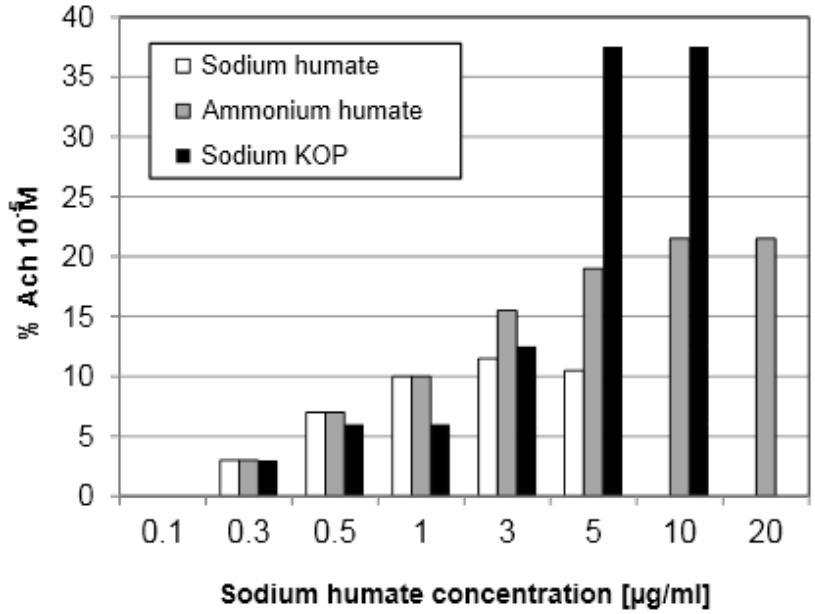

Fig. 2. Influence of different humic substances on the spontaneous contractile activity of smooth muscle strips of the guinea pig stomach. Results are expressed as a percentage of the activity of 10^{-5} M acetylcholine (Ach); n = 10.

Blood coagulation

The influence of HS on the early phase of coagulation was studied. Altteich peat HA and sodium humate at concentrations between 8 and 32 µg/ml developed a styptic effect and act as anticoagulants at higher concentrations. The influence of sodium humate on the reaction time of blood coagulation is demonstrated in Figure 3. Similar to its influence on TNF-α release, sodium humate also reveals a remarkable bimodal effect on blood coagulation.

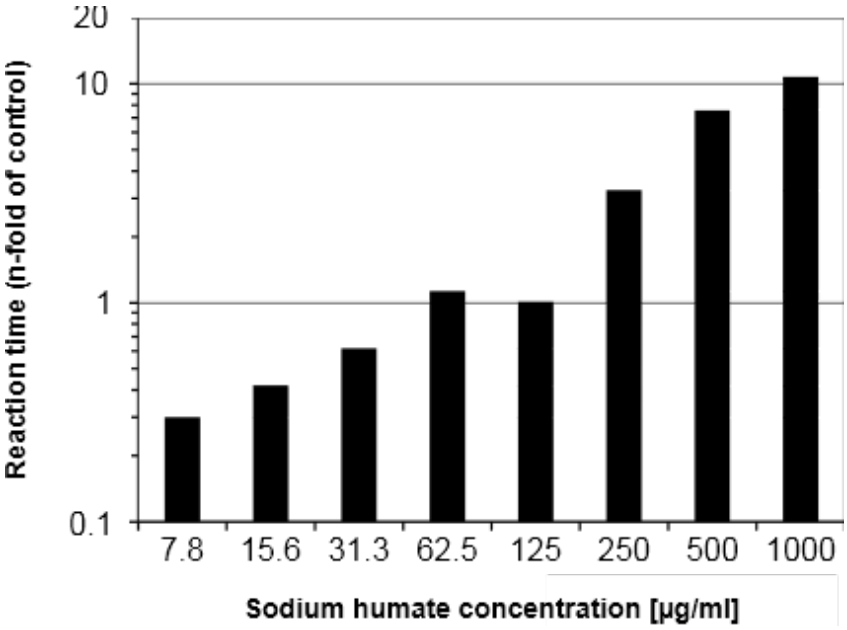

Fig. 3. Influence of sodium humate HD 76 on the thrombelastographically determined reaction time expressed as n-fold of untreated controls; n = 3.

CONCLUSION

The results show for all applied test systems that HS act in a dose-dependent manner. At low concentrations (1-10 µg/ml), they enhance the spontaneous contractile activity of smooth muscles by stimulating alpha(2)-adrenoceptors and dopamine D2 receptors, at intermediate concentrations (10-250 µg/ml), they inhibit TNF-α release, and at high concentrations (250-1000 µg/ml), they act as anticoagulants. Some HS show a bimodal dose-response curve, the cause of which has yet to be identified. The results should be considered in the assessment of HS intended for application in the medical field.

ACKNOWLEDGEMENTS

The financial support of the study by the German Federal Ministry for Education and Research (FH³ program, FKZ 1746 X06) is greatly acknowledged.

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