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Inhibitory Effect of Curcuminoids and Tetrahydrocurcuminoids on Equine Activated Neutrophils and Myeloperoxidase Activity

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Summary

In the horse, the inflammation response to various pathologies (intestinal strangulations, laminitis, etc.) involves an excessive stimulation of the polymorphonuclear neutrophils releasing reactive oxygen species (ROS) and myeloperoxidase (MPO). The aim of the present work was to study the effect of natural polyphenols, curcuminoids and tetrahydrocurcuminoids (THC) on isolated stimulated equine neutrophils and on the activity of purified MPO. The ROS production and the release of MPO by activated neutrophils were measured by chemiluminescence and ELISA techniques, respectively. The activity of purified MPO was measured by studying its nitration, chlorination or oxidation capacity and by using an original method called SIEFED allowing the study of drug interaction with the enzyme without interferences of the medium. Curcuminoids and THC had dose-dependent inhibitory effects on ROS production and MPO release by activated neutrophils and on purified MPO activity. We suggest that the higher efficacy of curcuminoids versus THC could be explained, at least partially, by its chemical structure: the conjugated double bounds and the plane structure of curcuminoids made easier the neutralization of the radical species generated by activated neutrophils and the interaction of the drug with the active site of MPO. These inhibitory effects of curcuminoids on the oxidant activity of equine neutrophils and on MPO activity open therapeutic perspectives in equine pathologies with excessive inflammatory reactions.

Key words:
Curcuminoids • Stimulated equine neutrophils • Myeloperoxidase activity

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Introduction

The polymorphonuclear leucocytes (neutrophils or PMN) are highly specialized for their primary function of phagocytosis and destruction of microorganisms (Borregaard and Cowland 1997, Klebanoff 2005). Their stimulation by endogenous (cytokines, lipid mediators) or exogenous agents (bacterial peptides, endotoxins) results in a sudden increase of their oxygen consumption (respiratory burst) with a cascade production of reactive oxygen species (ROS) (Klebanoff 2005). This ROS cascade starts with the production of the superoxide anion which is formed when NADPH oxidase is activated (Lee *et al.* 2003). In addition to the ROS production, the cytoplasmic granules of the neutrophils discharge hydrolytic and proteolytic enzymes, and myeloperoxidase (MPO; EC 1.11.1.7) a hemic enzyme specific of azurophilic granules. MPO is unique of its kind because it has a dual activity of peroxidase and chlorination. During the reaction with H_2O_2, ferric MPO (resting state) is oxidized to compound I (CpI) characterized by a π-cation radical state. When MPO acts as a peroxidase, the CpI is reduced back into ferric MPO by two monoelectronic oxidations of an electron donor *via* the formation of an intermediate non-radical state (CpII) of the enzyme (Deby-Dupont *et al.* 1999, Klebanoff 2005). The electron donors (e.g. tyrosine, ascorbate or urate) are oxidized into radical intermediates, which can diffuse and generate new active species (Podrez *et al.* 2000). Nitrite is involved in the peroxidasic cycle of MPO activity with the release of a reactive nitrogen species able to nitrate proteins and

cellular constituents (Sampson *et al.* 1998, Burner *et al.* 2000). The mechanism suggested for the nitration activity of MPO is a direct monoelectronic oxidation of nitrite into nitrogen dioxide ($\bullet NO_2$) by CpI. At physiological pH, nitrite is a poor substrate for CpII and a high nitrite concentration is needed to complete the peroxidasic cycle. However, in the presence of efficient electron donors for Cp II (e.g. tyrosine) nitration reactions are enhanced (Burner *et al.* 2000).

Besides its peroxidasic activity, the unique characteristic of MPO is its capacity to oxidize halide or pseudo-halide ions, mainly chloride anions (Harrison and Schultz 1976) because they are the most abundant halide ions *in vivo* (Furtmüller *et al.* 1998). Chloride ion is transformed by a one-step dielectronic oxidation into the potent oxidant, hypochlorous acid (HOCl), which plays an important role in microbial killing (Borregaard and Cowland 1997) and participates to both oxidation and chlorination reactions (Podrez *et al.* 2000). Owing to its important enzymatic activities, MPO is more and more considered as a marker of neutrophil activation and degranulation in an increasing number of pathologies (Brennan and Hazen 2003, Wu *et al.* 2005).

In acute and chronic inflammation, when neutrophils are stimulated by various mediators or during phagocytosis, they produce ROS and release MPO, exerting an oxidant activity on neighboring cells and tissues (Deby-Dupont *et al.* 1999, Kaminski *et al.* 2002, Hoy *et al.* 2002). In the horse, neutrophil activation takes place in inflammation diseases such as laminitis, recurrent airway obstructions and intestinal strangulation pathologies often associated to an irreversible endotoxic shock (Weiss *et al.* 1997, Grulke *et al.* 1999, Deaton *et al.* 2004b, Riggs *et al.* 2007). A therapeutic goal could be to lower the oxidant activity of stimulated neutrophils and the MPO activity. In this perspective, the use of natural polyphenols is promising. These compounds have antioxidant, anti-inflammatory and antitumor activities (Nakamura *et al.* 2000). Antioxidant supplementations (vitamins C and E) have been tested in horses affected by recurrent airway obstruction (Deaton *et al.* 2004a) or during endurance race (Williams *et al.* 2004), but there are few data about the beneficial effects of a nutritional supplementation by natural polyphenols.

Curcuminoids are natural yellow phenolic compounds found in rhizomes of *Curcuma longa* L. (*Zingiberaceae*) commonly known as turmeric, and widely used as food flavoring and coloring agent (Ukil *et al.* 2003, Sharma *et al.* 2005). Curcuminoids include curcumin, demethoxycurcumin and bisdemethoxycurcumin. Most of commercial curcuminoids sold as "curcumin" (eg by ICN, GNC, and Sigma-Aldrich), are mixtures of the 3 curcuminoids. The reduced and colorless analogs of curcuminoids, tetrahydrocurcuminoids (THC), include tetrahydrocurcumin, tetrahydrodemethoxycurcumin and tetrahydrobisdemethoxycurcumin (Fig. 1). Curcuminoids and THC differ at the level of the central seven-carbon chain. Curcumin and tetrahydrocurcumin are the major constituents (about 75 % of the total content) of the turmeric and reduced turmeric preparations, respectively. Tetrahydrocurcumin would have higher physiological and pharmacological activities than its parent compound (Nakaruma *et al.* 1998). The therapeutic interest for curcuminoids lies in their low toxicity and their large biological activities (Reddy *et al.* 2005, Maheshwari *et al.* 2006). In humans, they inhibit oxidative stress through ROS scavenging, suppress leukocyte infiltration into the inflamed regions and inhibit the activation of leukocytes including neutrophils (Nakaruma *et al.* 2000). We focused our research work on the effects of curcuminoids and THC on the oxidant activity of equine neutrophils as assessed by their ROS production and MPO release. We completed our investigations by studying the effect of curcuminoids and THC on the specific activity of purified equine MPO (by using an original immunocapture technique) and on the chlorination, nitration and oxidation activities of purified human MPO.

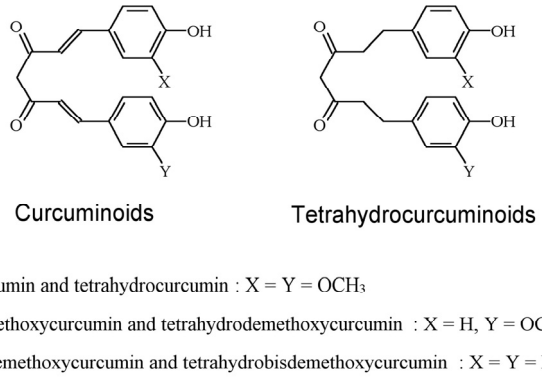

Fig. 1. Chemical structure of curcuminoids and tetrahydrocurcuminoids.

Methods

Reagents

Analytical grade Na and K salts, ethanol, dimethylsulfoxide (DMSO), hydrogen peroxide (30 %

v/v) and Tween 20 were from Merck (VWRI, Belgium). Tyrosine was from Acros (Geel, Belgium). Commercial grade curcumin (77 % curcumin, 17 % demethoxycurcumin and 3 % bisdemethoxycurcumin), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), bovine serum albumin (BSA), catalase, EDTA, lucigenin (Bis-N-methylacridinium nitrate), 2-nitro-5-thiobenzoic acid (TNB), percoll, phorbol 12-myristate 13-acetate (PMA) and taurine were purchased from Sigma-Aldrich (Bornem, Belgium). THC were from Sabinsa Corporation (Piscataway, USA). The product is synthesized in patent application WO 0061162 with the following specifications: minimum 95 % tetrahydro-curcuminoids with 75-90 % by weight of tetrahydro-curcumin, 15-20 % by weight of tetrahydrodemethoxy-curcumin and 1-4 % by weight of tetrahydrobis-demethoxycurcumin. For the study of the nitration, chlorination and oxidation activities of MPO, commercial human MPO from Calbiochem (Merck, USA) was used. Protein A Sepharose was from Amersham Bioscience Benelux (The Netherlands). Microtitration plates (Cliniplate EB) and White Combiplate 8 were from Thermo Labsystems (Finland). Amplex red substrate was purchased from Molecular Probes, Inc. (The Netherlands). Monovette blood collection systems were from Sarstedt Aktiegesellschaft & Co (Germany).

Experimental conditions for solubilizing and testing the polyphenols

Three stock solutions of curcuminoids and THC (10^{-2} M, 10^{-3} M, 10^{-4} M) were prepared in ethanol, and adequate volume of these solutions were added to the cell suspensions or the MPO activity assays to reach the final concentration of 10^{-4}, 10^{-5} or 10^{-6} M, respectively. By this way, the final ethanol concentration into the assays was always 1 % (172 mM) independently from the dilution of curcuminoids or THC. A control assay was made with 172 mM ethanol and compared to a control performed either with the buffer used for the cell suspensions (20 mM PBS pH 7.4) or with H_2O used to solubilize MPO. A 172 mM ethanol control was performed for all the assays except for the SIEFED assays (see below).

Isolation of equine neutrophils

Neutrophils were isolated from EDTA (1.6 mg/ml blood) blood drawn from the jugular vein of healthy horses, fed and bred in identical conditions and not under medical treatment (Faculty of Veterinary Medicine, University of Liège, Belgium). Each batch of neutrophils was obtained from 60 ml blood drawn from one horse, the cells were used immediately after isolation, the experiment was completed within 5 hours and each assay was performed in triplicate. Each experiment was repeated 2 to 4 times with different cell batches from different horses. The neutrophils were isolated at room temperature (18-22 °C) by centrifugation (400 x g, 30 min, 20 °C) on a discontinuous percoll density gradient according to the method of Pycock *et al.* (1987). The polymorphonuclear fraction was gently collected and washed in two volumes of physiological saline solution. The cell pellets were suspended in 20 mM phosphate buffer saline (PBS) at pH 7.4 containing 137 mM NaCl and 2.7 mM KCl. The cell preparation was ≥ 90 % neutrophils with a viability of 95 % as measured by the trypan blue exclusion test.

Measurement of ROS production by activated neutrophils

The ROS production by activated neutrophils was measured by chemiluminescence (CL) according to the method of Benbarek *et al.* (1996) with minor modifications. In our conditions, the assay was performed on microtiter plates and CL was read with a Fluoroscan Ascent FL (Thermo Labsystems).

In a first group of experiments, the neutrophil suspensions (10^6 neutrophils/ml PBS) were incubated for 10 min at 37 °C with curcuminoids or THC at the final concentration of 10^{-4}, 10^{-5} or 10^{-6} M. After the incubation, the neutrophil suspensions were centrifuged (450 x g, 10 min, 20 °C), the supernatant was removed and the neutrophils were resuspended to obtain 10^6 neutrophils in 200 µl of PBS. The neutrophil suspensions were distributed in the wells (10^6 neutrophils per well) of a 96 wells microtiter plate (White Combiplate 8, Thermo Labsystems) and 25 µl $CaCl_2$ (7.5 µM), 2 µl lucigenin (5 µM) and 10 µl PMA (1.6 x 10^{-5} M) were added. Just after PMA addition, the CL response of neutrophils was monitored during 30 min and expressed as the integral value of the total CL emission. In a second group of experiments, the neutrophil suspensions (10^6 neutrophils/ well) were incubated for 10 min in the wells with curcuminoids or THC at the final concentration 10^{-4}, 10^{-5} or 10^{-6} M and then the CL was monitored during 30 min in the presence of the curcuminoids or THC after addition of $CaCl_2$, lucigenin and PMA. Control assays were performed with neutrophils either preincubated or directly added with 172 mM ethanol and taken as 100 % of CL response.

Purification of equine MPO and anti-equine MPO polyclonal antibodies

The purification of equine MPO and the preparation of the anti-MPO antibodies used in the immunological techniques were described in detail previously (Mathy-Hartert *et al.* 1998, Franck *et al.* 2005). Briefly, MPO was extracted from isolated equine neutrophils and purified by two chromatographic steps (ion exchange and gel filtration) to reach a purity > 98 % (as established by electrophoresis with enzymatic detection on electrophoretic bands). Antisera were obtained from rabbit and guinea pig after their immunization against purified equine MPO. The polyclonal antibodies (IgG) were isolated from antisera by affinity chromatography on Protein A sepharose (Franck *et al.* 2005).

Measurement of total MPO released by activated neutrophils

The neutrophil suspensions (10^6 cells/ml) were incubated for 10 min at 37 °C with curcuminoids and THC at the final concentration of 10^{-4}, 10^{-5} or 10^{-6} M and then activated for 30 min at 37 °C with 8 x 10^{-7} M PMA (final concentration). After the activation, the neutrophil suspensions were centrifuged (450 x g, 10 min) and the supernatants collected for the measurement of MPO released by the neutrophils. An original ELISA assay was used to measure MPO (Franck *et al.* 2005) (Equine MPO ELISA, EqRK001, BIOPTIS, Belgium). Briefly, polyclonal antibodies against equine MPO were coated on 96 wells microtiter plates. Samples were added into the wells and incubated overnight at 4 °C. After washing, a second anti equine MPO polyclonal antibody raised in guinea pig and coupled to alkaline phosphatase was added and incubated 2 h at 37 °C. After washing, phosphatase activity was measured after incubation (25 °C, 30 min in the darkness) with a paranitrophenyl phosphate solution. Absorbance (405 nm) was read with the Multiscan Ascent (Labsystem). The samples were diluted 60 fold with PBS buffer pH 7.4 added with 5 g/l BSA and 0.1 % Tween 20. A control assay with 172 mM ethanol was taken as 100 % of MPO release.

Effect of curcuminoids and THC on neutrophil viability

Unstimulated neutrophils (10^6 cells/ml) were incubated with curcuminoids or THC at the different tested concentrations and with their ethanol vehicle, and the cell viability was estimated by the Trypan blue exclusion test.

Measurement of active MPO by SIEFED

An original method called SIEFED ("Specific Immunological Extraction Followed by Enzymatic Detection") was developed for the specific detection of active equine neutrophil MPO (Franck *et al.* 2006). The method is a three steps procedure: firstly, the extraction of MPO out of a solution or a biological sample by its capture on specific immobilized antibodies, secondly washings to eliminate unspecifically bound compounds or interfering substances and, thirdly, the detection of MPO enzymatic activity by using H_2O_2 as substrate, Amplex Red as fluorogenic electron donor and nitrite as enhancer of the reaction (Franck *et al.* 2006). The nitrite ions favor the peroxidase activity of the enzyme (Burner *et al.* 2000) increasing the amount of Amplex Red molecules transformed into fluorescent molecules.

The MPO solution (20 ng/ml) used for SIEFED was prepared with purified equine MPO diluted in PBS buffer at pH 7.4 with 5 g/l BSA and 0.1 % Tween 20. The stock solutions of curcuminoids or THC (10^{-2} M) were prepared in ethanol and the dilutions of the stock solution to reach 10^{-3} and 10^{-4} M were made in 20 mM PBS buffer, pH 7.4. Adequate volumes of the diluted polyphenol solutions were added to the MPO solution to reach the final polyphenol concentrations of 10^{-4}, 10^{-5} or 10^{-6} M. Control assays were made with the vehicle solution of polyphenols containing 1 % (172 mM), 0.1 % (17.2 mM), 0.01 % (1.72 mM) ethanol respectively. A control was also made with PBS alone and taken as 100 % MPO activity. The assays were incubated for 10 min at 37°C. After the incubation, the mixtures were loaded on the SIEFED microplate, and incubated (2 h, 37 °C) to allow the capture of MPO by the antibodies. After washings (elimination of the incubation milieu containing the tested compounds and their vehicle solution), the enzymatic activity of MPO was measured.

In vitro study of human MPO oxidation, nitration, and chlorination activity

For the reason of availability of the enzyme, this part of the study was performed with human MPO for the *in vitro* oxidation, nitration and chlorination activity. The effect of curcuminoids and THC were studied at the final concentration of 10^{-6}, 10^{-5} and 10^{-4} M.

Nitration activity of MPO on tyrosine

The experiments were carried out in 100 mM acetate buffer at pH 5.5 with tyrosine (1.5 mM), human

MPO (1 µg/ml, ie, 200 mU/ml), NaCl (150 mM), H_2O_2 (1 mM) and $NaNO_2$ (5 mM). Assays were performed in the presence of 10^{-6}, 10^{-5}, and 10^{-4} M of curcuminoids and THC. Control assays were made with the curcuminoids and THC vehicle solution (1 % ethanol, 172 mM) taken as 100 % activity.

The reaction was performed for 30 min at 37 °C. We monitored the formation of 3-nitrotyrosine by UV-visible spectroscopy at 450 nm (Multiskan Ascent Thermo Labsystem, Helsinki, Finland) after alkalinization with 100 µl NaOH (0.1 M).

Chlorination activity of MPO on taurine

Taurine is a good substrate for the chlorination activity of MPO, yielding chlorotaurine, the concentration of which is evaluated by a subsequent reaction with TNB absorbing at 412 nm (Nève *et al.* 2001).

The 2-nitro-5-thiobenzoate (TNB) solution was prepared following Ching *et al.* (1994): 1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 5 mM EDTA and 20 mM $NaBH_4$ were dissolved in phosphate buffer (50 mM) at pH 6.6 and allowed to react at 37 °C for 30 min. The TNB concentration was directly estimated by UV-visible spectroscopy at 412 nm (ε_{412} = 13600 M^{-1} cm^{-1}).

For the chlorination reaction, 10 µl of taurine (150 mM), 10 µl MPO (10 µg/ml, i.e. 20 mU), 20 µl NaCl (1.5 M) and 2 µl of curcuminoids and THC stock solutions were added in the wells of a microplate (96 wells) to reach the final polyphenol concentration of 10^{-4}, 10^{-5} and 10^{-6} M and the volume was adjusted to 190 µl with 100 mM acetate buffer (pH 5.5). Control assays were made with the vehicle solution of curcuminoids and THC (1 % ethanol, 172 mM) taken as 100 % activity. After addition of 10 µl H_2O_2 (5 mM), the plate was incubated for 30 min at 37 °C. Then, 35 µl of catalase (3 mg/ml) were added to stop the reaction and the solution was incubated for 15 min at 37 °C. Finally, 100 µl of the TNB solution (0.45 mM) were added and TNB reaction with chlorotaurine was followed by UV-visible spectroscopy (Multiskan Ascent Thermo Labsystem, Helsinki, Finland) at 405 nm (the closest available filter to 412 nm).

Oxidation activity of MPO on ABTS

ABTS is converted by peroxidases into the stable cation radical form $ABTS^{\bullet+}$ strongly absorbing around 700 nm. The experiments were carried in acetate buffer at pH 5.5 with the following reagents: ABTS (750 µM), MPO (0.5 µg/ml, ie, 100 mU/ml), NaCl (150 mM), H_2O_2 (0.5 mM) and $NaNO_2$ (5 mM), which were incubated for 30 min at 37 °C. The formation of $ABTS^{\bullet+}$was followed by UV-visible spectroscopic measurements at 690 nm (Multiskan Ascent Thermo Labsystem, Helsinki, Finland). The phenolic compounds curcuminoids and THC were used at 10^{-6}, 10^{-5}, and 10^{-4} M and their effects were compared to control assays performed with their vehicle solution (1 % ethanol, 172 mM) taken as 100 % activity.

Statistical analysis

Within an experiment, each point was repeated three times and each experiment was repeated 2 to 4 times with cell batches from different horses, so that the n value of one experimental point ranged from 6 to 12. Relative values (%) were used considering as 100 % the ethanol or buffer control. Data are given as mean ± SD. An unpaired non-parametric Mann Whitney test was performed with the GraphPad Instat 3.05 (GraphPad Software, San Diego California, USA). $P<0.05$ value was considered to be significant.

Results

Effect of the drugs on the cell viability

The neutrophil mortality in the PBS was 4.8 %. Ethanol, curcuminoids and THC, whatever the concentration, had no significant effect on neutrophils viability.

Effect of drugs on the chemiluminescence (CL) response (ROS production) of activated neutrophils

Curcuminoids and THC have dose-dependent inhibitory effects on the chemiluminescence response of stimulated neutrophils, either when the stimulation occurs in the presence of the drug (Fig. 2A) or after its removal (Fig. 2B). Curcuminoids and THC significantly inhibited the neutrophils response at all the tested concentrations when they were present during stimulation (Fig. 2A) and at 10^{-5} and 10^{-4} M when they were eliminated before stimulation (Fig. 2B). A more potent effect was observed for curcuminoids. No significant difference was observed between the PBS and ethanol controls (data not shown).

Effect of the drugs on the release of MPO by activated neutrophils (total MPO assay by ELISA)

Curcuminoids had inhibitory effects on the release of MPO by activated neutrophils at 10^{-4} and 10^{-5}

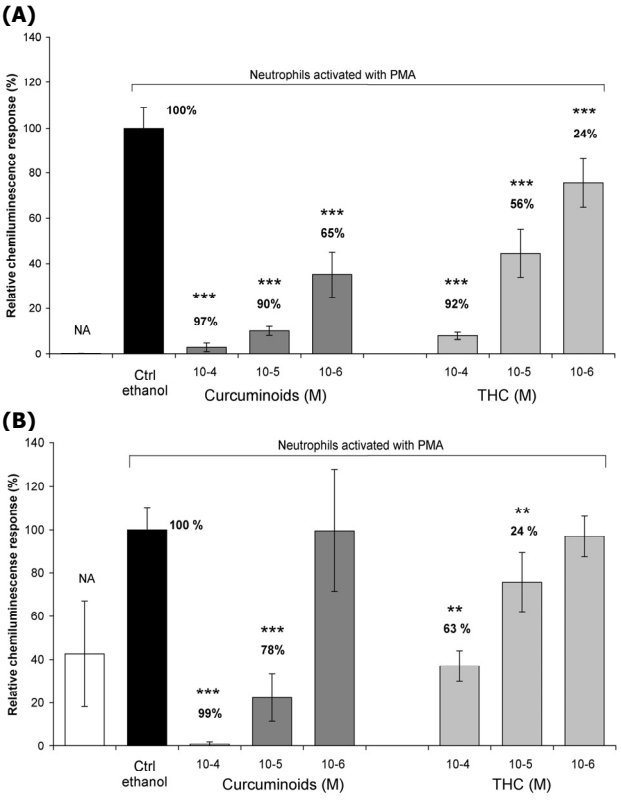

Fig. 2. Effect of curcuminoids and THC on the chemiluminescence response of $1x10^6$ equine neutrophils stimulated by PMA. The drugs were let in the cell suspension during stimulation (A) or pre-incubated 10 min with the cells and eliminated before stimulation (B). The percentages of inhibition indicated on the top of each column were calculated versus the ethanol (172 mM) vehicle control (Ctrl ethanol) taken as 100 %. Data are given as mean ± S.D. (n = 6 to 11), *** p<0.0001, ** p<0.001 versus control. NA: control with ethanol vehicle, but no stimulation.

M (Fig. 3). THC had a significant inhibitory effect only at 10^{-4} M. The ethanol vehicle was taken as the 100 % control value. No significant difference was observed between the PBS and ethanol controls (data not shown).

Effect of the drugs on the activity of equine MPO (active MPO assay by SIEFED)

The purified equine MPO used in this experiment was the same as that used by Franck *et al.* (2006) to develop the SIEFED technique. Its specific activity was 160.3 units/mg protein. The 172 and 17.2 mM ethanol vehicle solutions (ethanol controls) exerted an inhibitory effect compared to MPO alone taken as 100 % and this inhibitory effect was significant for 172 mM ethanol (Fig. 4). At 10^{-4}, 10^{-5} and 10^{-6} M curcuminoids and THC had significant inhibitory effects on the activity of MPO versus their respective controls with 172, 17.2 and 1.72 mM ethanol (Fig. 4). The most potent effects were observed for curcuminoids.

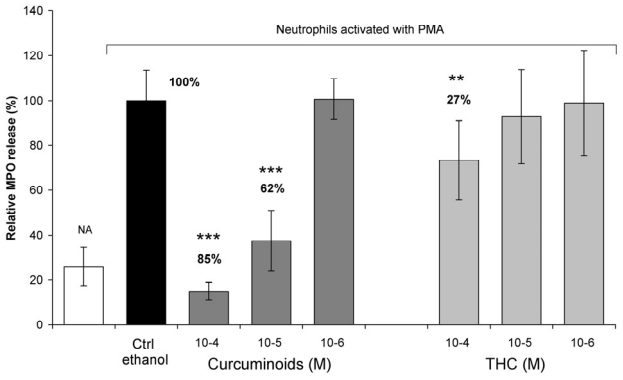

Fig. 3. Effect of curcuminoids and THC on the MPO released by $1x10^6$ neutrophils stimulated with PMA. The percentages of inhibition by curcuminoids and THC indicated on the top of each column were calculated versus the ethanol (172 mM) vehicle control (Ctrl ethanol) taken as 100 %. Data are given as mean ± S.D. (n = 6 to 9), *** p<0.0001, ** p<0.001 versus control. NA: control with ethanol vehicle, but no stimulation.

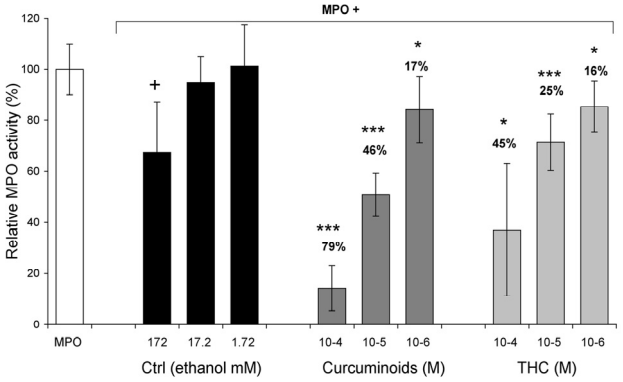

Fig. 4. Effect of curcuminoids and THC on MPO activity measured by SIEFED. The drugs were incubated with MPO during 120 min and eliminated before the detection of the MPO enzymatic activity. The percentages of inhibition indicated on the top of each column were calculated for each polyphenol concentration versus the corresponding ethanol vehicle control (Ctrl ethanol mM). Data are given as mean ± S.D. (n = 9 to 12), *** p<0.0001, * p<0.05 versus respective control; + p<0.05 versus MPO alone.

Influence of curcuminoids and THC on the oxidizing, chlorinating and nitrating activities of MPO

As this part of the study needed important amounts of MPO, the assays were performed with purified human MPO available from a commercial origin (human polymorphonuclear neutrophils MPO, Calbiochem) with a specific activity of 200 units/mg protein. The inhibition of the triple activity (oxidizing, chlorinating and nitrating activities) of human MPO was followed by UV-visible spectroscopy. The effect of ethanol used as the vehicle solvent for curcuminoids and THC was controlled: a slight non-significant decrease of the oxidation and the chlorination activities and a non-significant increase of the nitration activity were observed

Table 1. Inhibitory activity of curcuminoids and THC on the oxidation, nitration or chlorination activity of human MPO.

Reaction	Curcuminoids (M)			THC (M)		
	10^{-6}	10^{-5}	10^{-4}	10^{-6}	10^{-5}	10^{-4}
ABTS oxidation	10.3±9.2	30.2±7.0*	100.3±4.0*	+2.5±4.0	31.3±3.2*	102.5±3.6*
Tyrosine nitration	6.2±12.4	45.0±9.6*	ND	+2.7±4.6	7.3±4.7	71.9±6.3*
Taurine chlorination	28.9±14.9*	30.1±8.9*	69.3±19.5*	4.5±10.0	7.1±10.6	68.6±5.8*

Results are expressed as the percentage of inhibition versus the ethanol controls taken as 100 %. Data are given as mean ± S.D. (n = 5), * p <0.01 versus control. ND: not determined due to the strong absorption of curcuminoids.

at the highest concentration of ethanol compared to the control with an equivalent amount of H_2O (data not shown). The inhibitory rates reported in the Table 1 were calculated versus the corresponding ethanol controls taken as 100 %.

Curcuminoids at 10^{-5} and 10^{-4} M significantly inhibited all the activities of MPO, except for the effect of 10^{-4} M curcuminoids on tyrosine nitration, which was not measured due to the strong absorption of curcuminoids. The inhibitory effect of THC was significant at 10^{-4} M for the three reactions and at 10^{-5} M for the oxidation reaction. At 10^{-5} M, curcuminoids had a more pronounced effect on the nitration reaction compared to THC, an observation that was confirmed at 5.10^{-6} M. At this concentration, only curcuminoids significantly inhibited the nitration reaction: 15.38±7.19 % inhibition versus the ethanol control ($p<0.01$) (results not shown).

Discussion

In human, curcumin is reported to be a scavenger of ROS and an inhibitor of the neutrophils response (Srivastva 1989). Tetrahydrocurcumin, one of the main colorless metabolites of curcumin mainly present in its glucoronide conjugated form in the bile, is reported to exhibit antioxidant properties stronger than curcumin in several *in vitro* systems (Osawa *et al.* 1995, Pari and Amali 2005). But tetrahydrocurcumin has an inhibitory activity weaker than curcumin in bioassay related to tumor promotion and superoxide anion generation by PMA induced HL-60 cells (Nakamura *et al.* 1998). In our models, we tested curcuminoids and tetrahydrocurcuminoids (THC) containing about 75 % curcumin and tetrahydrocurcumin, respectively, and used freshly isolated equine neutrophils stimulated with PMA. When equine neutrophils were activated with PMA in the presence of curcuminoids or THC, a dose-dependent inhibitory effect was observed on the chemiluminescence response that could be attributed to the extracellular ROS scavenging activities of the drugs, in agreement with previous results (Osawa *et al.* 1995, Okada *et al.* 2001). However, a decrease of the chemiluminescence response of neutrophils was also observed when curcuminoids and THC were preincubated with the cells, but removed before the stimulation, suggesting that the two compounds were fixed to the cell surface or entered into the cells, modifying their reactivity to PMA. Liu *et al.* (1993) and Balasubramanyam *et al.* (2003) showed that curcumin may inhibit the phorbol ester-induced assembly of the NADPH oxidase subunits or the signal transduction systems, probably by inhibiting the activity of protein kinase C (PKC) and by interfering with the calcium regulation. Curcumin and tetrahydrocurcumin also affect cellular processes linked to the inflammatory response: they inhibit the inflammatory cytokines production in macrophages or *Chlamydia*-primed THP-1 cells (Abe *et al.* 1999, Deby-Dupont *et al.* 2005). By inhibiting the activation cascade of neutrophils, curcumin helps to reduce the excessive production of chemotactic factors, which plays an important role in chronic inflammation (Nakamura *et al.* 2000). Curcumin also affects the activity of lipoxygenases and cyclooxygenases, key enzymes of the arachidonic acid metabolism (Ammon *et al.* 1993). Salh *et al.* (2003) showed that the anti-inflammatory effects of curcumin involved a reduction of MPO activity by reducing the phagocyte infiltration in tissues, but they did not study its effects on the release of MPO by stimulated neutrophils or the activity of the enzyme. We demonstrated here that curcuminoids had an inhibitory effect on the MPO release by activated neutrophils, an observation that emphasizes the intracellular action of curcumin on the cascade of events leading to the NADPH oxidase activation and the neutrophil degranulation. According to Lee *et al.* (2003), PKC activation is essential for the generation of superoxide anion, but not strictly required for

degranulation. Independently from their activity on PKC, curcuminoids interfere with the degranulation process, probably by acting on the cytoskeleton as already hypothesized by Holy (2004).

Few studies reported the effect of curcuminoids directly on the MPO activity. Kato *et al.* (2003) showed an inhibitory effect of curcumin on MPO-catalyzed tyrosylation which they attributed to a quenching of the tyrosyl radical by the polyphenol and/or to an interaction between MPO and curcumin. Our results showed that curcuminoids and THC had almost the same effect on the oxidation activity of MPO, but that curcuminoids were more efficient than THC to inhibit the nitration and chlorination activities. We explain this inhibition by: 1) a scavenging of the reactive species (e.g. HOCl or $\bullet NO_2$) produced during the reaction, 2) a competition with the MPO substrates or 3) an inhibition of the enzyme. The more important capacity of curcuminoids to inhibit the nitration and chlorination reactions of MPO suggests that curcuminoids efficiently scavenged $\bullet NO_2$ and HOCl. However, we further investigated the inhibitory properties of curcuminoids on MPO activity by using the SIEFED method. This original method consists in the immunoextraction of MPO from complex aqueous samples by immobilized anti-MPO antibodies. The MPO capture is followed by washings to eliminate the rest of the sample (proteins, tested molecules, vehicle solvent) and by the measurement of the peroxidasic activity of MPO (Franck *et al.* 2006). The enzymatic detection is thus not biased by the molecules present in the sample or by the tested compounds since the washing eliminates them. Using the SIEFED method, we demonstrated a dose-dependent inhibitory effect of curcuminoids and THC on the activity of MPO with a better effect of curcuminoids. Under the SIEFED conditions, free curcuminoids and THC molecules (as well as other potentially interfering species) were removed before the detection of MPO activity and no chloride anions were added in the revelation step of the method, so that HOCl cannot be generated by MPO activity and thus cannot react directly with curcuminoids. Therefore, we hypothesized that the two drugs interacted with or bound to the MPO protein and either modified the enzyme structure or hindered the substrate access to the enzymatic active site or acted as a competitive substrate. It was shown that the active site of human MPO is located in a hydrophobic cavity with a narrow oval-shaped opening (Fiedler *et al.* 2000), and we can expect that the polyphenols could hinder or block the entry of this cavity. Recently, Jiao *et al.* (2006) showed that curcuminoids could act as iron chelators and we cannot exclude that a chelating activity of curcuminoids on the hemic iron of MPO played a role in the enzyme inhibition. However, the potent iron chelator, deferoxamine, does neither react with nor remove the iron of MPO (Klebanoff and Waltersdorph, 1988).

The better efficacy of curcuminoids could be related, at least partially, to their chemical structure: the conjugated double bounds of the central carbon chain and the coplanarity with the phenolic rings could facilitate the interaction with the radical species generated by the activated neutrophils and during the nitration and chlorination activities of MPO. The curcuminoid structure could also favor the interactions of the drug with the active site of MPO. On the oxidation reaction catalyzed by MPO, curcuminoids and THC had similar inhibitory effects. However, further experiments are needed to determine if the inhibitory effects that we observed in our experimental models are due to a synergy of the three compounds present in curcuminoids and THC or especially to curcumin or tetrahydrocurcumin mainly present (more than 75 %) in the turmeric and reduced turmeric.

Considering that curcuminoids and THC are innocuous and possess demonstrated therapeutic effects in a variety of models of human and animal diseases, it will be desirable to further investigate their potential use in equine medicine. As observed in different animal models (Ukil *et al.* 2003, Gukovsky *et al.* 2003, Pari and Amali 2005), there is an interest to use curcumin for treating horse gastrointestinal lesions or other pathologies associated with a local or systemic inflammation with the infiltration and activation of the neutrophils and the release of proteases and MPO. Obstructive intestinal conditions are associated with such an activation and degranulation of the neutrophils, and with high concentrations of MPO in the systemic blood flow (Grulke *et al.* 1999). Endotoxins absorbed from a damaged intestine activate equine neutrophils and stimulate monocytes to release stimulating mediators for neutrophils (Benbarek *et al.* 1998, Dagleish *et al.* 2003). Other horse pathologies such as joint diseases, laminitis, and recurrent airway obstruction involve a neutrophil activation and a release of MPO (Weiss *et al.* 1997, Art *et al.* 2006, Riggs *et al.* 2007). In this context, the inhibitory effects of curcuminoids and THC on neutrophil activation and MPO release with oxidative stress and cell injury arise therapeutic perspectives in equine pathologies with excessive inflammatory reactions. THC would even present some more advantages for use as

food additive. According to Nakamura *et al.* (1998), their preparation by hydrogenation of curcuminoids is easy and their absorption from the intestine would be better.

Conflict of Interest

There is no conflict of interest.

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