Journal of Inorganic Biochemistry 130 (2014) 84–91

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

(−)-Epicatechin regenerates the chlorinating activity of myeloperoxidase *in vitro* and in neutrophil granulocytes

Jörg Flemmig [a,b,*,1], Johannes Remmler [a,1], Fiete Röhring [a], Jürgen Arnhold [a,b]

[a] Institute for Medical Physics and Biophysics, Medical Faculty, University of Leipzig, Haertelstrasse 16–18, 04107 Leipzig, Germany
[b] Translational Centre for Regenerative Medicine, University of Leipzig, Philipp-Rosenthal-Strasse 55, 04103 Leipzig, Germany

ARTICLE INFO

Article history:
Received 20 July 2013
Received in revised form 2 October 2013
Accepted 2 October 2013
Available online 11 October 2013

Keywords:
Aminophenyl fluorescein
Peroxidases
Compound II accumulation
Myeloperoxidase
Neutrophils
(−)-epicatechin

ABSTRACT

The heme-containing enzyme myeloperoxidase (MPO) is mainly expressed in polymorphonuclear leukocytes (PMNs), the most abundant immune cell type in the blood. Accordingly, MPO is classically attributed to the innate immune response against pathogens. Yet, new results also show immune-regulatory functions of the halogenating MPO activity including the formation of anti-inflammatory mediators. In this work we tested the ability of the flavonoid (−)-epicatechin to regenerate this enzymatic activity both *in vitro* at the isolated MPO–H_2O_2–Cl^- system and *ex vivo* in human PMNs. For all experiments the non-fluorescent dye aminophenyl fluorescein (APF) was used. Upon oxidation by the MPO, the halogenation product hypochlorous acid (HOCl) fluorescein is formed which can be detected *e.g.* by flow cytometry. The *in vitro*- and *ex vivo*-results concordantly show that (−)-epicatechin is a suitable substrate to overcome a compound II accumulation of MPO which was experimentally forced by applying excess hydrogen peroxide. Thereby concentration-dependent effects of the flavan-3-ol were found in both cases and confirmed the proposed mode of action of (−)-epicatechin. The results are in accordance with previous stopped-flow kinetic studies which showed a high reactivity of the polyphenol with MPO compound II. The obtained data may contribute to the explanation of the well-known health promoting effects of (−)-epicatechin. Moreover, the presented study provides new insights into the role of MPO during inflammation.

1. Introduction

The function of myeloperoxidase (MPO) is classically attributed to the innate host response against pathogens as this heme peroxidase is mainly expressed in myeloid immune cells, namely polymorphonuclear leukocytes (PMNs, also called neutrophils) and, to a minor degree, in monocytes [1,2]. Furthermore, at neutral pH values MPO two-electronically oxidizes chloride to form the bactericidal product hypochlorous acid (HOCl), a strong oxidant which is also responsible for MPO-derived tissue modifications at chronic and/or severe inflammatory conditions [1,3,4].

Yet, more recent studies also indicate immune-regulatory functions of HOCl like the limiting of the bactericidal PMN activity, apoptosis induction in these cells and the inhibition of pro-inflammatory pathways [5–7]. Thus it can be assumed that the HOCl-producing activity of MPO during acute inflammation helps to limit pathological side effects and to down-regulate the immune response [8]. In contrast, under chronic inflammatory conditions the ongoing PMN recruitment/activation and subsequent cell necrosis lead to MPO-catalyzed pro-inflammatory processes and HOCl-derived tissue destructions [1].

The formation of hypochlorous acid from chloride (halogenation cycle) is catalyzed by the MPO redox intermediate compound I which is formed upon reaction of the resting enzyme with hydrogen peroxide [9]. As a strong oxidant compound I also one-electronically oxidizes several substrates [8]. Thereby the MPO redox intermediate compound II is formed which is converted back to the native enzyme by a second one-electron step (peroxidase cycle) [10,11]. The enzymatic cycles of MPO as well as the APF detection system used in this work are shown in Fig. 1. Yet, as compound II is a much weaker oxidant than compound I many substrates readily react with the latter but much slower with the former. This leads to an accumulation of compound II which is unable to oxidize chloride [12,13]. The half-life time of this MPO redox intermediate is estimated to be about 1 h [14].

An example of this mechanism is H_2O_2 which, at high concentration, forces a compound II accumulation [15]. An elevated production of hydrogen peroxide occurs upon pro-inflammatory PMN activation [16]. Another pathway for the compound II accumulation is the direct conversion of the native enzyme by peroxynitrite ($ONOO^-$), which is also formed at sites of inflammation [17,18]. It can be hypothesized that especially at inflammatory conditions the halogenating MPO

* Corresponding author at: Institute for Medical Physics and Biophysics, Medical Faculty, University of Leipzig, Haertelstrasse 16–18, 04107 Leipzig, Germany. Tel.: +49 341 9715772; fax: +49 341 9718959.
E-mail address: joerg.flemmig@medizin.uni-leipzig.de (J. Flemmig).
[1] These authors contributed equally to this work.

0162-0134/$ – see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.jinorgbio.2013.10.002

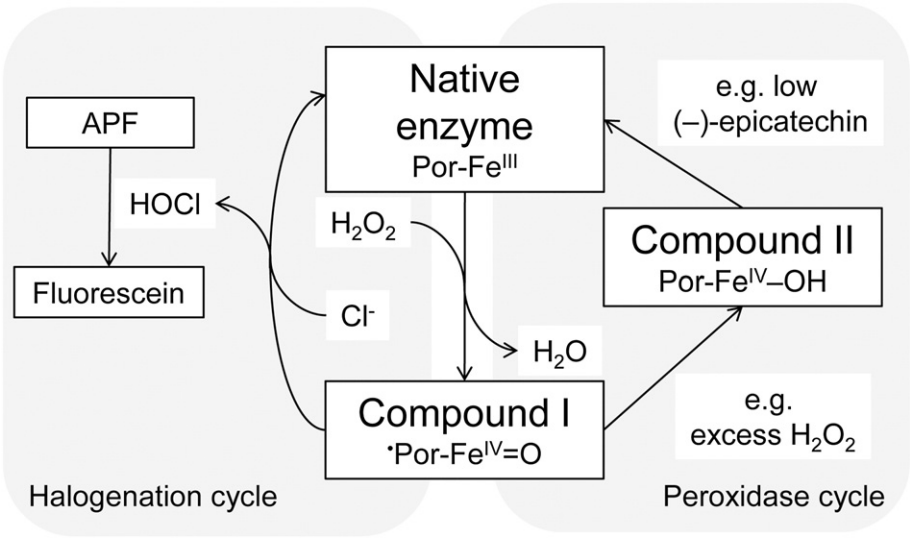

Fig. 1. Enzymatic activity of MPO. The enzyme can be activated from its native ferric form by hydrogen peroxide. Thereby compound I is formed which can subsequently undergo both one- and two-electron redox reactions. In the halogenation cycle compound I is directly reduced back to native MPO by two electron oxidation of (pseudo-)halides. Upon oxidation of chloride, hypochlorous acid (HOCl) is formed which is able to oxidize the non-fluorescent dye APF to fluorescein. Yet, compound I is also able to oxidize excess hydrogen peroxide by abstracting one electron. By adding low amounts of (−)-epicatechin compound II can be one electronically reduced back to the native enzyme which restores the ability of the enzyme to produce HOCl.

activity is impaired by an increased accumulation of compound II. Consequently less HOCl is produced which may lead to impairments regarding the termination of acute inflammatory reactions.

A further hint for the immune regulatory functions of the halogenating MPO activity comes from the fact that certain substances with well-known anti-inflammatory properties are also good substrates for compound II and thus regenerate native MPO [8]. In fact, for the reaction between (−)-epicatechin and compound II the highest second order rate constant known so far was determined ($4.5 \times 10^6\,M^{-1}\,s^{-1}$ at pH 7.0 and 25 °C) suggesting this flavonoid as a good candidate for a MPO compound II resolution [19]. (−)-Epicatechin can be found *e.g.* in green tea and was shown to exhibit numerous beneficial health effects [20–22]. Subsequent *in vivo*-studies clearly showed regulatory properties of this flavonoid on the immunological activity of PMNs [23].

Accordingly, our *in vitro*-kinetic studies on isolated MPO clearly showed an enhancing effect of (−)-epicatechin on the halogenating enzyme activity [24]. Thereby we applied monochlorodimedon (MCD), a well-established dye for the spectrophotometric detection of HOCl [24,25]. Yet for HOCl detection in PMNs we used aminophenyl fluorescein (APF) which is well applicable for flow cytometric measurements [26,27]. In this paper we now used APF to investigate the effect of (−)-epicatechin on the isolated MPO–H_2O_2–Cl^- system as well as on the chlorinating MPO activity in isolated human PMNs. Reactivating effects of this flavonoid regarding the HOCl-producing MPO activity would give new insights into the mode of action of this well-known anti-inflammatory polyphenol.

2. Material and methods

2.1. Materials

Neutrophil MPO (E.C 1.11.2.2) was purchased from Planta GmbH, Vienna, Austria. APF was obtained from Biomol GmbH, Hamburg, Germany. All other chemicals were obtained from Sigma-Aldrich, Taufkirchen, Germany.

Working solutions of H_2O_2 were prepared by dilution of a corresponding 30% stock solution. Their concentrations were determined using $\varepsilon_{240} = 43.6\,M^{-1}\,cm^{-1}$ [28]. The solutions were stored on ice and used within 3 h.

For the *in vitro*-experiments stock solutions of 100 mM (−)-epicatechin (E4018, 98% purity, Sigma Aldrich) were prepared in DMSO/water (50/50 v/v) and further diluted in water. For the experiments on isolated PMNs 1 M (−)-epicatechin stock solutions were prepared in 100% DMSO. In both cases the final DMSO concentration did not exceed 0.013%.

2.2. Kinetic studies of the APF oxidation by MPO

If not stated otherwise, the measurements were performed in 10 mM phosphate buffered saline (PBS) at pH 7.4. For each measurement 10 nM MPO were pre-incubated with 10 μM aminophenyl fluorescein (APF) either in the presence or absence of (−)-epicatechin (0 to 32 μM) at 37 °C. To start the chlorinating activity H_2O_2 (0 to 250 μM) was added to the samples *via* an injection device and the formation of fluorescein from APF was detected within the next 15 to 30 min.

All measurements were performed using a fluorescence microplate reader Tecan Infinite 200 PRO, Männedorf, Switzerland. The fluorescence intensity was monitored at 522 nm using an excitation at 488 nm.

Control experiments were performed either in the absence of MPO, H_2O_2, APF, or chloride or in the additional presence of 4-aminobenzoic acid hydrazide (4-ABAH). This compound is a well-known MPO inhibitor [29].

All measurements were performed at least in triplicate. From the obtained kinetic data an averaged curve was created for each sample composition. Afterwards these data were fitted to an exponential function using the equation $y = A_1 - A_2 e^{-kx}$ (exponential function "MnMolecular1", Origin Pro 8G SR2, OriginLab Corporation, Northampton, MA, USA). Thereby y corresponds to the observed fluorescence intensity value (arbitrary units, a.u.) at time point x (s).

If not stated otherwise, the data used for the curve fitting correspond to the data recorded between 0.5 and 6.5 min after the addition of H_2O_2. The value for the observed rate constant (k_{obs}, s^{-1}) obtained from the curve fits reflects the rate of the APF oxidation by HOCl and was used as a parameter for the chlorinating activity of MPO. The standard error (SE) given in the corresponding results section reflects the deviation of the fitted exponential function from the averaged kinetic curve of the experimental data. In the supplemental tables with the complete fitting parameters the number of iterations (I) used for the curve fittings is also given.