JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Resveratrol Inhibits the Activity of Equine Neutrophil Myeloperoxidase by a Direct Interaction with the Enzyme

STEPHAN KOHNEN,*,†,‡ THIERRY FRANCK,†,‡ PIERRE VAN ANTWERPEN,|| KARIM ZOUAOUI BOUDJELTIA,⊥ ANGE MOUITHYS-MICKALAD,‡ CAROL DEBY,‡ NICOLE MOGUILEVSKY,# GINETTE DEBY-DUPONT,‡,§ MAURICE LAMY,‡,§ AND DIDIER SERTEYN†,‡

Department of Clinical Sciences, Large Animal Surgery, Faculty of Veterinary Medicine B41, University of Liège, Sart Tilman, 4000 Liège, Belgium, Centre for Oxygen Research & Development (C.O.R.D.), Institute of Chemistry B6a, University of Liège, Sart Tilman, 4000 Liège, Belgium, Department of Anesthesia and Intensive Care Medicine, CHU B35, University of Liège, Sart Tilman, 4000 Liège, Belgium, Laboratory of Pharmaceutical Chemistry, Institute of Pharmacy, Université Libre de Bruxelles, Campus Plaine 205-5, B-1050 Brussels, Belgium, Laboratory of Experimental Medicine, CHU Charleroi-Vésale, Université Libre de Bruxelles, Montigny-le-Tilleul, Belgium, and Technology Transfer Office, University of Namur, 5000 Namur, Belgium

Resveratrol is a polyphenolic antioxidant present in beverage and food known for its multiple protective effects. We report the inhibitory effects of resveratrol on equine myeloperoxidase (MPO), a hemic peroxidase present in the granules of the neutrophils involved in the inflammatory response. Resveratrol inhibited the production of reactive oxygen species (ROS) by stimulated equine neutrophils by acting as a direct scavenger of the ROS released by the cells but did not modify the degranulation of the stimulated neutrophils as the amounts of released MPO were unchanged. Resveratrol strongly inhibited the chlorination, oxidation, and nitration activities of MPO in a dose-dependent manner. By an original technique of specific immunological extraction followed by enzymatic detection (SIEFED), we demonstrated that resveratrol inhibited the peroxidasic activity of the MPO measured by a direct interaction such as the fixation of resveratrol on the enzyme. The observation of a decrease of the accumulation of compound II suggested that resveratrol acts as an electron donor for MPO reduction.

KEYWORDS: Myeloperoxidase; resveratrol; neutrophils; SIEFED

INTRODUCTION

Resveratrol (*trans*-3,5,4′-trihydroxystilbene), a phytoalexin present in various fruits and beverages, is a polyphenolic stilbene, devoid of the toxic properties generally ascribed to the members of the stilbene family. It has generated significant interest in biomedical research because of its various biological activities (for review, see *1*): cardiovascular protection (*2, 3*), neuroprotective effects (*4, 5*), regulation of cell proliferation and anticancerous activity (*2, 6*), decrease of inflammatory response, and reduction of the production of reactive oxygen species (ROS) by neutrophils (*7*). We demonstrated that resveratrol lowered the activity of NADPH oxidase in monocytes infected by the intracellular microorganism *Chlamydia pneumoniae* (*8*), a kind of chronic inflammatory process involved in the development of atherosclerosis (*9*). Cavallaro et al. showed that resveratrol dose-dependently inhibited the generation of hypochlorous acid (HOCl) and nitric oxide by isolated neutrophils and suggested that resveratrol acted by preventing the release of myeloperoxidase (MPO; EC 1.11.1.7) by a strictly intracellular pathway (*10*). MPO is abundant in neutrophils, present in lesser amount in the primary granules of monocytes, but absent in mature macrophages (*11*). It uses hydrogen peroxide (H_2O_2) and chloride anion (Cl^-) to produce hypochlorous acid (HOCl), an important microbial killer by both oxidation and chlorination reactions (*11*). The capacity to oxidize halide and pseudo-halide ions, mainly chloride anions that are the most abundant in plasma (*12–14*), is a unique characteristic of MPO. The chloride ion is oxidized by a one-step dielectronic oxidation into hypochlorous acid (HOCl). But MPO also acts

* Corresponding author. Tel: 32-4-3663366. Fax: 32-4-3662866. E-mail: S.Kohnen@ulg.ac.be.
† Department of Clinical Sciences, University of Liège.
‡ Centre for Oxygen Research & Development (C.O.R.D.), University of Liège.
§ Department of Anesthesia and Intensive Care Medicine, University of Liège.
|| Laboratory of Pharmaceutical Chemistry, Université Libre de Bruxelles.
⊥ Laboratory of Experimental Medicine, Université Libre de Bruxelles.
University of Namur.

10.1021/jf071741n CCC: $37.00
Published on Web 09/11/2007

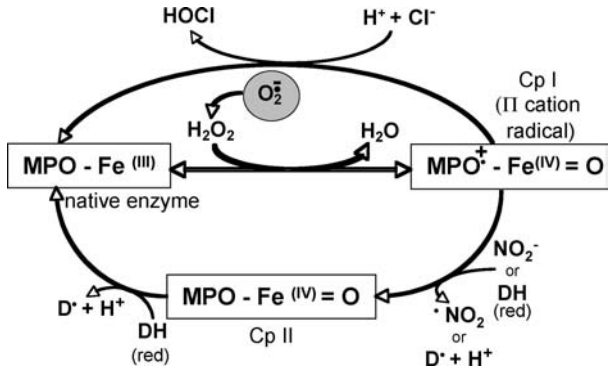

Figure 1. Simplified representation of the main steps summarizing the enzymatic activity of MPO (MPO-Fe(III) is the native enzyme) and the formation of the intermediate compounds (Cp). In CpI (oxoferryl π-cation radical), the free radical is localized on the porphyrin ring of the protein. Nitrite (NO_2^-) intervenes as an electron donor. DH, reducing substrate; D•, radical derived from DH (with loss of •H) (*20*).

as a peroxidase. In this case, ferric MPO (resting state) is oxidized to compound I (CpI) characterized by an oxoferryl π-cation radical state (see **Figure 1**). CpI is reduced back into ferric MPO by 2 monoelectronic oxidations of an electron donor (such as tyrosine, ascorbate, urate, catecholamines, etc.) via the formation of an intermediate oxoferryl nonradical compound (compound II, CpII) (*15*). The protein nitration of tyrosyl residues by neutrophils and macrophages (*16*) is now explained by this peroxidasic activity of MPO, and the suggested mechanism is a direct monoelectronic oxidation of nitrite into nitrogen dioxide ($\bullet NO_2$), responsible for tyrosine nitration (*17*).

A neutrophil activation with MPO release is involved in various acute and chronic inflammatory pathologies in humans (*15, 18, 19*), and excessive stimulation of the neutrophils producing ROS and releasing MPO leads to damaging oxidant activity on neighbouring cells, tissues, and molecules (*20*). MPO is reported as responsible for the oxidative modification of low-density lipoproteins (LDL), producing oxidized derivatives, which accumulate in the vascular wall and promote a local inflammatory process involved in the development of atheromatous plaque (*21*). It was also demonstrated that neutrophil activation with MPO release occurred in inflammation pathologies in horses, such as laminitis, recurrent airway obstructions and intestinal strangulation pathologies (*22–25*).

Some drugs have been tried to limit the deleterious effects of excessive activity of MPO (*26–28*), and resveratrol is a valuable candidate. Its effects have been demonstrated on neutrophil oxidant activity but not directly on MPO. We were interested to study the effects of resveratrol not only on the global oxidant activity of isolated equine neutrophils but also on their capacity to release MPO and on the activity of the enzyme after degranulation. The effects of resveratrol were tested on the oxidant activity of purified equine MPO on its in vitro enzymatic activity of chlorination, nitration, and oxidation. The mechanism of MPO inhibition by resveratrol was explored by an original technique (the SIEFED technique) able to detect a direct enzyme–inhibitor interaction and confirmed by a spectrophotometric kinetic study of compound II formation (*29*).

MATERIALS AND METHODS

Chemicals. Sodium, potassium, and calcium chloride, sodium and ammonium acetate, acetic acid, ethanol, hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), KOH, KI, HCl, $EDTANa_2H_2$, H_2SO_4, trypan blue, and NaOH were analytical grade products from Merck (VWRI, Leuven, Belgium). 5,5′-Dithiobis(2-nitrobenzoic acid) (TNB), sodium borohydride ($NaBH_4$), and tyrosine were from Acros (Geel, Belgium). Paranitrophenyl phosphate, resveratrol, sodium nitrite, catalase, α-keto-γ-methylthiobutyric acid (KMB), lucigenin, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, USA). Amplex Red was purchased from Molecular Probes (Invitrogen, Merelbeke, Belgium). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and taurine were from Aldrich (St. Louis, USA). De-oxygenated milliQ water or ultrapure water (EasyPure UV purification system) was used for the preparation of all of the solutions.

Isolation of Equine Neutrophils. Neutrophils were isolated from EDTA (1.6 mg/mL) anticoagulated blood drawn from the jugular vein of healthy horses, fed and bred in identical conditions and under no medical treatment (Faculty of Veterinary Medicine, University of Liège, Belgium). The neutrophils were isolated at room temperature (18–22 °C) by centrifugation (400*g*, 30 min, 20 °C) on a discontinuous percoll density gradient according to the method of Pycock et al. (*22, 30*). The cells were gently collected, washed in 2 volumes of physiological saline solution, and the cell pellets resuspended in 20 mM phosphate saline buffer (PBS) at pH 7.4 with 137 mM NaCl and 2.7 mM KCl. The preparation contained ≥ 90 % neutrophils with a viability of 98% as measured by trypan blue exclusion test. Each batch of neutrophils was prepared from 60 mL of blood from one horse. The cells were used immediately after isolation, the experiment was completed within 5 h, and each assay was performed in triplicate. Each experiment was repeated at least twice with different cell batches.

Purification of Equine MPO and Antiequine 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 (*22, 31*). 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). The purified equine MPO used in this experiment was the same as that used by Franck et al. (*29*) to develop the SIEFED technique. Its specific activity was 160.3 units/mg protein.

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 (*22*).

Preparation of Resveratrol Solutions. Resveratrol was dissolved either in ethanol (ethanol solution) or in 1/1 DMSO/H_2O (DMSO solution). An adequate volume of resveratrol in ethanol solution was used to reach final concentration of 10^{-4}, 10^{-5} and 10^{-6}M. Resveratrol in DMSO solution was used at the final concentrations of 10^{-4} to 10^{-6}M by dilution in phosphate buffer. Control assays were performed either with an equivalent volume of the DMSO solution or with an equivalent volume of ethanol.

Effect of Resveratrol on Neutrophils Viability. Neutrophils (10^6 cells/ml) were incubated with the DMSO vehicle or resveratrol at the different tested concentrations. After the incubation period, the cell viability was estimated by the trypan blue exclusion test.

Measurement of ROS Production by Activated Neutrophils. *Lucigenin-Enhanced Chemiluminescence.* The ROS production by activated neutrophils was followed by chemiluminescence, according to the method adapted from Benbarek et al. (*32*). The assays were performed on microtiter plates, and chemiluminescence was measured with a Fluoroscan Ascent FL (Thermo Labsystems) at 37 °C. Neutrophil suspensions (10^6 neutrophils/ 200 μL PBS) were distributed in the wells of a 96 well microtiter plate (White Combiplate 8, Thermo Labsystems) and incubated for 10 min at 37 °C with resveratrol at the final concentrations of 10^{-4} M, 10^{-5} M, or 10^{-6}M. After the incubation, 25 μL $CaCl_2$ (7,5 μM), 2 μL lucigenin (5 μM) and, just before the chemiluminescence measurement, 10 μL of a $1.6.10^{-5}$ M PMA stock solution were added to the cell suspensions. The chemiluminescence response of the neutrophils was monitored for 30 min (Multiscan Ascent, Thermo Labsystem, Helsinki, Finland) and expressed as the peak value or as the integral value of the total chemiluminescence emission. Control assays made with the vehicle solution of resveratrol (50% DMSO solution) were taken as 100 % of chemiluminescence response.

Ethylene Production from KMB Oxidation. The neutrophils that are excited with PMA in the presence of α-keto-γ-methylthiobutyric acid (KMB) release ROS in the extracellular medium. The ROS react with KMB, which decomposes to produce ethylene, measured in the gaseous phase by gas chromatography. This technique has been reported in detail in previous papers (*8*). Isolated equine neutrophils were suspended in PBS with glucose (30 mg/20 mL PBS) at a concentration of 10^6 cells/mL. One milliliter of the neutrophil suspension was preincubated for 10 min at room temperature with resveratrol (10^{-4}, 10^{-5}, and 10^{-6} M) or the resveratrol vehicle (10 μL 50% DMSO solution) in a 10 mL vial. Then, KMB and PMA were added at final concentrations of 10^{-3} M and 8.10^{-7} M, respectively. Immediately afterward, the vials were sealed and incubated for 1 h at 37 °C. The level of KMB oxidation was estimated by measuring the ethylene amount released into the gaseous phase. Ethylene was quantified by gas chromatography on a Porapak T column (1 m length; ID 1/8 in; supplied by Supelco, Belgium) at 80 °C using nitrogen as vector gas (40 mL/min), with flame ionization detector at 120 °C. The gas chromatography column was standardized with pure ethylene (C_2H_4-25 quality, Air–Liquide, Belgium). The gas samples (0.5 mL) were obtained by puncture through the sealing membrane with a 1 mL Hamilton gas syringe A-2 (Vici Precision Sampling Inc.).

Measurement of the Total Amount of MPO Released by the Activated Neutrophils. The neutrophil suspensions (10^6 cells/mL) were incubated for 10 min at 37 °C with resveratrol and then activated for 30 min at 37 °C with 8×10^{-7} M PMA (final concentration). After activation, the neutrophil suspensions were centrifuged (450*g*, 10 min), and the supernatants were collected. MPO released by the neutrophils was measured in the supernatants by an original ELISA assay raised against equine MPO as previously described (*22*) and developed by Biocode-Hycel (Liège, Belgium; kit AEE032).

Briefly, polyclonal antibodies obtained in rabbit against purified equine MPO (*31*) were coated on 96 well microtiter plates. Samples were added into the wells and incubated overnight at 4 °C. After washing, a second antiequine MPO polyclonal antibody raised in guinea pig and coupled to alkaline phosphatase was added and incubated for 2 h at 37 °C. After washing, phosphatase activity was measured after 30 min of incubation (25 °C, in darkness) with a paranitrophenyl phosphate solution. Absorbance (405 nm) was read with Multiscan Ascent. The control assay taken as 100 % of MPO release was made with the supernatant obtained from neutrophils activated by PMA after 10 min of incubation with the vehicle solution of resveratrol.

Interaction of Resveratrol with MPO Studied by the SIEFED Technique and by a Spectrometric Study of Compound II. *Study by the SIEFED Technique.* SIEFED is an original method allowing in the first step the capture of MPO out of a solution or a biological sample by specific immobilized antibodies (immunoextraction step). The second step consists of washings to eliminate all the compounds (proteins, potential modulating or interfering substances, etc.) of the sample that do not bind to the antibodies. The third step is an *in situ* detection of MPO activity (revelation step) by using a fluorogenic substrate (amplex red 40 μM), H_2O_2 (10 μM), and $NaNO_2$ (10 mM) as the enhancer of the reaction (*29*). The activity of MPO transforms amplex red into a fluorescent compound, and after an incubation time of 30 min at 37 °C in the dark, the fluorescence is read (Multiscan Ascent, λ excitation, 544 nm; λ emission, 590 nm).

Resveratrol in DMSO solution at final concentrations of 10^{-4}, 10^{-5}, or 10^{-6} M was incubated for 10 min with equine MPO (9 ng/mL) before the immunoextraction step. After the incubation, the mixture was loaded on the SIEFED microplate and incubated (2 h, 37 °C) to allow the capture of MPO by the antibodies, and after washing, the activity of MPO was measured. Resvratrol was thus washed out before the measurement of MPO activity.

Controls were performed with equivalent concentrations of the DMSO solution. The fluorescence value measured for MPO without resveratrol (PBS control) was taken as 100 % activity.

Spectrophotometric Study of the Accumulation of Compound II. The interaction of resveratrol with equine MPO was assessed by measuring the lifetime of compound II in the presence or the absence of Cl^-. The method was adapted from Nève et al. (*33*). PBS buffer (pH 7.4) was prepared at a final concentration of 10 mM phosphate ions (KH_2PO_4/KOH) with or without 150 mM of NaCl. In a 1.5 mL quartz cell, the following reagents were introduced, at the final concentration stated within parentheses, for a final volume of 1.0 mL: 50 μL of MPO (23 μg/ml), 400 μL of PBS with or without NaCl (150 mM), 10 μL of resveratrol solution in ethanol (10 or 50 μM), and 440 μL of water. The reaction was initiated by the addition of 100 μL of H_2O_2 (30 μM), and absorbances were simultaneously monitored with a diode-array spectrophotometer (Agilent 8453, Palo Alto, CA, USA) at the wavelengths characteristic of compound II (456 nm) and of the native enzyme (430 nm). During the reaction, the two absorbance curves evolved in opposite ways and intersected at two different times. The lapse of time between the two intersections corresponded to the lifetime of compound II. This time corresponds to the complete conversion of compound II into the native enzyme, which is demonstrated by a shift in the enzyme spectrum.

In Vitro Study of the Oxidation, Nitration, and Chlorination Activities of Equine MPO. *Nitration Activity of Equine MPO on Tyrosine.* The experiments were carried out at pH 5.5 (100 mM acetate buffer) with tyrosine (1.5 mM), equine MPO (1 μg/mL, i.e., 200 mU/mL), NaCl (150 mM), H_2O_2 (1 mM), and $NaNO_2$ (5 mM). Assays were performed in the presence of 0, 10^{-6}, 10^{-5}, and 10^{-4} M of resveratrol in ethanol solution. The reaction was performed for 30 min at 37 °C.

The formation of 3-nitrotyrosine was monitored by UV–visible spectroscopy at 405 nm (Multiskan Ascent) after alkalination with 100 μL of 0.1 M NaOH.

Chlorination Activity of Equine MPO on Taurine. Taurine is a good substrate for MPO; it is transformed into chlorotaurine, the yield of which is evaluted by a subsequent reaction with 2-nitro-5-thiobenzoate (TNB), which absorbs at 412 nm (*33*).

(a) Preparation of the 2-Nitro-5-thiobenzoate (TNB) Solution. One millimolar 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 at 37 °C for 30 min. The TNB concentration was measured by UV–visible spectroscopy at 412 nm ($\varepsilon_{412} = 13600\ M^{-1}\ cm^{-1}$).

(b) Chlorination Reaction. Ten microliters of taurine (150 mM), 10 μL of equine MPO (0.1 μg/mL, i.e., 20 mU), 20 μL of NaCl (1.5 M), and 2 μL of resveratrol in ethanol solution (0, 10^{-2}, 10^{-3} or 10^{-4} M) were added in each well of a microplate (96 wells, Thermo Labsystems). The volume was adjusted to 190 μL with 100 mM acetate buffer (pH 5.5), and 10 μL of 10 mM H_2O_2 solution was added. The plate was incubated for 30 min at 37 °C. The reaction was then stopped by the addition of 35 μL of catalase (3 mg/ml) and an incubation of 15 min at 37 °C. Finally, 100 μL of the TNB solution (0.45 mM) was added, and the absorbance was measured at 405 nm (the closest filter available to 412 nm) (Multiskan Ascent).

Oxidation Activity of Equine MPO on ABTS. ABTS is converted by the peroxidasic activity of MPO into the stable radical cation form $ABTS^{\bullet+}$, which strongly absorbs around 700 nm. The experiments were carried out at pH 5.5 (100 mM acetate buffer) with the following reagents: ABTS (750 μM), equine MPO (0.5 μg/mL, i.e., 100 mU/mL), NaCl (150 mM), H_2O_2 (0.5 mM), $NaNO_2$ (5 mM), and resveratrol in ethanol solution (0, 10^{-4}, 10^{-5}, and 10^{-6} M). The solutions were incubated for 30 min at 37 °C. The formation of $ABTS^{\bullet+}$ was monitored by UV–visible spectroscopy at 690 nm (Multiskan Ascent) after an incubation period of 30 min.

Statistical Analysis. Within an experiment, each point was repeated three times, and each experiment was repeated two or three times with cell batches from different horses so that the *n* value of one experimental point ranged from 6 to 10. Data are presented as the mean ± SD, and statistical analysis was performed with GraphPad Instat 3.05 (GraphPad Software, San Diego California, USA). The assays with resveratrol were compared to control assays (DMSO solution) using a Welch's corrected *t*-test. A p value < 0.05 was considered as significant.

RESULTS

Effects of Resveratrol on Total ROS Production by Equine Neutrophils. The influence of resveratrol on the ROS production of PMA-activated equine neutrophils was studied by using two techniques: a chemiluminescence assay with lucigenin (**Figure 2**) and the measurement of KMB oxidation by gas chromatography

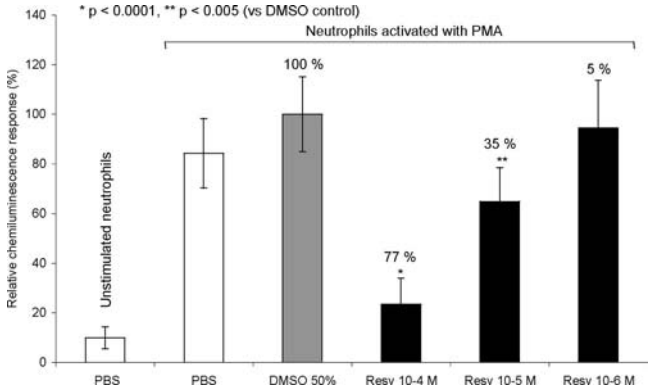

Figure 2. Inhibitory effect of resveratrol (10^{-4}, 10^{-5}, and 10^{-6} M) on the chemiluminescence of the reactive oxygen species produced by PMA stimulated equine neutrophils. Control with the DMSO solution is taken as 100% (mean ± SD, $n = 4$).

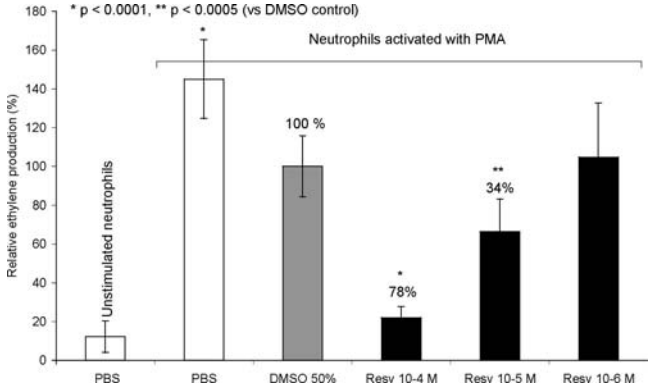

Figure 3. Inhibitory effect of resveratrol (10^{-4}, 10^{-5}, and 10^{-6} M) on the ethylene released from the KMB oxidation by the reactive oxygen species produced by PMA stimulated equine neutrophils. Control with the DMSO solution is taken as 100% (mean ± SD, $n = 10$).

measurement of the ethylene release (**Figure 3**). But first, we checked by the Trypan blue exclusion test that resveratrol in the range of concentrations used for these experiments was not cytotoxic. We found 90–93 % living cells, and the same values were found for the control (PBS and DMSO solution).

Chemiluminescence Assay with Lucigenin. Resveratrol showed significant dose-dependent inhibition except at 10^{-6} M (**Figure 2**). We reached 50% inhibition for a concentration around 2.10^{-5} M resveratrol. This effect of resveratrol observed by chemiluminescence is a real inhibitory effect on ROS production and not a toxic effect on equine neutrophils. The addition of 2 μL of the DMSO solution, the vehicle of resveratrol, slightly increased the luminescence response.

Gas Chromatography Assay. Resveratrol had exactly the same inhibitory effect on ethylene release that it had on chemiluminescence response (**Figure 3**). We also reached 50% of inhibition for a concentration of 2.10^{-5} M resveratrol. The addition of the DMSO solution significantly decreased the ethylene production.

Effects of Resveratrol on the Total MPO Released by Activated Neutrophils. The effect of resveratrol on the total amount of MPO released by activated neutrophils was measured by an ELISA assay (**Figure 4**). The activation of neutrophils by PMA doubled the release of MPO in the supernatants, as compared to unstimulated cells. The addition of the DMSO solution (resveratrol vehicle) led to an insignificant decrease in MPO release, and resveratrol did not add any significant modification of MPO release versus the DMSO solution alone.

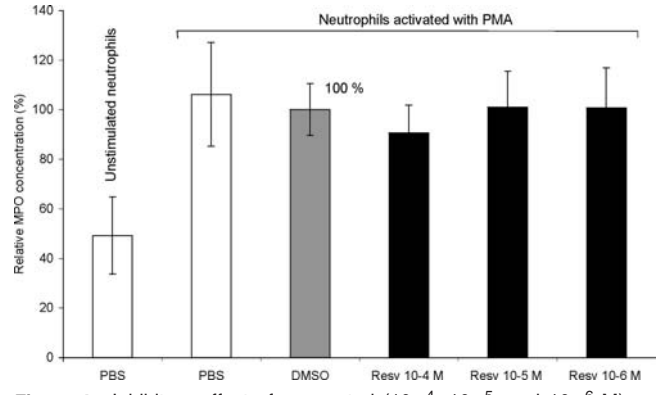

Figure 4. Inhibitory effect of resveratrol (10^{-4}, 10^{-5}, and 10^{-6} M) on the amount of MPO released by PMA stimulated equine neutrophils (original ELISA technique). Control with the DMSO solution is taken as 100% (mean ± SD, $n = 4$).

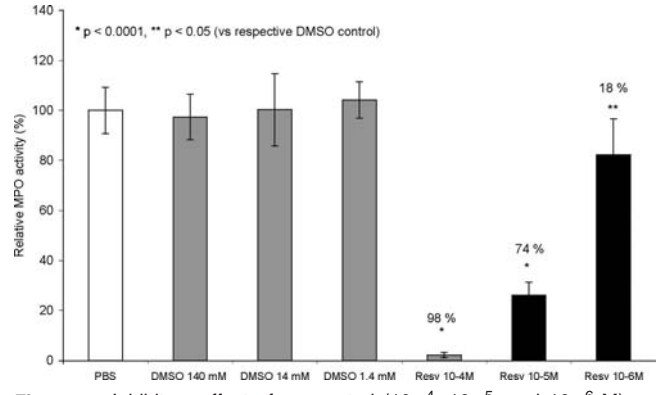

Figure 5. Inhibitory effect of resveratrol (10^{-4}, 10^{-5}, and 10^{-6} M) on the peroxidasic activity of equine MPO (original SIEFED technique). Control with the DMSO solution is taken as 100% (mean ± SD, $n = 3$).

Resveratrol thus had no effect on the neutrophil degranulation leading to MPO release.

Effects of Resveratrol on the Peroxidasic Activity of MPO (SIEFED Technique). We observed a strong inhibition of the peroxidasic activity of MPO by resveratrol at 10^{-4} and 10^{-5} M (**Figure 5**). At 10^{-5} M, it inhibited almost 75% of the MPO activity, and at 10^{-4} M, it totally suppressed the enzyme activity. We reached 50% of inhibition for a concentration around 5.10^{-6} M resveratrol. These observations suggested that resveratrol was fixed on MPO or altered the enzyme structure, leading to MPO inactivation.

Effects of resveratrol on the in vitro activities of MPO. *Influence of Resveratrol on the Oxidizing, Chlorinating, and Nitrating Activities of MPO (***Figure 6***).* The three activities of equine MPO by UV–visible spectroscopy were studied. The experiments were performed at pH 5.5, the optimal pH value to reach the highest yield of reaction.

Nitration, chlorination, and oxidation activities of MPO were strongly inhibited by resveratrol. Interestingly, at 10^{-5} M, resveratrol was a strong inhibitor (≥80% inhibition), while no significant effect was observed at 10^{-6} M. Such an important jump in the effect of resveratrol confirmed what we already observed in the SIEFED experiment at the same concentrations. At 10^{-4} M resveratrol, equine MPO activities were completely suppressed. The ethanol vehicle had a slight activating effect on nitration and oxidation and a slight inhibitory effect on chlorination.

Accumulation of Compound II. The lifetime of equine MPO compound II was measured with and without the addition of chloride anions (**Table 1**). No accumulation of compound II

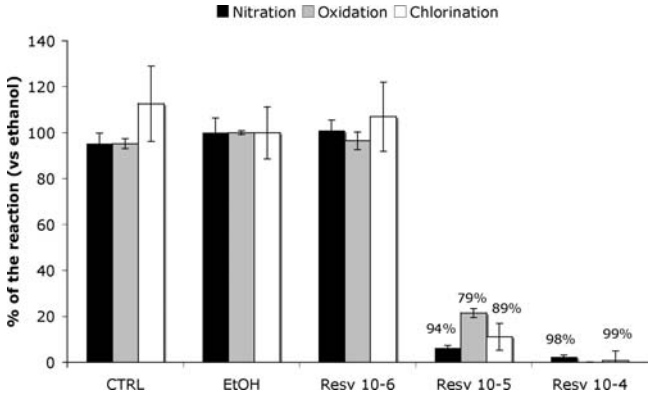

Figure 6. Inhibitory effect of resveratrol (10^{-4}, 10^{-5}, and 10^{-6} M) on the 3 activities (nitration, oxidation, and chlorination) of equine MPO (measurements performed by UV–visible spectroscopy. Control with ethanol (EtOH) is taken as 100% (mean ± SD, $n = 3$). CTRL: assays performed without resveratrol or ethanol.

Table 1. Accumulation of Compound II Measured by Spectroscopic Changes at the Wavelengths Characteristic of Compound II (456 nm) and of the Native Enzyme (430 nm)

	lifetimes of compound II (s)	
drug	with chloride	without chloride
blank (1 % ethanol)	ND[a]	2066
resveratrol (10 mM)	ND	280
resveratrol (50 mM)	ND	ND

[a] ND: compound II not detected.

was observed in the presence of chloride anions, and resveratrol did not induce any change, which suggested the absence of inhibition of HOCl synthesis by resveratrol. However, in the absence of chloride anions, resveratrol inhibited the accumulation of compound II. The lifetime of compound II was reduced by more than 85 % in the presence of 10 mM resveratrol, and no more compound II was detected in the presence of 50 mM resveratrol, which demonstrated that it was able to interact directly with compound II.

DISCUSSION

Resveratrol was reported to have an inhibitory effect on all of the phases of the inflammatory response, from the initial recruitment of neutrophils to their activation and the subsequent release of inflammatory mediators (*2*). Resveratrol can decrease the MPO activity according different pathways: it can act as an inhibitor of neutrophil degranulation or the enzyme itself by scavenging the reactive species produced by MPO activity (HOCl, $\bullet NO_2$, etc.) or by acting as a competitive electron donor for MPO reduction.

We studied the effect of resveratrol on the responses of equine neutrophils to the activation by PMA and observed a strong dose-dependent inhibitory effect of resveratrol on ROS production using two different techniques (**Figures 2** and **3**) in agreement with previous studies on activated human monocytes/macrophages (*8*). The similarity of the results obtained with the two techniques confirms the effect of resveratrol on ROS production. The inhibition can be attributed to the direct scavenging effect of ROS by resveratrol in the plasma or to an intracellular inhibition. Several works showed that resveratrol can inhibit the phorbol ester-induced intracellular ROS production (*7*), the NAPDH oxidase activity (*7, 8*), the protein kinase C (PKC) (*35*), and the iNOS induction (*36*). Resveratrol can also decrease various cellular processes linked to the inflammatory response such as the release and expression of pro-inflammatory cytokines by *Chlamydia*-primed THP-1 cells (*8*). In our working conditions, when the neutrophils were activated in the presence of resveratrol, the dose-dependent inhibitory effect of this compound on ROS chemiluminescence confirmed its extracellular ROS scavenging activity. Indeed, this dose-dependent inhibition was not observed anymore when the neutrophils were activated after the removal of the drug, suggesting that resveratrol did not enter into the cells or bind to the cell surface (data not shown). This last point is in opposition to the results previously reported by Cavallaro et al. suggesting that resveratrol entered into the cells to inhibit ROS production by the neutrophils (*10*). In our point of view, this difference of behaviour can easily be explained by the short duration of the incubation of neutrophils with resveratrol (only 10 min) in comparison with the experiments of Cavallaro's group (until 60 min). The hydroxyl groups are very hydrophilic and slow down the penetration of resveratrol in the membranes. Therefore, resveratrol does not have enough time before the washing step to significantly enter the neutrophils in our conditions.

Moreover, by ELISA measurement, we confirmed that resveratrol did not influence the release of MPO in the plasma (**Figure 4**). The amount of released MPO remained, with or without resveratrol, suggesting that resveratrol did not modify the degranulation of the neutrophils and did not act on the neutrophils directly but interacted with the ROS and the enzymes released by the neutrophils. Resveratrol thus plays the role of ROS scavenger and potential MPO inhibitor but does not inhibit the formation or release by the cells as previously reported by Leonard et al. (*37*).

In order to understand the mechanism of inhibition of equine neutrophils by resveratrol, we designed *in vitro* experiments to study the effect of resveratrol on the activity of isolated equine MPO. First, the peroxidasic activity of equine MPO in the presence of resveratrol was followed by the SIEFED technique. The original aspect of this technique is the ability to measure the peroxidasic activity of MPO after an immunological extraction of the enzyme without artifactual effect of the milieu (biological or otherwise): if an inhibition of MPO activity is observed in these conditions, it can only be attributed to a direct interaction of the tested compound with the enzyme before the revelation step and not to a neutralizing effect on the products of MPO activity because the free molecules of the drugs have been discarded by the washing steps. We showed that resveratrol is an inhibitor of MPO activity (**Figure 5**). In the SIEFED conditions, there are no chloride anions so that HOCl can not be generated by MPO. The inhibition that we observed can thus not be related to a direct reaction or a scavenger effect of resveratrol on HOCl. The peroxidasic activity of MPO is also strongly lowered by low concentrations of resveratrol (5 μM leads to 50% inhibition). The effect of resveratrol observed in SIEFED measurement can only be attributed to resveratrol molecules immobilized on the enzyme structure of MPO: resveratrol seems to interact with equine MPO by changing the configuration of the enzyme or by blocking the access to the active site, which is supposed to be located in a hydrophobic cavity with a narrow oval-shaped opening as observed for human MPO (*38*). This irreversible binding could be related to the mechanism-based inactivation reported for the peroxidase activity of COX-1, induced by resveratrol and other *m*-hydroquinones (*39, 40*).

We compared the SIEFED results with the results obtained by the direct measurement of equine MPO activity in solution

by using the same fluorogenic substrate, Amplex Red. In these conditions, we observed that the inhibitory effect of resveratrol was higher than that in SIEFED conditions: we measured 30% and 91% compared to 10% and 73% in SIEFED. From these compared data, we can deduce that resveratrol in solution acted as a competitive substrate for MPO and also by interaction with the enzyme explaining why its inhibitory capacity was higher in SIEFED. The inhibitory activity of resveratrol was still higher when the enzymatic activity of MPO was revealed by the SIEFED technique after immunocapture but without performing the washing step: the inhibition rates were then 63% and 97% for 10^{-6} and 10^{-5} M resveratrol, respectively, which were the sum of the activity of resveratrol as competitive substrate and its inhibitory interaction with the enzyme, more pronounced on the immobilized MPO than on free MPO in the solution.

We also studied the effect of resveratrol on the nitration, oxidation, and chlorination activity of equine MPO *in vitro* by direct spectroscopic techniques. We observed the strong inhibitory effect of resveratrol, which was expected as resveratrol is known as a powerful antioxidant species. Resveratrol was very effective on nitration, chlorination, and oxidation activities: a 10 μM concentration was enough to inhibit 90 % of the 2 first reactions and 80 % of the latter. Various hypotheses can explain these observations: resveratrol can act as a scavenger of the reactive species (HOCl, $\bullet NO_2$, etc.), as a competitive substrate or as a inhibitor of the enzyme, keeping MPO in its resting state. The last hypothesis does not agree with our observations because in this case we would not observe a dose-dependent effect of resveratrol on MPO activities. In our opinion, the effect of resveratrol should result from the combination of a scavenger effect (as observed in chemiluminescence experiments) and a competition effect.

In order to confirm this hypothesis, we followed the accumulation of compound II of MPO. No accumulation of compound II was detected in the presence of chloride anions, suggesting the absence of antagonism between resveratrol and chloride anions in the synthesis of HOCl such as that reported with flufenamic acid or several oxicams (*33*, *34*). This observation could lead us to think that resveratrol only acts by scavenging HOCl. However, in the experiment in the absence of chloride anions, resveratrol drastically reduces compound II accumulation, an observation that suggests that resveratrol has the capacity to reduce compound II into the resting state, probably by behaving as an electron donor for compound II but also for compound I (see **Figure 1**), competing with chloride for compound I reduction. This can explain the inhibition observed during the nitration and oxidation reactions. Resveratrol thus acts, at least in part, as a competitive substrate: preliminary HLPC studies confirmed that oxidized, chlorinated, and nitrated resveratrol derivatives were formed, but these experiments need further precise identifications of these derived compounds (data not shown). The inhibitory action of resveratrol (*41*) could be related to the *m*-hydroquinone ring, whereas its action as peroxidase substrate (*42*) could be due to its monophenolic ring since many monophenols have been reported as substrates of peroxidases (*43–46*). In fact, resveratrol and derivatives have been characterized as substrates and inhibitors of other oxidases such as laccase (*47*, *48*) and tyrosinase (*49–51*).

In conclusion, we explored the effects of resveratrol, a natural polyphenolic antioxidant, on ROS and MPO released by equine neutrophils activated by PMA and on the activities of isolated equine MPO. We always observed a strong dose-dependent inhibitory effect of resveratrol except on the total amount of MPO released by neutrophils. We suggest that resveratrol is both a scavenger of ROS and an inhibitor of the pro-inflammatory enzyme released by degranulating equine neutrophils but that it does not act in the cell but mainly in the extracellular milieu. Resveratrol strongly inhibited the peroxidasic activity of purified equine MPO, and its inhibitory effect was conserved when resveratrol was discarded before the measurement of the peroxidasic activity. Therefore, we suggest that by binding on MPO resveratrol hinders the acces to the enzymatic active site for the substrates. The absence of compound II accumulation also suggested the role of resveratrol as an electron donor for compound II reduction. Resveratrol is thus active in ROS scavenging, blocks heme access, and is a competitive substrate for MPO. The sum of all these activities makes resveratrol an important candidate for MPO inhibition.

ABBREVIATIONS USED

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CpI, MPO intermediate compound I; CpII, MPO intermediate compound II; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; KMB, α-keto-γ-methylthiobutyric acid; LDL, low density lipoprotein; MPO, myeloperoxidase; $ONOO^-$/peroxynitrite; PBS, phosphate buffer saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SIEFED, specific immunological extraction followed by enzymatic detection; TNB, 2-nitro-5-thiobenzoate.

LITERATURE CITED

(1) Frémont, L. Biological effects of resveratrol. *Life Sci.* **2003**, *66*, 663–673.
(2) Pervaiz, S. Resveratrol: from grapevines to mammalian biology. *FASEB J.* **2003**, *17*, 1975–1985.
(3) Das, D. K.; Maulik, N. Resveratrol in cardioprotection: a therapeutic promise of alternative medicine. *Mol. Interventions* **2006**, *6*, 36–47.
(4) Kaplan, S.; Bisleri, G.; Morgan, J. A.; Cheema, F. H.; Oz, M. C. Resveratrol, a natural red wine polyphenol, reduces ischemia-reperfusion-induced spinal cord injury. *Ann. Thorac. Surg.* **2005**, *80*, 2242–2249.
(5) Mokni, M.; Elkahoui, S.; Limam, F.; Amri, M.; Aouani, E. Effect of resveratrol on antioxidant enzyme activities in the brain of healthy rat. *Neurochem. Res.* **2007**, *32*, 981–987.
(6) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N.R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218–220.
(7) Martín, A. R.; Villegas, I.; Sánchez-Hidalgo, M.; Alarcón de la Lastra, C. The effects of resveratrol, a phytoalexin derived from red wines, on chronic inflammation induced in an experimentally induced colitis model. *Br. J. Pharmacol.* **2006**, *147*, 873885..
(8) Deby-Dupont, G.; Mouithys-Mickalad, A.; Serteyn, D.; Lamy, M.; Deby, C. Resveratrol and curcumin reduce the respiratory burst of *Chlamydia*-primed THP-1 cells. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 21–27.
(9) Saikku, P.; Leinonen, M.; Mattila, K.; Ekman, M. R.; Nieminen, M. S.; Makela, P. H.; Huttunen, J. K.; Valtonen, V. Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* **1988**, *2*, 983986..
(10) Cavallaro, A.; Ainis, T.; Bottari, C.; Fimiani, V. Effect of resveratrol on some activities of isolated and in whole blood human neutrophils. *Physiol. Res.* **2003**, *52*, 555–562.
(11) Klebanoff, S. J. Myeloperoxidase: friend and foe. *J. Leukocyte Biol.* **2005**, *77*, 598–625.
(12) Furtmüller, P. G.; Burner, U.; Obinger, C. Reaction of myeloperoxidase compound I with chloride, bromide, iodide, and

thiocyanate. *Biochemistry* **1998**, *37*, 17923–17930.
(13) Furtmuller, P. G.; Obinger, C.; Hsuanyu, Y.; Dunford, H. B. Mechanism of reaction of myeloperoxidase with hydrogen peroxide and chloride ion. *Eur. J. Biochem.* **2000**, *267*, 5858–5864.
(14) Furtmuller, P. G.; Burner, U.; Regelsberger, G.; Obinger, C. Spectral and kinetic studies on the formation of eosinophil peroxidase compound I and its reaction with halides and thiocyanate. *Biochemistry* **2000**, *39*, 15578–15584.
(15) Podrez, E. A.; Abu-Soud, H. M.; Hazen, S. L. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radical Biol. Med.* **2000**, *28*, 1717–1725.
(16) Sampson, J. B.; Ye, Y. Z.; Rosen, H.; Beckman, J. S. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch. Biochem. Biophys.* **1998**, *356*, 207–213.
(17) Burner, U.; Furtmuller, P. G.; Kettle, A. J.; Koppenol, W. H.; Obinger, C. Mechanism of reaction of myeloperoxidase with nitrite. *J. Biol. Chem.* **2000**, *275*, 20597–20601.
(18) Mathy-Hartert, M.; Damas, P.; Nys, M.; Deby-Dupont, G.; Canivet, J. L.; Ledoux, D.; Lamy, M. Nitrated proteins in bronchoalveolar lavage fluid of patients at risk of ventilator-associated bronchopneumonia. *Eur. Respir. J.* **2000**, *16*, 296–301.
(19) Zhang, R.; Brennan, M. L.; Shen, Z.; MacPherson, J. C.; Schmitt, D.; Molenda, C. E.; Hazen, S. L. Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation. *J. Biol. Chem.* **2002**, *277*, 46116–46122.
(20) Deby-Dupont, G.; Deby, C.; Lamy, M. Neutrophil myeloperoxidase revisited: it's role in health and disease. *Intensivmed.* **1999**, *36*, 500–513.
(21) Malle, E.; Waeg, G.; Schreiber, R.; Gröne, E. F.; Sattler, W.; Gröne, H.-J. Immunohistochemical evidence for the myeloperoxidase/H_2O_2/halide system in human atherosclerotic lesions: colocalization of myeloperoxidase and hypochlorite-modified proteins. *Eur. J. Biochem.* **2000**, *267*, 4495–4503.
(22) Franck, T.; Grulke, S.; Deby-Dupont, G.; Deby, C.; Duvivier, H.; Peters, F.; Serteyn, D. Development of an enzyme-linked immunosorbent assay for specific equine neutrophil myeloperoxidase measurement in blood. *J. Vet. Diagn. Invest.* **2005**, *17*, 412–419.
(23) Grulke, S.; Benbarek, H.; Caudron, I.; Deby-Dupont, G.; Mathy-Hartert, M.; Farnir, F.; Deby, C.; Lamy, M.; Serteyn, D. Plasma myeloperoxidase level and polymorphonuclear leukocyte activation in horses suffering from large intestinal obstruction requiring surgery: preliminary results. *Can. J. Vet. Res.* **1999**, *63*, 142–147.
(24) Weiss, D. J.; Evanson, O. A.; McClenahan, D.; Fagliari, J. J.; Jenkins, K. Evaluation of platelet activation and platelet-neutrophil aggregates in ponies with alimentary laminitis. *Am. J. Vet. Res.* **1997**, *58*, 1376–1380.
(25) Deaton, C. M.; Marlin, D. J.; Smith, N. C.; Smith, K. C.; Newton, R. J.; Gower, S. M.; Cade, S. M.; Roberts, C. A.; Harris, P. A.; Schroter, R. C.; Kelly, F. J. Breath condensate hydrogen peroxide correlates with both airway cytology and epithelial lining fluid ascorbic acid concentration in the horse. *Free Radical Res.* **2004**, *38*, 201–208.
(26) Jantschko, W.; Furtmüller, P. G.; Zederbauer, M.; Neugschwandtner, K.; Lehner, I.; Jakopitsch, C.; Arnhold, J.; Obinger, C. Exploitation of the unusual thermodynamic properties of human myeloperoxidase in inhibitor design. *Biochem. Pharmacol.* **2005**, *69*, 1149–1157.
(27) Hirota, S.; Takahama, U.; Ly, T. N.; Yamauchi, R. Quercetin-dependent inhibition of nitration induced by peroxidase/H_2O_2/nitrite systems in human saliva and characterization of an oxidation product of quercetin formed during the inhibition. *J. Agric. Food Chem.* **2005**, *53*, 3265–3272.
(28) Schewe, T.; Sies, H. Myeloperoxidase-induced lipid peroxidation of LDL in the presence of nitrite. Protection by cocoa flavanols. *Biofactors* **2005**, *24*, 49–58.
(29) Franck, T.; Kohnen, S.; Deby-Dupont, G.; Grulke, S.; Deby, C.; Serteyn, D. A specific method for measurement of equine active myeloperoxidase in biological samples and in in vitro tests. *J. Vet. Diagn. Invest.* **2006**, *18*, 326–334.
(30) Pycock, J. F.; Allen, W. E.; Morris, T. H. Rapid, single-step isolation of equine neutrophils on a discontinuous Percoll density gradient. *Res. Vet. Sci.* **1987**, *42*, 411–412.
(31) Mathy-Hartert, M.; Bourgeois, E.; Grulke, S.; Deby-Dupont, G.; Caudron, I.; Deby, C.; Lamy, M.; Serteyn, D. Purification of myeloperoxidase from equine polymorphonuclear leucocytes. *Can. J. Vet. Res.* **1998**, *62*, 127–32.
(32) Benbarek, H.; Deby-Dupont, G.; Deby, C.; Caudron, I.; Mathy-Hartert, M.; Lamy, M.; Serteyn, D. Experimental model for the study by chemiluminescence of the activation of isolated equine leucocytes. *Res. Vet. Sci.* **1996**, *61*, 59–64.
(33) Nève, J.; Parij, N.; Moguilevsky, N. Inhibition of the myeloperoxidase chlorinating activity by non-steroidal anti-inflammatory drugs investigated with a human recombinant enzyme. *Eur. J. Pharmacol.* **2001**, *417*, 37–43.
(34) Van Antwerpen, P.; Zouaoui Boudjeltia, K.; Babar, S.; Legssyer, I.; Moreau, P.; Moguilevsky, N.; Vanhaeverbeek, M.; Ducobu, J.; Nève, J. Thiol-containing molecules interact with the MPO/H_2O_2/Chloride system to inhibit LDL oxidation. *Biochem. Biophys. Res. Commun.* **2005**, *337*, 82–88.
(35) Slater, S. J.; Seiz, J. L.; Cook, A. C.; Stagliano, B. A.; Buzac, C. J. Inhibition of protein kinase C by resveratrol. *Biochem. Biophys. Acta* **2003**, *1637*, 59–69.
(36) Tsai, S. H.; Lin-Shiau, S. Y.; Lin, J. K. Suppression of nitric oxide synthase and the down-regulation of the activation of NFkappaB in macrophages by resveratrol. *Br. J. Pharmacol.* **1999**, *126*, 673–680.
(37) Leonard, S. S.; Xia, C.; Jiang, B.-H.; Stinefelt, B.; Klandorf, H.; Harris, G. K.; Shi, X. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. *Biochem. Biophys. Res. Commun.* **2003**, *309*, 1017–1026.
(38) Fiedler, T. J.; Davey, C. A.; Fenna, R. E. X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 Å resolution. *J. Biol. Chem.* **2000**, *275*, 11964–11971.
(39) Szewczuk, L. M.; Penning, T. M. Mechanism-based inactivation of COX-1 by red wine m-hydroquinones: A structure-activity relationship study. *J. Nat. Prod.* **2004**, *67*, 1777–1782.
(40) Szewczuk, L. M.; Forti, L.; Stivala, L. A.; Penning, T. M. Resveratrol is a peroxidase-mediated inactivator of COX-1 but not COX-2: A mechanistic approach to the design of COX-1 selective agents. *J. Biol. Chem.* **2004**, *279*, 22727–22737.
(41) Fan, X.; Mattheis, J. P. Inhibition of oxidative and antioxidative enzymes by trans-resveratrol. *J. Food Sci.* **2001**, *66*, 200–203.
(42) Takaya, Y.; Terashima, K.; Ito, J.; He, Y. H.; Tateoka, M.; Yamaguchi, N.; Niwa, M. Biomimic transformation of resveratrol. *Tetrahedron* **2005**, *61*, 10285–10290.
(43) Gilabert, M. A.; Hiner, A. N. P.; Garcia-Ruiz, P. A.; Tudela, J.; Garcia-Molina, F.; Acosta, M.; Garcia-Canovasa, F.; Rodriguez-Lopez, J. N. Differential substrate behaviour of phenol and aniline derivatives during oxidation by horseradish peroxidase: kinetic evidence for a two-step mechanism. *Biochim. Biophys. Acta* **2004**, *1699*, 235–243.
(44) Gilabert, M. A.; Fenoll, L. G.; Garcia-Molina, F.; Tudela, J.; Garcia-Canovas, F.; Rodriguez-Lopez, J. N. Kinetic characterization of phenol and aniline derivates as substrates of peroxidase. *Biol. Chem.* **2004**, *385*, 795–800.
(45) Gilabert, M. A.; Fenoll, L. G.; Garcia-Molina, F.; Garcia-Ruiz, P. A.; Tudela, J.; Garcia-Canovas, F.; Rodriguez-Lopez, J. N. Stereospecificity of horseradish peroxidase. *Biol. Chem.* **2004**, *385*, 1177–1184.
(46) Fenoll, L. G.; Garcia-Mollina, F.; Gilabert, M. A.; Varon, R.; Garcia-Ruiz, P. A.; Tudela, J.; Garcia-Canovas, F.; Rodriguez-Lopez, J. N. Interpretation of the reactivity of peroxidase compound II with phenols and anilines using the Marcus equation. *Biol. Chem.* **2005**, *386*, 351–360.
(47) Pezet, R.; Perret, C.; Jean-Denis, J. B.; Tabacchi, R.; Gindro, K.; Viret, O. delta-viniferin, a resveratrol dehydrodimer: One of the major stilbenes synthesized by stressed grapevine leaves. *J. Agric. Food Chem.* **2003**, *51*, 5488–5492.

(48) Nicotra, S.; Cramarossa, M. R.; Mucci, A.; Pagnoni, U. M.; Riva, S.; Forti, L. Biotransformation of resveratrol: synthesis of trans-dehydrodimers catalyzed by laccases from *Myceliophtora thermophyla* and from *Trametes pubescens*. *Tetrahedron* **2004**, *60*, 595–600.

(49) Espin, J. C.; Wichers, H. J. Study of the oxidation of resveratrol catalyzed by polyphenol oxidase. Effect of polyphenol oxidase, laccase and peroxidase on the antiradical capacity of resveratrol. *J. Food Biochem.* **2000**, *24*, 225–250.

(50) Kim, Y. M.; Yun, J.; Lee, C. K.; Lee, H.; Min, K. R.; Kim, Y. Oxyresveratrol and hydroxystilbene compounds: Inhibitory effect on tyrosinase and mechanism of action. *J. Biol. Chem.* **2002**, *277*, 16340–16344.

(51) Ohguchi, K.; Tanaka, T.; Kido, T.; Baba, K.; Iinuma, M.; Matsumoto, K.; Akao, Y.; Nozawa, Y. Effects of hydroxystilbene derivatives on tyrosinase activity. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 861–863.

Received for review June 13, 2007. Revised manuscript received August 3, 2007. Accepted August 6, 2007. This work was supported by a grant (MPOEQUI n°011/4695) from the Région Wallonne (Direction Générale des Technologies, de la Recherche et de l'Energie) Belgium.

JF071741N