Inhibition by Polyphenolic Phytochemicals and Sulfurous Compounds of the Formation of 8-Chloroguanosine Mediated by Hypochlorous Acid, Human Myeloperoxidase, and Activated Human Neutrophils

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Received June 21, 2012; Accepted August 23, 2012; Online Publication, December 7, 2012 [doi:10.1271/bbb.120482]

Hypochlorous acid (HOCl) produced by myeloperoxidase (MPO) of activated neutrophils can react with nucleic acid bases to form chlorinated nucleosides such as 8-chloroguanosine (Cl-Guo). Chlorination is enhanced by nicotine. We investigated the effects of various natural antioxidants including polyphenolic phytochemicals on the formation of Cl-Guo by HOCl in the presence and the absence of nicotine. Polyphenols, including catechins, curcumin, resveratrol, silibinin, and sulfurous compound α-lipoic acid, were found to inhibit both HOCl- and human MPO-induced Cl-Guo formation dose-dependently. Among the test compounds, (−)-epigallocatechin gallate (EGCG) showed the strongest inhibitory effect. Cl-Guo formation, mediated by activated human neutrophils in the presence of nicotine, was inhibited by EGCG, silibinin, and α-lipoic acid. These results suggest that polyphenols and sulfurous compounds have the potential to inhibit the induction of nucleobase damage mediated by chlorination, with possible application to reducing DNA damage associated with inflammation and cigarette-smoke inhalation.

Key words: hypochlorous acid; chlorination; nicotine; neutrophils; polyphenolic phytochemicals

In inflamed and infected tissues, the host immune system is stimulated to produce and activate enzymes such as myeloperoxidase (MPO) and NADPH oxidase (NOX). These enzymes generate reactive oxygen species (ROS) such as hypochlorous acid (HOCl) and superoxide anion (O2−). These ROS can react with various important biological molecules such as protein and lipids.1,2

Recently, HOCl was reported to react with nucleic acid bases to form chlorinated and oxidized compounds, e.g., 2-chloroguanosine (Cl-Guo), 8-chloroadenine, 8-chloro-2′-deoxyguanosine, 5-chloro-2′-deoxyxytidine, and 8-oxo-deoxyGua.3,4 In addition, tertiary amines such as nicotine and trimethylamine at physiologically relevant concentrations have been found strongly to enhance Cl-Guo formation mediated by HOCl.5 The G-463A polymorphism of the mpo gene, which reduces the expression of MPO mRNA, has been reported to be associated with reduced risk of lung cancer in cigarette smokers.6 Hence DNA damage induced by MPO of activated neutrophils and enhancement of it by nicotine may be important in the pathophysiology of human diseases associated with cigarette smoking (e.g., chronic obstructive pulmonary disease and lung cancer). Chlorinated nucleosides such as Cl-Guo can be used as new biomarkers related to diseases (inflammation and cancer) mediated by activated neutrophils in vivo.

Epidemiological studies indicate that the consumption of polyphenolic compounds such as green tea catechins decreases the risk of cancer, cardiovascular disease, and hepatic inflammation.10 Various phytochemicals, especially plant polyphenolic compounds, have received much attention for their unique chemopreventive activities against cancer.10,11 Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, indomethacin, and other natural pharmaceutical products, are used to treat inflammation and rheumatic disease. The chemoprevention exhibited by NSAIDs against several types of cancer has been discussed,2,13,14 but little attention has been paid to the activities of phytochemicals and oxidant that plays an important role in the host defense system owing to its bacteria killing abilities, it can also react with various important biological molecules such as protein and lipids.1,2

Abbreviations: Cl-Guo, 8-chloroguanosine; DMFA, dimethylformamide; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; Hanks’ BSS, Hanks’ buffered salt solution; H2O2, hydrogen peroxide; HOCl, hypochlorous acid; MPO, myeloperoxidase; NOX, NADPH oxidase; NSAIDs, non-steroidal anti-inflammatory drugs; O2−, superoxide anion; PMA, β-phorbol myristate acetate; ROS, reactive oxygen species

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NSAIDs in MPO/NOX-mediated nucleic acid base damage in vivo.

In this study, we examined various polyphenolic phytochemicals, NSAIDs, and sulfurous compounds for their effects on the formation of Cl-Guo mediated by HOCl, human MPO in the presence of its substrates (NaCl and H₂O₂), and activated human neutrophils.

Materials and Methods

Materials. CI-Guo was purchased from the Biolog Life Science Institute (Bremen, Germany). Polymorphprep™ and human MPO were from Nycomed (Tordhov, Oslo, Norway) and Alexis (San Diego, CA) respectively. All the other reagents were commercially available and of analytical grade, and were from Sigma-Aldrich (St. Louis, MO) and Merck (Darmstadt, Germany).

Chemical reaction of Guo with HOCl. Chloride ion-free sodium hypochlorite was prepared by a previously reported method.⁵ The reaction mixture (0.5 mL) contained 0.5 mM Guo, 0.05 mM HOCl with and without 0.05 mM nicotine in 50 mM sodium phosphate buffer (pH 7.4). This was incubated at 37°C for 30 min in the presence of 0.5, 5, and 50 μM of test compounds. The reactions were initiated by adding HOCl, and were terminated by adding 5 mM methionine. The test compounds were dissolved in N,N-dimethylformamide (DMFA), which was diluted with distilled water (final DMFA containing 0.1%). Controls containing 0.1% DMFA were incubated in the absence of the test compounds.

Enzymatic reaction of Guo with human MPO. The concentration of MPO was assayed spectrophotometrically (E₂₈₀ = 178 mM⁻¹ cm⁻¹). The reaction mixture (0.5 mL) contained 0.5 mM Guo, 0.1 mM H₂O₂, 100 mM sodium chloride, and 0.05 mM nicotine in 50 mM sodium phosphate buffer (pH 7.4). It was incubated at 37°C for 30 min in the presence of 0.5, 5, and 50 μM of the test compounds in 0.1% DMFA. The reactions were started by the addition of 80 mM MPO, and stopped by the addition of 5 mM methionine.

Reaction of Guo with human neutrophils. Human neutrophils were isolated from the fresh blood of healthy volunteers by the density gradient centrifugation method with Polymorphprep™ following the manufacturer’s instructions. Residual erythrocytes were removed by hypotonic lysis at 4°C. The cells were resuspended, rinsed with Hanks’ BSS, and counted using a hemocytometer. The viability of the cells was determined by the trypan blue exclusion method. During the experiments, neutrophils were freshly prepared each time. Neutrophils (5 x 10⁵ cells), activated with 0.2 μM β-phorbol myristate acetate (PMA) were incubated with 0.5 mM Guo and 0.05 mM nicotine in Hanks’ BSS at 37°C for 60 min in the presence of 0.5, 5, and 50 μM of the test compounds. The reactions were initiated by adding neutrophils. After a reaction, the cells were pelleted by centrifugation and the supernatant was transferred to a new polypropylene microtube. Then 5 mM methionine was added immediately to the supernatant to terminate the reaction.

All the protocols as to the collection and treatment of human blood were approved by the Ethics Committee of the International Agency for Research on Cancer (IARC/WHO, Lyon, France).

Measurement of Cl-Guo. Levels of CI-Guo were measured according by the procedures previously described.⁶ CI-Guo was separated with a Spectrophycis HPLC (model SP 8810) equipped with a reverse-phase column (4.6 × 250 mm Ultrasound ODS column, 5 μm; Beckman, Fullerton, CA) under isocratic conditions. The mobile phase was 20 mM ammonium formate buffer (pH 5.0) containing 15% methanol at a flow rate of 1 mL/min. CI-Guo was detected with a Waters Lambda Max model 481 UV/VIS spectrophotometer at 260 nm (Waters, Milford, MA).

Statistical analysis. All measurements expressing mean ± SD were analyzed statistically by Student’s t-test. Differences were considered statistically significant at p < 0.05.

Results

Effects of polyphenols, NSAIDs, and sulfurous compounds on the formation of CI-Guo mediated with HOCl

The average concentrations of Cl-Guo formed by the reaction of Guo with HOCl in the presence and the absence of nicotine were 7.8 and 33.6 μM respectively. Thus nicotine resulted in increased by about four-fold the amounts of CI-Guo formed with HOCl. Typical HPLC chromatograms are shown in Fig. 1. As shown in Fig. 2, most of the tested polyphenolic compounds dose-dependently inhibited CI-Gu formation mediated with HOCl. In particular, green tea polyphenol catechins such as (+)-catechin, (--)-epigallocatechin (EGC), (--)-epi catechin gallate (ECG) and (--)-epigallocatechin gallate (EGCG) were found strongly to inhibit HOCl-mediated CI-Guo formation in both the absence and the presence of nicotine (Fig. 2A and B). On the other hand, the low levels (0.5 and 5 μM) of both resveratrol and silybin rose significantly, but 50 μM of both compounds strongly suppressed CI-Guo formation mediated by HOCl in the presence of nicotine. Sulfurous compounds, taurine and α-lipoic acid, were less effective than phenolic compounds for inhibition of CI-Guo formation. Unfortunately, aspirin, one of the most popular NSAIDs, did not inhibit CI-Guo formation mediated by both HOCl and HOCl/nicotine.

Effects of polyphenols, NSAIDs, and sulfurous compounds on the formation of CI-Guo mediated with MPO

The average concentration of CI-Guo formed by the reaction of Guo with MPO in the presence of H₂O₂, NaCl, and nicotine was 3.4 μM. Similarly to the results obtained for the reaction of Guo with HOCl and nicotine, polyphenols effectively inhibited the MPO-mediated chlorination of Guo in the presence of nicotine (Fig. 3). The presence of 50 μM catechins, resveratrol, and α-lipoic acid strongly inhibited the formation of CI-Guo mediated with MPO. Similarly, 0.5 μM p-aminobenzoxyhydrate, a potent MPO inhibitor, also suppressed MPO-mediated chlorination of Guo under the conditions imposed (inhibition rate, 93.7 ± 1.1%). On the other hand, the strength of the inhibitory activity of
5 μM catechins against Cl-Guo formation in MPO/nicotine system was different from that in the HOCl and the HOCl/nicotine system (Figs. 2 and 3).

**Effects of EGCG, silibinin, and α-lipoic acid on the formation of Cl-Guo induced by activated neutrophils**

The effects of several compounds on Cl-Guo formation was examined using human neutrophils stimulated by PMA. The average concentration of Cl-Guo formed by the reaction of Guo with activated human neutrophils in the presence of nicotine was 0.08 μM. As shown in Fig. 4, the presence of 5 and 50 μM test compounds (EGCG, silibinin, and α-lipoic acid) inhibited the formation of Cl-Guo by activated human neutrophils in a dose-dependent manner. Especially, α-lipoic acid markedly blocked neutrophil-induced chlorination of Guo.

**Discussion**

HOCl is a major ROS produced by activated neutrophils.1) Nucleosides such as Guo, adenosine, and cytidine have been reported to be easily chlorinated to form Cl-Guo, 8-chloroadenosine, and 5-chlorocytidine.
respectively by HOCI. Chlorination of nucleosides might occur in the nucleotide pool, the RNA, and the DNA. In addition, tertiary amines such as nicotine dramatically enhance the chlorination of nucleoside by HOCI under physiological conditions. Tertiary amines react with HOCI to form reactive quaternary chlorammonium ions that chlorinate secondary amines to form N-chloro secondary amines. Consequently, the quaternary chlorammonium ions might chlorinate guanosine at the N-7 position to form its N-7 chlorine adduct, followed by migration of chlorine to produce 8-Chl-Guo. Chlorinated nucleosides that might induce genotoxicity have been identified in several tissues and are often discussed with regard to diseases such as inflammation and atherosclerosis. Taken together, chlorinated nucleosides can be regarded as useful specific biomarkers for HOCI- (and also activated neutrophil-) induced oxidative damage in vivo. In the present study, we examined the effects of several antioxidative compounds on the formation of biomarker Cl-Guo in vitro for the first time using three reaction systems, the HOCI-induced chemical reaction, the human MPO-induced enzymatic reaction, and activated human neutrophil-induced cellular reaction system.

Green tea has very specific polyphenolic compounds, including catechins. Many of the biological and pharmacological effects of green tea consumption are thought to be due to catechins. EGCG is found abundantly in green tea, and has been recognized as the main active compound of tea catechins. Curcumin, resveratrol, and silibinin used in this study, are known as NSAID. They are found in the rhizomes of turmeric, grape (red wine), and milk thistle (Silybum marianum) seed respectively. Silymarin is a mixture of flavonolignans. Silibinin is one of the constituents of silymarin and a well-documented flavonolignan showing positive health effects. In our study, catechins and natural NSAIDs were found effectively to suppress the Cl-Guo formation induced by HOCI and HOCI/nicotine (Figs. 1 and 2). Catechins are known to have excellent antioxidative and HOCI scavenging activity. Natural NSAIDs such as curcumin, resveratrol, and silibinin (silymarin) are also known to have cancer chemopreventive and antioxidative actions. Consequently, the main reason for the suppression of HOCI- and HOCI/nicotine-induced Cl-Guo formation (the chemical reaction of Guo with HOCI) by phytochemicals is their excellent antioxidative activity. Many phytochemicals contain phenolic hydroxyl groups. The strengths of the inhibitory activities of catechins and catechin-gallate esters against chlorination were observed to fall in the order EGC > ECG > EGC > catechin (Fig. 2B). The antioxidative properties of those compounds depend upon the phenolic hydroxyl group. Accordingly, our results, and data reported in the literature, suggest that the numbers and the positions of the phenolic hydroxyl groups in the skeleton of a compound is an important factor in the chlorination inhibitory activity of phytochemicals.

Synthesized NSAIDs such as aspirin have been studied for their cancer-preventive effects besides their anti-inflammatory action. Some synthesized NSAIDs also show antioxidative activity, but the activity of aspirin is reported to be low compared to other NSAIDs such as indomethacin and sulindac. The reaction between HOCI and Guo has been found to be very rapid. This observation with regard to aspirin can be explained by its low antioxidative activity and slow reaction rate in the scavenging of ROS (Figs. 2 and 3).

As shown in Fig. 3, catechins and natural NSAIDs were also observed to inhibit the formation of Cl-Guo mediated by MPO (Fig. 3). The inhibitory effects of 5 µM catechins against Cl-Guo formation in the MPO/nicotine system were different from those in the HOCI and the HOCI/nicotine system (Figs. 2 and 3). For example, inhibition rate for 5 µM EGCG was higher than that for 5 µM EGC (Fig. 3). Polyphenolic compounds inhibit the activity of MPO by interaction of the compound with the active site of the enzyme. The explanation of the inhibitory action shown by catechins in this study is unknown, and further study is needed.

In the case of activated neutrophils, the HOCI-induced chlorination of nucleosides is modulated by both NOX and MPO, and hence the mechanisms of the inhibitory action of polyphenols against Cl-Guo formation are complicated. We also found suppression effects of EGCG, silibinin, and α-lipoic acid on the Cl-Guo formation using human neutrophils stimulated by PMA (Fig. 4). The synthesis of NOX and MPO, and ROS generation in activated neutrophils have been reported to be affected by certain polyphenols. Accordingly, the suppression of MPO- and activated neutrophil-induced chlorination of Guo shown by plant polyphenols must relate to the actions of NOX or MPO. These phenomena might have to do with the reduction of neutrophil functioning as in respiratory burst, ROS production, MPO release, and the assembly of enzymes by phenolic compounds. Phenolic compounds are also observed to modulate the signal transduction pathway related to inflammation and cancer in neutrophils.

Taurine and α-lipoic acid are typical sulfurous compounds, inhibited Cl-Guo formation mediated by HOCI and human MPO/nicotine (Figs. 2A and 3). On the other hand, HOCI/nicotine-induced Cl-Guo formation was not inhibited by sulfurous compounds (Fig. 2B). As explained in “Results,” the amount of HOCI/nicotine-induced Cl-Guo formation was observed to be 4-fold and 10-fold of the concentrations of Cl-Guo.

Fig. 4. Effects of EGCG, Silibinin, and α-Lipoic Acid on Activated Neutrophil and Nicotine-Induced Cl-Guo Formation.

The reactions were carried out with human neutrophils in the presence of nicotine. Results are presented as percentage of control (means, n = 2).
in the HOCl system and the MPO/nicotine system respectively. The antioxidative strength of sulfuric compounds was lower than that of catechins (Fig. 2A). Hence, neither sulfurous compound tested in this study effectively suppressed HOCl/nicotine-induced Cl-Guo formation as compared with catechins. HOCl reacts with thiols and sulphhydril groups of proteins faster than with H₂O₂. Taurine reacts with HOCl and protects tissues from injury induced by activated neutrophils. α-Lipoic acid has been found to suppress Cl-Guo formation by activated neutrophils (Fig. 4). α-Lipoic acid is soluble in both lipid and aqueous environments, and can easily be incorporated into cells. Further, it can improve oxidative stress-induced damage in vivo by antioxidative action.

Collectively, based on the present results and studies of the literature, α-lipoic acid acts in both extracellular and intracellular neutrophils and scavenges ROS or inhibits certain enzymes, which are related to ROS production. As shown in Fig. 4, the strength of the inhibitory activity of α-lipoic acid in activated neutrophils was higher than that of the hydrophilic compounds such as EGCG. Additional studies are needed to clarify the mechanism of the action of polyphenolic and sulfurous compounds on the function of neutrophils.

The nicotine in cigarette smoke can enhance tissue damage through HOCl-induced chlorination mediated by MPO secreted from activated human neutrophils. Cigarette smoke inhalation is often discussed with reference to DNA damage, inflammation, and several diseases. Cigarette smoking reduces antioxidant defensive ability and produces lipid peroxides in smokers. Hence susceptibility to oxidative stress is increased and oxidative damage to nucleotides can occur in smokers. In addition, a tertiary amine, trimethylamine, is known to be the origin of typical fishy odors in rotating fish as a result of bacterial degradation of choline and the reduction of trimethylamine N-oxide to trimethylamine. High levels of trimethylamine are also known to present in salted and dried fish products whose consumption is associated with increased risk of stomach cancer. In a stomach with a Helicobacter pylori infection, tissue damage induced by activated neutrophils can accelerated by dietary trimethylamine, and it might contribute to gastric cancer. When chlorinated nucleosides associated with nicotine or trimethylamine are formed in the DNA, they can show genotoxicity and mutagenicity in the tissue.

In conclusion, this study suggests that small-molecule antioxidative substances such as polyphenolic phytochemicals and sulfuric compounds have the potential to suppress several diseases, including inflammation and cancer, mediated by the chlorination of nucleosides in vivo. Ascorbic acid is known a scavenger of HOCl in vivo, and can regenerate antioxidants such as α-tocopherol and polyphenols. Although the scavenging activity of ascorbic acid against HOCl is lower than that of polyphenols, the synergistic actions of ascorbic acid and polyphenols on chlorinated nucleosides formation have been confirmed.

Most of the compounds tested in this study have been observed to show beneficial effects in mammals without cytotoxicity. Though the bioavailability and inhibitory activities against HOCl-induced chlorinated nucleoside formation of the tested compounds in vivo should be analyzed, we believe that these compounds have special properties that no other drugs show. We plan to determine the effects of polyphenolic and sulfuric compounds on the regulation of cellular activities such as the expression of gene and protein, and activities regarding MPO and NOX in activated neutrophils. Further investigation is required to determine the relationships among natural antioxidants, the chlorination of DNA, and inflammation- and cigarette smoke inhalation-related disease.

Acknowledgments

Most of this study was done at the International Agency for Research on Cancer, World Health Organization (IARC/WHO), Lyon, France, when the authors were at the Laboratory of Endogenous Cancer Risk Factors (ECR). The authors thank Mrs. P. Collard at IARC for secretarial assistance and the staff at ECR, IARC, for valuable discussion and support. Financial support was provided by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (to T.N.).

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