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Scavenging of peroxynitrite by a phenolic/peroxidase system prevents oxidative damage to DNA

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Abstract We examined the ability of horseradish peroxidase (HRP), an analog of human myeloperoxidase, to protect DNA against oxidative damage caused by peroxynitrite in the presence of chlorogenic acid (CGA), a naturally occurring polyphenol. Chlorogenic acid inhibits the formation of single strand breaks in supercoiled pBR322 DNA by acting as a scavenger of peroxynitrite. Horseradish peroxidase markedly enhances the extent of DNA protection by catalyzing the decomposition of peroxynitrite in the presence of CGA. Horseradish peroxidase alone does not inhibit peroxynitrite-induced DNA strand breaks, indicating that CGA is required as an electron donor to regenerate the active enzyme. The apparent second order rate constant for the HRP-mediated oxidation of CGA in the presence of peroxynitrite at pH 6.9 is 3.4×10^7 M^{-1} s^{-1}. This high rate suggests that CGA and other dietary polyphenols might efficiently scavenge peroxynitrite in peroxidase-containing systems in vivo.

Key words: Antioxidant; Chlorogenic acid; DNA damage; Horseradish peroxidase; Inflammation; Myeloperoxidase; Peroxynitrite scavenging; Polyphenol

1. Introduction

The superoxide anion ($O_2^{\bullet-}$) rapidly reacts with the biological mediator nitric oxide ($^{\bullet}NO$) to produce peroxynitrite, a potent oxidant [1]. Activated macrophages and neutrophils release significant quantities of $O_2^{\bullet-}$ and $^{\bullet}NO$ during the inflammatory response and are therefore considered major sources of peroxynitrite in vivo [2–5]. Neutrophils also produce hypochlorous acid (HOCl) through a reaction mediated by myeloperoxidase and H_2O_2. Hypochlorous acid is a potent bactericidal agent that may interact with metabolites of $^{\bullet}NO$ to enhance the oxidation and nitration of cellular targets [6]. The formation of leukocyte-derived oxidants has long been implicated in the pathophysiology of inflammation, and more recently peroxynitrite itself has been shown to be an important factor in several inflammatory disorders, including atherosclerosis, rheumatoid arthritis, myocardial dysfunction and autoimmune diabetes [7–10].

In biological fluids devoid of peroxidases, peroxynitrite rapidly reacts with CO_2 to form several reactive intermediates [11–13]. These intermediates undergo fast secondary reactions with sensitive biotargets such as tyrosine, which may explain the formation of nitrotyrosines in peroxynitrite-producing systems in vivo [14]. Peroxynitrite also rapidly reacts with various heme proteins, including horseradish peroxidase and human myeloperoxidase, and converts these enzymes to the quasi-stable and catalytically inactive form Compound II [15]. Based on published rate data, the reaction of peroxynitrite with myeloperoxidase will take precedence over the CO_2 reaction under physiological conditions in vivo [11,14]. Since activated neutrophils release high concentrations of myeloperoxidase in addition to peroxynitrite [16], peroxynitrite produced at sites of inflammation may react predominantly with myeloperoxidase rather than CO_2.

DNA is a sensitive biotarget for peroxynitrite-mediated oxidative damage. In both supercoiled plasmid DNA [17–21] and mammalian cellular DNA [22–25], peroxynitrite causes extensive base modification as well as single strand breaks. Oxidative damage to DNA has long been considered an important underlying event in chronic inflammation leading to carcinogenesis [26–28]. Therefore, it is essential to identify and characterize compounds that show pharmacological activity against peroxynitrite.

Polyphenols are plant secondary metabolites that exhibit a broad spectrum of biological and pharmacological activities. Among their desirable properties, polyphenols, and particularly flavonoids, are an important class of naturally occurring antioxidants [29–32]. Phenylpropanoids are a structurally related group of phenolic compounds, and chlorogenic acid (CGA), an ester of quinic acid and caffeic acid (Fig. 1), is a prominent phenylpropanoid constituent of the human diet [33]. Like other *o*-dihydroxy phenolics, chlorogenic acid possesses powerful radical scavenging properties in a variety of in vitro model systems [34–37], as well as strong electron donating properties to guaiacol-type peroxidases such as horseradish peroxidase [38].

Although the antioxidant properties of CGA and other dietary polyphenols have been well characterized, there have been few studies to assess whether these compounds can protect DNA against oxidative damage caused by peroxynitrite. In the present study we investigated whether CGA alone, and in the presence of horseradish peroxidase, can scavenge peroxynitrite and inhibit peroxynitrite-mediated strand breaks in supercoiled pBR322 DNA. Horseradish peroxidase is a typical heme peroxidase with kinetic properties like those of myeloperoxidase and lactoperoxidase [15,39] and therefore was used as a model peroxidase to study the interaction of CGA and peroxynitrite.

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2. Materials and methods

2.1. Reagents

Plasmid DNA (pBR322) was purchased from GibcoBRL (Gaithesburgh, MD). Agarose and chlorogenic acid were from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (EIA grade) was purchased from Boehringer Mannheim (Indianapolis, IN).

2.2. Synthesis of peroxynitrite

Peroxynitrite was synthesized by the azide-ozone reaction according to the method of Uppu et al. [40]. Briefly, a 100 mM solution of alkaline sodium azide (pH 12) was ozonated with a Sander model 200 ozonator at 4°C for approximately 3 h. To reduce contamination by unreacted azide, ozonation was prolonged for 10–15 min after a maximal yield of peroxynitrite was obtained. This procedure ensured that HRP activity was not inhibited by contaminating azide in peroxynitrite-containing solutions (data not shown). The concentration of peroxynitrite was calculated from the absorbance at 302 nm (ε=1670 M^{-1} cm^{-1}). Stock solutions of peroxynitrite were stored at −20°C and used within 3–4 weeks.

2.3. Peroxynitrite treatment and analysis of DNA strand breaks

Single and double strand breaks in supercoiled DNA were analyzed by agarose gel electrophoresis as described previously [17]. Plasmid pBR322 DNA (0.5 μg) was treated with 50 μM peroxynitrite at room temperature in a buffered reaction containing 50 mM sodium phosphate, 10 mM NaCl, 0.1 mM DTPA, pH 6.9 and various concentrations of CGA and HRP. The solution was vigorously vortexed during addition of peroxynitrite, since under these conditions peroxynitrite spontaneously decays with a half-life of less than 2 s [13]. The DNA was precipitated with ethanol after 5 min and left at −80°C for 3 h. Samples were centrifuged and the DNA pellet was resuspended in 10 μl 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, mixed with 5 μl of electrophoresis loading buffer (0.05% bromophenol blue, 50% sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and loaded onto a 0.9% agarose gel prepared in 89 mM Tris-borate/2 mM EDTA containing 0.5 μg/ml ethidium bromide. Electrophoresis was carried out at 60–70 mV for approximately 3 h. Gels were de-stained overnight, then visualized under UV light and photographed.

2.4. Stopped-flow kinetic analysis

The reaction between CGA and peroxynitrite was monitored as the loss of CGA at 323 nm using a stopped-flow instrument (On-Line Instrument Systems, Jefferson, GA) as described elsewhere [13]. Chlorogenic acid (50 μM) was allowed to react with peroxynitrite (50–500 μM) at 25°C in 50 mM sodium phosphate, 10 mM NaCl, 0.1 mM DTPA, pH 6.9. The peroxynitrite was added from a stock prepared in 5 mM NaOH (pH 11–12) which did not affect the final pH of the solution. When present, HRP (0–100 nM) was pre-mixed with the CGA solution. The concentration of HRP was calculated from its absorbance at 402 nm ($\varepsilon = 2\times10^5$ M^{-1} cm^{-1}) [41]. The data were analyzed for pseudo-first order rates using the OLIS-RSM software.

3. Results

3.1. Chlorogenic acid is oxidized by peroxynitrite

Fig. 2 shows the effects of peroxynitrite on changes in the UV absorbance profile of CGA. Increasing concentrations of peroxynitrite abolished the 325 nm absorbance peak associated with reduced CGA, and the absorbance changes coincided with a yellowing of the solution. To assess the nature of the products, UV spectra were also recorded after exposure of CGA to either an oxidizing system (H_2O_2 plus HRP) [38] or a nitrating system (sodium nitrite at pH 4.0) [42]. As in the case of peroxynitrite, both treatments caused a loss of absorbance at 325 nm due to oxidation of the catechol function in CGA. However, the spectrum of the nitrated product also showed a new peak at 444 nm, similar to other nitrated aromatic compounds such as tyrosine [43] and 3-hydroxyphenyl acetic acid [44]. Since a peak in this region was not observed in the products of the reaction of CGA with peroxynitrite, we conclude that peroxynitrite oxidizes but does not nitrate CGA at pH 6.9. Similar behavior has been reported for the reaction between peroxynitrite and catecholamines [45].

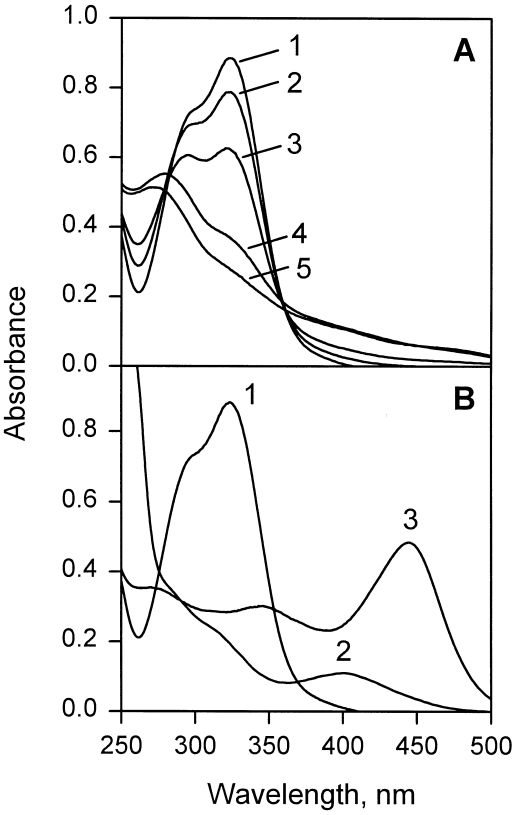

Fig. 2. Spectral changes of CGA caused by peroxynitrite. A: Chlorogenic acid (50 μM) was treated with peroxynitrite in a buffered reaction containing 50 mM phosphate, 10 mM NaCl, and 0.1 mM DTPA, pH 6.9. Peroxynitrite concentrations ($\times10^{-5}$ M) were as follows: 0 (1), 8 (2), 16 (3), 32 (4), and 64 (5). Absorbance spectra were recorded 5 min after the addition of peroxynitrite. B: Comparison of UV spectra of CGA following no addition (1), treatment with 200 μM H_2O_2 plus 20 nM HRP (2), or treatment with 20 mM sodium nitrite in 50 mM acetate buffer (pH 4.0) (3).

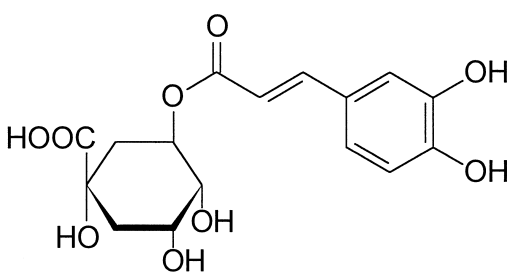

Fig. 1. Structure of chlorogenic acid.

3.2. Chlorogenic acid protects DNA against peroxynitrite-mediated strand breaks

As shown in previous studies [17,20], peroxynitrite causes extensive strand breaks in plasmid DNA. Exposure of pBR322 DNA to 50 μM peroxynitrite converted more than 90% of the native supercoiled (SC) form to a relaxed open circular (OC) form with single strand breaks plus a lower yield of a linearized (LIN) form caused by double strand breaks

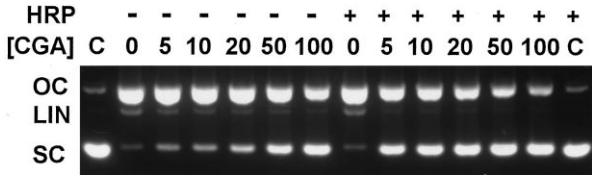

Fig. 3. Inhibition of peroxynitrite-induced strand breaks in DNA by chlorogenic acid in the absence (lanes 3–7) or presence (lanes 9–13) of horseradish peroxidase. Supercoiled (SC) pBR322 DNA (0.5 μg) was exposed to 50 μM peroxynitrite in 50 mM sodium phosphate, 10 mM NaCl, 0.1 mM DTPA, pH 6.9, and analyzed for single (OC, open circular) and double (LIN, linear) strand breaks by agarose gel electrophoresis. Chlorogenic acid was added to the reaction at the concentrations indicated above each lane ($\times 10^{-5}$ M). Horseradish peroxidase (100 nM) was included in the reaction mixture as indicated above each lane. Lanes 1 and 14 represent untreated controls.

(Fig. 3). In the presence of CGA the extent of peroxynitrite-mediated DNA damage decreased in a dose-dependent manner (lanes 3–7). At the highest CGA concentrations used in these experiments (1 mM, lane 7), approximately 40% of the DNA was recovered in the supercoiled form after exposure to peroxynitrite.

3.3. Horseradish peroxidase catalyzes the reaction between chlorogenic acid and peroxynitrite

Horseradish peroxidase markedly enhances the ability of CGA to prevent peroxynitrite-mediated strand breaks in pBR322 DNA (Fig. 3, lanes 9–13). By itself, HRP does not prevent oxidative damage to DNA (lane 8); however, HRP in the presence of CGA led to a significant increase in the fraction of DNA recovered in the supercoiled form after treatment with peroxynitrite. The enhanced DNA protection in the presence of HRP was apparent at all CGA concentrations but was especially pronounced at low concentrations of the polyphenol (compare lanes 3 and 9). At high concentrations of CGA, the direct reaction with peroxynitrite supersedes the enzymatic reaction and provides sufficient scavenging activity to protect DNA. These results indicate that HRP catalyzes the decomposition of peroxynitrite in the presence of CGA.

To further investigate the involvement of HRP in the CGA/peroxynitrite reaction, the kinetics of the reaction were analyzed using a stopped-flow system. The high molar absorbance of CGA in the 300–330 nm region precluded the direct analysis of peroxynitrite loss in the stopped-flow system, so instead we monitored the loss of CGA at 323 nm. Chlorogenic acid is stable in the presence of HRP (data not shown). However, upon addition of peroxynitrite CGA is rapidly oxidized, and HRP accelerates this reaction in a dose-dependent manner (Fig. 4, inset). The pseudo-first order rate constant for the reaction (k_{obs}) is linearly dependent on HRP concentration (Fig. 4). Thus, the apparent second order rate constant (k_{app}) for the enzyme-catalyzed decomposition of CGA by peroxynitrite at pH 6.9 obeys the following equation:

$$k_{obs} = k_{app}[HRP] + k_{off} \quad (1)$$

where k_{off} is the pseudo-first order rate constant for the uncatalyzed oxidation of CGA by peroxynitrite. From the slope an apparent second order rate constant (k_{app}) of 3.4×10^7 M^{-1} s^{-1} was calculated for the enzyme-catalyzed reaction, which is in good agreement with the rate constant reported for the reaction of peroxynitrous acid with both HRP and human myeloperoxidase [15].

To assess the ability of the HRP-phenolic system to inhibit peroxynitrite-mediated DNA damage under more physiological conditions, pBR322 DNA was exposed to 50 μM peroxynitrite and an equal concentration of CGA over a range of HRP concentrations. As shown in Fig. 5, in the absence of HRP, 50 μM CGA was only moderately effective in preventing DNA strand breaks caused by peroxynitrite (lane 3). However, increasing concentrations of HRP significantly increased the fraction of DNA recovered in the supercoiled form after treatment with peroxynitrite (lanes 4–8). In the absence of CGA, HRP did not inhibit peroxynitrite-induced DNA damage, even at relatively high concentrations of the enzyme (lanes 9–13). However, in the presence of 1 μM HRP and 50 μM CGA, almost complete DNA protection was observed (lane 8). Since activated leukocytes can release myeloperoxidase at concentrations exceeding 10 μM [11], these results suggest that CGA can efficiently scavenge peroxynitrite in peroxidase-containing systems in vivo.

4. Discussion

4.1. Peroxynitrite scavenging by chlorogenic acid

In this study we have demonstrated that chlorogenic acid, an important dietary polyphenol, inhibits DNA damage caused by the physiological oxidant peroxynitrite. In previous studies, CGA has been shown to be a potent scavenger of free radicals, similar to other *o*-dihydroxy polyphenolics, as well as artificial phenols such as Trolox, the water soluble analog of Vitamin E [34–37]. Similarly, CGA was oxidized by peroxynitrite at pH 6.9 (Figs. 2 and 4), indicating a possible scavenging role against this oxidant, as was recently suggested for catechins, the major polyphenolic constituents of green tea [46].

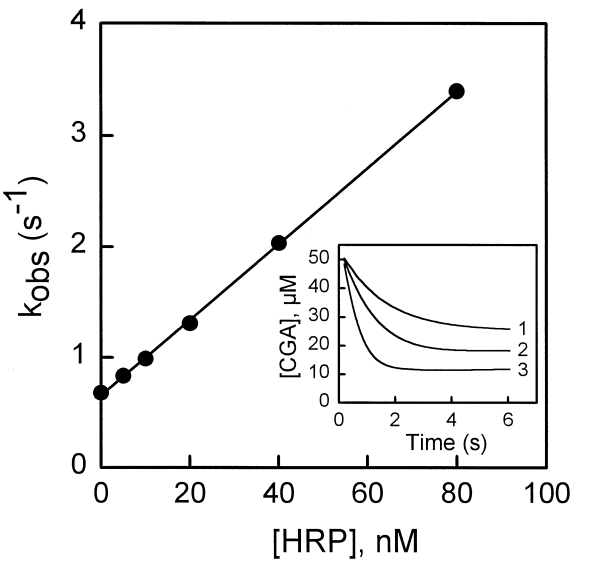

Fig. 4. Pseudo-first order rate constants for the HRP-catalyzed decomposition of CGA by peroxynitrite. The inset shows typical kinetic traces of CGA decomposition in the presence of 0 nM (1), 10 nM (2), or 80 nM (3) HRP. The reaction contained 50 μM CGA, 200 μM peroxynitrite and various concentrations of HRP in 50 mM phosphate buffer, 10 mM NaCl, 0.1 mM DTPA, pH 6.9. The second order rate constant was calculated from the slope. Data points represent the mean (±S.D.) of three individual measurements.

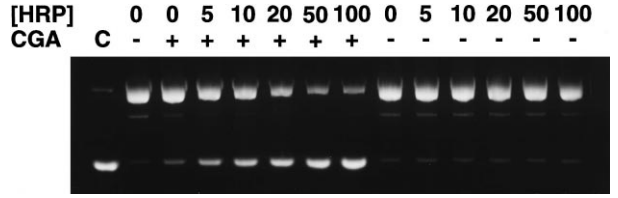

Fig. 5. Inhibition of peroxynitrite-induced strand breaks in plasmid DNA by the CGA/HRP peroxynitrite scavenging system. Supercoiled pBR322 DNA was exposed to 50 μM peroxynitrite in the presence (lanes 3–8) or absence (lanes 9–14) of 50 μM CGA. Horseradish peroxidase was included in the reaction at the concentrations indicated above each lane ($\times 10^{-8}$ M). Lane 1 represents untreated control DNA and lane 2 represents a sample treated with 50 μM peroxynitrite alone. All other conditions as in Fig. 3.

The DNA nicking activity of peroxynitrite is thought to arise from the ability of either an activated form of peroxynitrous acid [22,47], or a peroxynitrite/CO_2 adduct [14], to abstract hydrogen atoms from deoxyribose groups in DNA. By acting as a scavenger of this intermediate, CGA was able to reduce the extent of strand breakage in supercoiled plasmid DNA (Fig. 3).

4.2. Catalysis of the CGA/peroxynitrite reaction by horseradish peroxidase

The ability of CGA to protect DNA against peroxynitrite-induced oxidative damage is markedly enhanced in the presence of the heme-containing enzyme horseradish peroxidase. In the absence of the enzyme, a 10- to 20-fold molar excess of CGA over peroxynitrite is required to observe significant DNA protection (Fig. 3). However, in the presence of catalytic concentrations of HRP (<1 μM), CGA provides significant DNA protection when present at equivalent concentrations of peroxynitrite (Figs. 3 and 5).

Peroxynitrous acid readily reacts with various peroxidases, including HRP, to form the stable one-electron oxidation product Compound II, as shown below.

$$HRP^{3+} + HOONO \rightarrow \text{Compound II} + NO_2^{\bullet} + H^+. \quad (2)$$

This reaction proceeds rapidly ($k = 3\times10^6$ M^{-1} s^{-1}), and the product $NO_2^{\bullet}$ radical decays primarily through disproportionation and hydrolysis to produce NO_2^- and NO_3^- [15]. Compound II is catalytically inactive toward peroxynitrite and slowly decays to the native enzyme in the absence of external electron donors [15]. Although there is evidence that NO_2^- can donate electrons to Compound II [48], our results indicate that HRP is a poor scavenger of peroxynitrite in the absence of added reductants (Figs. 3 and 5).

Chlorogenic acid is an efficient electron donor to HRP in the presence of H_2O_2, and the CGA radical has recently been detected as an intermediate [38], indicating that CGA is a 'classical' peroxidase substrate that donates a single electron to both Compounds I and II:

$$\text{Compound I} + CGA \rightarrow \text{Compound II} + CGA^{\bullet}, \quad (3)$$

$$\text{Compound II} + CGA \rightarrow HRP^{3+} + CGA^{\bullet}. \quad (4)$$

Thus, in the peroxynitrite/HRP system, the active form of the enzyme is rapidly regenerated by CGA, allowing the scavenging of peroxynitrite to proceed in the presence of catalytic concentrations of HRP. The CGA radical evidently does not cause DNA nicks under these conditions, unlike the catechol radical associated with cigarette tar [49].

4.3. Biological implications of the peroxynitrite/peroxidase reaction

Neutrophils and macrophages simultaneously generate both $^{\bullet}NO$ and $O_2^{\bullet-}$ during the inflammatory response, and these species rapidly react to yield peroxynitrite in vivo [1–5]. Recent studies have shown that CO_2 is the principal catalyst for peroxynitrite decomposition in biological fluids [11–14]. However, stimulated neutrophils also release high concentrations of the enzyme myeloperoxidase [16], which reacts with peroxynitrite more rapidly than does CO_2 at physiological pH [11,14]. The relative rate of these competing biological pathways can be calculated from their *reactivity products*, defined as the second order rate constant times the physiological concentration of the reactants [14]. Biological fluids contain approximately 1 mM CO_2, which can react with peroxynitrite at a rate of 5.8×10^4 M^{-1} s^{-1} to give a reactivity product of $k\times[CO_2] = 58$ s^{-1} [14]. The reaction of peroxynitrite with myeloperoxidase is considerably faster ($k = 2\times10^7$ M^{-1} s^{-1}) [15], and the extracellular MPO concentration within phagosomal sites of infection is in the order of 10–100 μM [11], which yields a reactivity product of 200–2000 s^{-1} for the MPO/peroxynitrite reaction. Thus, a significant amount of peroxynitrite generated at sites of infection and inflammation could react directly with MPO rather than CO_2.

Myeloperoxidase also plays a fundamental role in neutrophil oxidant production by catalyzing the formation of hypochlorous acid (HOCl) from hydrogen peroxide and chloride. Hypochlorous acid is considered the predominant cytotoxic and bactericidal species released during the immune response [16]. In addition, HOCl is a powerful oxidant that reacts with endogenous thiols and other cellular targets and therefore is implicated in oxidative stress associated with chronic infection and inflammation [16,50]. Unlike the typical peroxidase cycle, HOCl is formed by a direct two-electron reaction between chloride and the MPO Compound I [16], as shown below.

$$MPO^{3+} + H_2O_2 \rightarrow \text{Compound I} + H_2O, \quad (5)$$

$$\text{Compound I} + Cl^- \rightarrow MPO^{3+} + HOCl. \quad (6)$$

Peroxynitrite inhibits HOCl formation, since chloride cannot be oxidized by Compound II [16]. However, the chlorinating activity of MPO is strongly modulated by $O_2^{\bullet-}$, which is produced in higher concentrations than $^{\bullet}NO$ by activated neutrophils [2]. Superoxide reacts with Compound II to regenerate the ferric enzyme, and also reduces MPO to the ferrous enzyme Compound III, the major form found in extracellular fluids [16]. In addition, the MPO/H_2O_2 system oxidizes cellular aromatics such as tyrosine to form highly reactive tyrosyl radicals [40,51]. Chlorogenic acid, by acting as both a radical scavenger and an alternative one-electron donor for MPO, could potentially inactivate neutrophil oxidants by several mechanisms.

4.4. Conclusions

We suggest that cellular damage caused by the simultaneous formation of peroxynitrite and release of MPO during chronic infection and inflammation can be mitigated in the presence of

CGA. That is, in the presence of CGA as an electron donor, MPO could be diverted from promoting oxidative stress to scavenging endogenous peroxynitrite. A variety of plant polyphenols other than CGA, including the common flavonoids quercetin and rutin, are also efficient donors to heme peroxidases [52]. Many of these compounds have also been reported to possess anti-inflammatory properties. Thus, CGA and other plant polyphenols could be therapeutic agents, reducing oxidative damage caused by peroxynitrite in chronic inflammation and other forms of stress. More research is needed on the uptake and metabolism of these compounds in living cells to assess whether they might fulfil such a function in vivo.

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