Polyphenols, Inflammatory Response, and Cancer Prevention: Chlorination of Isoflavones by Human Neutrophils\textsuperscript{1,2}

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ABSTRACT An important aspect of the risk of cancer is the involvement of the inflammatory response. Currently, antiinflammatory agents are used in chemopreventive strategies. For example, aspirin is recommended for the prevention of colon cancer as well as breast and other cancers. The inflammatory response involves the production of cytokines and proinflammatory oxidants such as hypochlorous acid (HOCl) and peroxynitrite (ONO\textsubscript{2}\textsuperscript{−}). These oxidants react with phenolic tyrosine residues on proteins to form chloro- and nitrotyrosine. Diets rich in polyphenols (green tea catechins, soy isoflavones) have also been shown to be chemopreventive. The aromatic nature of polyphenols makes them potential targets of HOCl and ONO\textsubscript{2}\textsuperscript{−}. These reactions may create novel pharmacophores at the site of inflammation. Previous studies in the neutrophil-like cell line, differentiated HL-60 cells, demonstrated the formation of chlorinated and nitrated isoflavones. In this study we have examined whether similar reactions occur in freshly isolated human neutrophils. After induction of a respiratory burst with a phorbol ester, isoflavones and their metabolites were identified by liquid chromatography-tandem mass spectrometry and then quantitatively measured by LC-mass spectrometry using multiple-reaction ion monitoring. The data obtained indicate that both chlorinated and nitrated genistein are formed by human neutrophils. The extent of chlorination of genistein was markedly increased by the phorbol ester whereas the low level of nitration of genistein was constitutive and unaffected. These data imply a potential role for modified forms of genistein that would be produced in the inflammatory environment in and around a tumor. J. Nutr. 133: 3773S–3777S, 2003.

KEY WORDS: polyphenols genistein neutrophils chlorination nitration

The importance of inflammation as a component of the development of cancer has become increasingly clear. Recruitment of inflammatory cells into the tissue sites of tumors leads to pro- and antitumor growth effects. The activation status as well as environmental factors influence the inflammatory cell’s diverse functions, such as angiogenesis, neoplastic cell mitogenesis, antigen presentation, matrix degradation, and cytotoxicity (1). Indeed, the medical profession has become so convinced of the role of antiinflammatory agents that a daily dose (one tablet) of aspirin is strongly recommended as a way to prevent colon cancer as well as heart disease.

The immune response is a complex system of soluble agents and cells whose principal purpose is to protect the human body from infectious agents, chemical and physical damage, and tumors (2). A feature of aging is that the immune response declines, thereby increasing the risk of infection as well as potentially allowing tumor cells to escape immune surveillance. Inflammatory cells have specialized biochemical pathways that can locally generate reactive compounds that are used to attack foreign cells. Typically, inflammatory cells convert oxidants such as singlet oxygen, superoxide anion, and hydrogen peroxide into more highly reactive compounds, such as
hydroxyl radicals (OH), hypochlorous acid (HOCl), hypobromous acid (HOBr), and peroxynitrite (ONO$_2^-$) (2). These compounds react with DNA, lipids, and proteins, resulting in the disruption of the target’s structure as well as its function. Although these compounds are reactive, their copious amounts (locally in the millimolar range) result in their diffusion away from the inflammatory focus into the local normal environment. This causes collateral damage to otherwise uninvolved cells and may contribute to lesion formation, thereby creating a separate disease process. Investigators have proposed that this is a significant event in the evolution of atherosclerosis.

Antinflammatory agents fall into two broad categories—those that inhibit the biosynthesis of prostanooids [non-steroidal antiinflammatory drugs (NSAID) such as aspirin] and those that alter the production and the action of proinflammatory oxidants (antioxidants). The activity of aspirin, although now made by chemical synthesis, was originally discovered 2000 years ago from a natural plant source and is a cyclooxygenase (COX) inhibitor. Other pharmacologically derived COX inhibitors to prevent colon cancer have been developed because of the relatively low efficacy of NSAID (~50% reduction in incidence and mortality) and their wide spectrum of side effects, some of which are fatal (3). Examples of these new COX inhibitors are the NO-NSAID, which are traditional NSAID linked to an NO-releasing group via a chemical spacer. The addition of the NO-releasing group is believed to reduce gastric toxicity seen with traditional NSAID (3).

Many epidemiologic studies and preclinical laboratory experiments have suggested roles of dietary antioxidants in the prevention of cancer. Fruits and vegetables contain large amounts of these antioxidants, and higher intakes of fruits and vegetables are the subject of federal dietary recommendations. However, the reluctance of the public to make a change in diet has led to an industry-based focus on dietary supplements to deliver these compounds, where either foods and edible plants enriched in these compounds are added to the regular diet or the compounds are extracted (or synthesized as occurred for aspirin) to create a pill form. This latter step may have unforeseen consequences in that the original food may have had more than one bioactive compound or a compound-food matrix effect may exist.

Conventional thinking about antioxidants has been that they react with either free radicals or the chemically reactive species generated by inflammatory cells. This has caused concern for some investigators because it appears to imply that the normal function of the oxidant reactive species in terminating invading cells or viruses would be compromised. However, the antioxidants may have a role in preventing the collateral damage associated with the chemically reactive species. This may occur at the perimeter of the inflammatory cells, not in the termination zone in the phagosome. Of course, at very high doses of the antioxidants, a progressive decrease in tissue oxidants may occur and unwanted effects on immune function could result. This emphasizes the importance of using foods and not pills in the context of preventing chronic disease risk. Therapeutic drugs may have greater bioactivity but they are rarely used chronically because of their substantial side effects.

An alternative role for antioxidants may occur as a result of their reaction with the proinflammatory oxidants. The products should not be considered as being biologically neutral and may have properties of locally produced, and hence active, novel pharmacophores. In our research program at the University of Alabama at Birmingham, we are interested in the properties and mechanisms of action of several types of polyphenols that are present in foods and plants whose consumption is associated with lowered risk of breast and prostate cancer as well as several other chronic diseases. In this article we describe our investigations of the reactions between certain polyphenols and reactive chemical species (HOCl and ONO$_2^-$). We (4) previously showed that human leukemia HL-60 cells differentiated with dimethylsulfoxide (DMSO) (a renewable source and model of human neutrophils) and induced to have a respiratory burst rapidly convert isoflavones to chlorinated and nitrated products. In this study we investigate whether freshly isolated human neutrophils carry out similar reactions.

### MATERIALS AND METHODS

#### Materials

Isoflavones were obtained both from biological sources and chemical suppliers, as described elsewhere (5). Chlorinated isoflavones standards were chemically synthesized using selected chlorinated starting materials (6). The 3'-chlorinated genistein derivative was synthesized from commercially available 3-chloro-4-hydroxyphenylacetic acid. They were fully characterized by H and 13C nuclear magnetic resonance spectroscopy and their purity was established by reverse-phase HPLC, using acetonitrile-water as solvent.

DMSO and sodium nitrite (NaNO$_2$) were purchased from Fisher (Fair Lawn, NJ). RPMI 1640, fetal bovine serum (FBS), and ACK Lysis Buffer were purchased from Mediatech, Inc. (Herdon, VA). Histopaque-1077, Histopaque-1119, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Krebs-Henseleit buffer (K-H; 118.0 mmol NaCl/L, 27.2 mmol NaHCO$_3$/L, 4.8 mmol KCl/L, 1.75 mmol CaCl$_2$/L, 1.0 mmol KH$_2$PO$_4$/L, 1.2 mmol MgSO$_4$/L, 11.1 mmol glucose/L, pH 7.4) was used for incubations with HL-60 cells and human polymorphonuclear cells (PMN).

#### Analysis of chlorinated genistein

HL-60 cell differentiation was induced with 1.3% (v/v) DMSO-supplemented 10% FBS/RPMI-1640 media for 7 d with media refreshment on d 4. Cell medium was exchanged with K-H buffer, and cells were suspended at 1 × 10$^6$ cells/mL. Cells were activated with PMA (10 μmol/L) in the presence of genistein (10 μmol/L) and NaNO$_2$ (50 μmol/L) for 60 min at 37°C. Each sample was treated with catalase (5 U/mL) to stop the respiratory burst by scavenging any remaining H$_2$O$_2$ and placed on ice for 10 min. The cells were centrifuged for 5 min at 800 × g at 4°C and the supernatant was removed and extracted as follows: the cell supernatant (~950 μL) was added to diethyl ether (2 mL). The samples were vortex mixed and centrifuged at 2000 × g, whereupon the ethereal top layer was removed. The same steps were repeated until a total volume of 6 mL of ether was added. Ether layers were combined and dried to evaporation under air. Before analysis by liquid chromatography-multiple reaction ion monitoring-mass spectrometry (LC-MRM-MS), 80% methanol (100 μL) was added to redissolve the dried residues.

Human PMN were isolated from freshly drawn human blood from volunteers using Histopaque according to manufacturer’s instructions. This study was approved by the University of Alabama at Birmingham Institutional Review Board for Human Use. Contaminating red blood cells were lysed with ACK lysis buffer for 5 min at room temperature. The cells were centrifuged at 800 × g for 5 min at room temperature. The buffer was aspirated leaving the PMN pellet. The PMN were suspended in K-H at 1 × 10$^6$ cells/mL. Genistein (10 μmol/L) was

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4 Abbreviations used: COX, cyclooxygenase; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HOCl, hypochlorous acid; K-H, Krebs-Henseleit; LC-MRM-MS, liquid chromatography-multiple reaction ion monitoring-mass spectrometry; NSAID, nonsteroidal anti-inflammatory drugs; ONO$_2^-$, peroxynitrite; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear cells; TAM, tumor-associated macrophages.
added to 1 mL of cell suspension. Cells were activated with PMA (10 μmol/L) and NaNO2 (50 μmol/L) and incubated for 60 min at 37°C. Each sample was treated with catalase (5 U/mL) and placed on ice for 10 min. The cells were centrifuged for 5 min at 800 × g at 4°C and the supernatant was removed and extracted in the same manner as described above.

**HPLC analysis of differentiated HL-60 reaction products**

Analyses of differentiated HL-60 reaction products were performed on a HP1100 series HPLC module with diode array detector. Reaction products were separated by reversed-phase HPLC as previously described (7).

**Mass spectrometry analysis of reaction products**

Reaction mixtures were separated by HPLC using a 10 cm × 4.6 mm i.d., C-8 Aquapore reverse-phase column preequilibrated with 10 mmol/L ammonium acetate (NH₄OAc). The mobile phase composition was 6 min isocratically with 40% acetonitrile in 10 mmol/L NH₄OAc at 1 mL/min flow rate. The column eluant was passed into the Ionspray™ ionization interface operating in the negative ion mode and passed into a PE-Sciex (Concord, Ontario, Canada) API III triple quadrupole mass spectrometer. The voltage on the Ionspray™ interface needle was 4900 V and the orifice potential was set at −70 V. Negative ion mass spectra were recorded over an m/z range of 20–400. Selected [M-H]⁻ molecular ions were analyzed by collision-induced dissociation mass spectrometry with 100% argon as the collision gas, and the product ion mass spectra were recorded. To obtain quantitative data, specific parent ion–product ion combinations were used in LC-MRM-MS analysis (4). Daidzein was added as the internal standard for experiments with genistein. Data were analyzed using software provided by the manufacturer on Macintosh Quadra 950 and PowerPC 950 computers (Apple Computers, Cupertino, CA). A series of samples prepared with several known concentrations of the varying genistein and a single concentration of the internal standard were analyzed to generate an area response-concentration curve. These typically gave correlation coefficients of 0.98 for a five-point curve.

**RESULTS**

Previously, when genistein was reacted with either HOCl or ONO₂⁻, the products were analyzed by ¹H nuclear magnetic resonance spectroscopy to determine the positions of chlorination and nitration (7). Chlorination took place in positions 6, 8, and 3', whereas the nitrated product was found only in the 3' position (Fig. 1). HPLC-diode array analysis detected chlorinated and nitrated forms of genistein in differentiated HL-60 cell medium after the addition of genistein to culture medium in the presence or absence of PMA and NaNO2 (Fig. 2). In the presence of genistein without PMA activation, chlorinated and nitrated genistein were not detected (Fig. 2A). However, in the presence of genistein with PMA activation, monochlorogenistein was detected (Fig. 2B) with peak enhancement upon the addition of NaNO2 (Fig. 2C). Nitrated genistein was not detectable by HPLC analysis. At this time, it is not known whether genistein was nitrated under these conditions because of the decreased sensitivity of HPLC analysis compared with LC-MRM-MS analysis.

To validate and verify the use of differentiated HL-60 cells as an experimental model for human neutrophils, chlorinated and nitrated genistein were measured after exposure of genistein to human neutrophils before and after activation with PMA and NaNO2 (Fig. 3). LC-MRM-MS analysis revealed that in the absence of PMA-induced activation, large amounts of unreacted genistein were detected (Fig. 3A) with small amounts of monochlorogenistein (Fig. 3B). The low level of chlorinated genistein is probably due to a subpopulation of activated PMN isolated from the blood. Upon activation with

![FIGURE 1](https://via.placeholder.com/150)
**FIGURE 1** Structure of genistein and the sites of chlorination and nitration (modified from reference 6).

![FIGURE 2](https://via.placeholder.com/150)
**FIGURE 2** Detection of chlorinated derivatives of genistein using differentiated HL-60 cells. HPLC chromatograms of differentiated HL-60 cell media reaction products in the presence and absence of genistein (10 μmol/L), PMA (10 μmol/L), and NaNO2 (50 μmol/L). (A) Unreacted genistein was detected (retention peak at 9.174 min). (B, C) Unreacted genistein (retention peak at 9.176 min) and monochlorogenistein (retention peak at 10.398 min) were detected after PMA-induced stimulation in the absence of NaNO2, respectively. Nitrated genistein was not detectable.
PMA, higher levels of mono- and dichlorogenistein were detected with approximately the same levels with the addition of NaNO2 (Fig. 3 B, C, respectively). Nitrated genistein was only detected in samples containing NaNO2 in the presence or absence of PMA-induced stimulation (Fig. 3D).

**Determination of specific isomers of chlorinated isoflavones**

We previously demonstrated that chlorination of isoflavones results in isomers with substitution at the 3', 6, and 8 positions (Fig. 1). Figure 4 shows the fragmentation patterns for both authentic compounds and those synthesized de novo. Figure 4 shows the MS-MS fragmentation patterns of cell extracts of genistein that had been incubated with PMA-activated PMN for 60 min. Panel A shows the MS/MS spectrum of monochlorogenistein extracted from activated PMN cell medium. The presence of the m/z 133 ion suggests that the chlorine resides in the A ring of genistein. Panel B shows the MS/MS spectrum of dichlorogenistein extracted from the same medium as the monochlorogenistein. The presence of chlorine in the molecule is also corroborated by the m/z 35 ion. A previous study in our laboratory gave a detailed description of the structural information obtained from the MS/MS spectrum of monochloro- and dichlorogenistein (8).

**DISCUSSION**

In this study we show that reactive oxygen and reactive nitrogen species generated by a respiratory burst in human neutrophils chemically modify isoflavones. Through the use of LC-MS methods, the isoflavones were demonstrated to be...
chlorinated and in the presence of NO$_2^-$ also nitrated. Conversion of the native isoflavones to modified products was rapid and complete within 60 min of incubation with human neutrophils. By 30 min, >95% of the added genistein was recoverable as chlorinated products in the cell medium. These data are comparable with those we previously obtained using DMSO-differentiated HL-60 cells (4). The present study confirms our previous hypothesis that isoflavones and other polyphenols will undergo rapid reaction with chemical oxidants produced by inflammatory cells. As we had presumed, human neutrophils and DMSO-differentiated HL-60 cells when stimulated with phorbol esters to have a respiratory burst both generate large amounts of HOCl that react with genistein to form mono- and dichlorinated products. This reaction is rapid and within 20–30 min the medium surrounding the cells contains <5% of the genistein. Interestingly, we observed that even the unstimulated neutrophils were capable of genistein chlorination, albeit at a lower rate. Unlike differentiated HL-60 cells, neutrophils freshly isolated from human blood may have already undergone some stimulation. This may vary from subject to subject.

Genistein also underwent some 3′-nitration of genistein. This was dependent on the addition of nitrite to the incubation medium. However, there was no enhancement of nitration as a result of stimulation with phorbol ester.

These data have important implications for cancer prevention because it has been suggested that tumors are wounds that will not heal (1). The presence of macrophages has been seen in carcinomas of the breast, cervix, and bladder and there have been conflicting reports for prostate, lung, and brain tumors (9). These macrophages are referred to as tumor-associated macrophages (TAM). TAM are recruited to tumor sites by the tumor cells through the release of chemokines monocyte chemotactic protein-1, macrophage colony stimulating factor, and vascular endothelial growth factor (9). It is the recruitment of TAM and their subsequent further production of chemokines and reactive oxygen and nitrogen species that create a heterogeneous microenvironment around the tumor. One study suggested that colorectal adenocarcinoma cell lines were capable of suppressing production of reactive oxygen intermediates by human phenotypes (10). However, the measurements were detecting superoxide production, which can be a measurement of superoxide or one of its metabolites, and did not clarify which reactive oxygen intermediates were being affected. Interestingly, genistein has been noted to decrease the production and release of vascular endothelial growth factor, which is a potent stimulator of angiogenesis (11). Further studies need to be performed looking into genistein’s effect on tumor microenvironment. We have shown that genistein reacts with reactive species generated by human neutrophils to form chlorinated and nitrated products; therefore, genistein could be directly affecting the tumor microenvironment by altering its oxidant status. One possibility could be that there is an indirect effect of the native, chlorinated, or nitrated species on chemokine production and release and TAM recruitment. This would affect the tumor’s ability to grow and spread because the antitumorigenic properties of TAM would no longer be suppressed.

LITERATURE CITED