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Original article

Dual effects of (+)-catechin on hemin-induced oxidative reactions: A potential pharmacological implication

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ABSTRACT

Introduction: Although the anti- and pro-oxidant properties were the well-known phenomenons for flavonoids, the influence of flavonoids on free heme-dependent redox reactions was not definitely elucidated.
Methods: By spectrophotometry and Western blotting, the influence of (+)-catechin (a polyphenol identified in red wine, tea, and cocoa) on hemin-induced oxidative reactions was investigated in this study.
Results: (+)-Catechin was found to act as an efficient reducing agent for the reduction of ferryl heme to ferric state, demonstrating novel anti-oxidant pathway. However, this antioxidant had the ability to trigger hemin oxidation through producing additional hydrogen peroxide (H_2O_2). On the other hand, the effects of (+)-catechin on hemin–H_2O_2–induced protein (or methylene blue) oxidation depended on the concentrations of H_2O_2 and antioxidant. (+)-Catechin exerted significant anti-oxidant ability when lower concentration of H_2O_2 was used. However, in the presence of higher concentration of H_2O_2, (+)-catechin at low concentrations could significantly aggravate oxidative reactions and exhibit protective effects at high concentrations.
Conclusions: The anti- and pro-oxidant effects of catechin on heme-dependent redox reactions at different concentration, may provide new insights into the dietary intake and therapeutic implications of catechins with free heme and heme proteins.

1. Introduction

As the prosthetic group of numerous heme proteins, heme carries out physiological functions as a transporter for oxygen and electrons.[1] However, excess free heme have deleterious effects on DNA, polyunsaturated fatty acids, and proteins by generating reactive radicals. Evidence to support the important role of free heme in oxidative stress and a variety of diseases is widely present.[1–4] The pathologies of many diseases are linked to the interaction between the heme groups and peroxides to initiate oxidative reactions, including the generation of highly reactive ferryl heme (Fe^{4+}).[1,3,4]

Due to the deleterious effects of oxidation on biological molecules,[5,6] flavonoids have been widely used as the classic antioxidants to ameliorate oxidative damage *in vitro* and *in vivo*. Flavonoids are polyphenolic compounds which are present in plants, vegetables and fruits. Due to their anti-oxidant activities, including free radical-scavenging, lipid peroxidation-inhibiting and metal-chelating, they exert a wide range of beneficial effects on human health.[7,8]

As one of the phenolic compounds present in red wine, tea, and cocoa, (+)-catechin is a major contributor to the high anti-oxidant capacity.[7–10] Therefore, the therapeutic use and dietary intake of catechins can be associated with significant health benefits.[7,8,11] Several studies have attributed the anti-oxidant properties of tea extracts to the presence of catechins. However, catechin could react with dissolved oxygen in aqueous solution and generate hydrogen peroxide (H_2O_2),[12] which might lead to the accelerated oxidation of heme proteins.[10,13] Although the anti- and pro-oxidant properties were the well-known phenomenons for flavonoids,[7,8,10,13,14] the

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influence of catechin on free heme-dependent redox reactions was not definitely elucidated.

2. Materials and methods

2.1. Materials

Ferriprotoporphyrin IX chloride (hemin; which is referred to as "heme"), bovine serum albumin (BSA), catalase, (+)-catechin (Catechin), rabbit polyclonal antibody against dinitrophenol (DNP) and 2, 4-dinitrophenylhydrazine (DNPH) were purchased from Sigma (Aldrich Co.; St. Louis, MO, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and RPMI-1640 (for cell culture) were purchased from Gibco BRL (Gaithersburg, MD, USA).

2.2. Ferryl hemin reduction by (+)-catechin

Ferryl hemin was generated by addition of H_2O_2 to hemin in a 1:1 ratio at pH 7.4.[10,13] After 15 min, catalase was added to remove excess H_2O_2, and then antioxidant was added. A Hitachi U-3310 spectrophotometer was used for the optical spectra measurement of samples.

2.3. Detection of H_2O_2 in the reaction between (+)-catechin and hemin

In order to investigate the potential roles of widespread H_2O_2 in oxidation process, catalase (H_2O_2 scavenger) was used.[2,15] In the absence or presence of catalase, hemin (20 μM, final concentration, the same below) was treated with (+)-catechin (20 μM) or H_2O_2 (20 μM) at 37 °C for 20 min. The obtained reaction samples were used in optical spectra determination.

2.4. Hemin and (or) H_2O_2-induced protein (or methylene blue) oxidation in the presence of (+)-catechin

Samples of BSA in phosphate buffered saline (PBS, 0.1 M, pH 7.4) were pre-incubated (5 min at 37 °C) with flavonoid, and then treated with hemin (20 μM) and (or) H_2O_2 (0.04 or 0.5 mM) at 37 °C for 20 min. The final concentration of BSA was 0.5 mg/ml. Significant protein oxidative modifications were observed in short time incubation when high concentrations of hemin-H_2O_2 were used in many *in vitro* experiments.[10,13] These high concentrations were, therefore, chosen in our studies to conveniently compare the different effects of catechin. The obtained reaction mixtures were used in protein oxidation assays.

Due to its applicability for the simple and rapid detection of free radicals, methylene blue (MB) was selected as the substrate to further study the role of (+)-catechin in hemin−H_2O_2−induced oxidative damage.[16] In the absence or presence of (+)-catechin, samples (0.01 mg/ml MB) in PBS were incubated with hemin-H_2O_2 for 2 h. Subsequently, the concentrations of MB were determined spectrophotometrically at 665 nm.

2.5. Western blotting analysis for protein oxidation

As the widely used marker of protein oxidation, the formation of protein carbonyl groups is always accompanied with oxidative damage of protein.[5,6] For detection of protein oxidation, the carbonyl groups in proteins were first derivatized with DNPH,[6] resulting in the formation of DNP. Antibody against DNP was used for the detection of protein carbonyl groups. Western blotting analysis for protein oxidation was processed as previously described.[6,10] Alpha Imager 2200 software was used to analyze the optical density in Western blotting.

2.6. Cell culture and cell viability determination

Human hepatoma HepG2 cells were grown in RPMI-1640. Different concentrations of (+)-catechin were added to the cells for 5 min. Hemin-H_2O_2 system then was added, and the cells were incubated further at 37 °C for up to 2 h. Cell viability was quantified by using MTT assay.[14]

2.7. Statistical analysis

All data were expressed as the means ± SD of three independent experiments. Significance was assessed by using the one-way ANOVA ($P < 0.05$ as significant).

3. Results and discussion

3.1. Anti-oxidant effects of (+)-catechin on hemin oxidative damage

In addition to its ability as radical scavengers, some flavonoids had the capacity to reduce highly reactive ferryl heme (Fe^{4+}) formed in the reaction of heme with H_2O_2.[10,13] The efficiency of this polyphenol compound in functioning as ferryl hemin reducing agent was examined at physiological pH. Addition of (+)-catechin to ferryl hemin led to optical changes shown in Fig. 1A. These changes were typical of the reduction of ferryl heme to ferric heme, the increase in the absorbance from 380 to 400 nm and the decrease about 420 nm assigned to the Soret bands of ferric heme and ferryl heme, respectively.[10,13,15] Therefore, this polyphenol compound was found to act as an efficient reducing agent for the reduction of ferryl heme to ferric state.

As a reducing agent for ferryl species, (+)-catechin could exert anti-oxidant effect on heme oxidative damage. Fig. 1B showed significant increase in hemin oxidation in the presence of H_2O_2. The addition of antioxidant greatly slowed down the oxidation process. Therefore, (+)-catechin exhibited significant protective activity in ameliorating heme-related oxidative damage through the reduction of ferryl heme to native heme.

3.2. Pro-oxidant effect of (+)-catechin on hemin oxidative damage

Moreover, the influence of (+)-catechin on normal heme redox transition was investigated. Fig. 2A showed the spectra changes of hemin after the addition of (+)-catechin. After 20 min of incubation with flavonoid, the spectra of hemin shifted to resemble that of oxidized heme.[10,13,15,17,18] Therefore, (+)-catechin exhibited pro-oxidant ability in hemin oxidation.

In the presence of anti-oxidant enzyme (Fig. 2A and B), catalase could efficiently inhibit catechin-triggered hemin oxidation as indicated by the absorbance increase close to 400 nm. Based on these results, it was demonstrated that H_2O_2 was the important intermediate on the pro-oxidant mechanism of catechin. A possible explanation for the pro-oxidant effect of catechin on hemin oxidation is that itself can be the source of H_2O_2 which may lead to the accelerated oxidation of heme. Moreover, catechin would bind to both intact and degraded forms of heme proteins through its metal chelating effect,[12,19] which was convenient for catechin to react with dissolved oxygen in aqueous solution, and generates H_2O_2. Also, metal-chelating ability would make the spectral changes of hemin after the addition of (+)-catechin different from that after the treatment of H_2O_2 (Fig. 2).