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The antioxidant activity of genistein in vitro

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Genistein, a flavonoid isolated from soy beans, has been studied with respect to its antioxidative characteristics. The material proved to be effective against UVA and UVB or peroxyl radical–induced lipid peroxidation in liposomes. Genistein was however ineffective in preventing conjugated diene formation in linoleic acid micelles. Other peroxidative systems involving hydrogen peroxide, such as metmyoglobin peroxidase activity and Fe/ ascorbate/hydrogen peroxide oxidation of liposomes, were inhibited by genistein. As measured by catechol decolorization, genistein did not appear to chelate iron. Genistein removed hydrogen peroxide efficiently when phenol red was coupled with peroxidase; however, when o-dianisidine was used as the color reagent there was no apparent loss of hydrogen peroxide, possibly due to oxidation of the dye by the product of genistein and hydrogen peroxide. This study provides further evidence that genistein is an effective scavenger of hydrogen peroxide but is less effective against other peroxidative systems. (J. Nutr. Biochem. 6:481–485, 1995.)

Keywords: genistein; isoflavonoid; antioxidant; soybean

Introduction

Epidemiological evidence from several studies has demonstrated that in Asia soybean consumption is often associated with a decreased risk of esophageal, breast, and colorectal cancer.[1,2] Experimentally there is much more conclusive evidence that soy products can afford some degree of protection. Barnes et al.[3] showed that soybeans could inhibit mammary tumors in rodents. Webb et al.[4] noted that dietary toasted soybean meal reduced ornithine decarboxylase activity and increased the activities of glutathione transferase, catalase, and protein kinase C, all favorable changes with respect to cancer chemoprevention.

Genistein, the major isoflavone in soy beans, has been shown to have a wide range of effects, all of which could contribute to its ability to protect against cancer. It inhibits the growth of *ras* oncogene-infected cells[5] and cytochrome P-450 activation of benzo[a]pyrene.[6] Genistein inhibits tyrosine kinase and topoisomerase II and arrests cell cycle progression at the G2-M boundary[7] and angiogenesis in vitro.[8] Recently it has been reported that genistein inhibited the tumor promoter-induced formation of hydrogen peroxide in vitro and in vivo in mouse skin.[9] Prior to this, other workers had also reported on the antioxidant potential of genistein. Pratt and Birac[10] found that in a coupled linoleic acid/β-carotene system genistein had antioxidative properties. Jha et al.[11] found antioxidative activity in a microsomal system using Fe^{2+}/ADP/NADPH. Kusonoki et al.[12] also found that superoxide production by neutrophils was inhibited by genistein.

There is an increasing body of evidence to implicate reactive oxygen species (ROS) in tumor initiation and promotion.[13] The aim of this study was to examine some of the antioxidant properties of genistein in order to understand its role in the prevention of ROS-induced damage in human disease.

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Received October 6, 1994; revised March 15, 1995; accepted April 12, 1995.

Methods and materials

Reagents

Turkey-egg phosphatidylcholine (PC), metmyoglobin, genistein, sodium dodecyl sulphate, horseradish peroxidase, and o-dianisidine were obtained from Sigma Ltd. (St. Louis, MO USA). Thiobarbituric acid was purchased from Merck Ltd. (Germany), and 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) was supplied by Polysciences Ltd. (USA). Trolox, linoleic acid and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were from Aldrich Ltd. (USA). All other chemicals were from BDH Ltd. (UK) or the best quality available.

Nutritional Biochemistry 6:481–485, 1995
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655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(95)00076-C

Liposomal peroxidation

Liposomes were prepared in phosphate-buffered saline (pH 7.4) to form multilamellar liposomes at a final concentration of 20 mg/mL and exposed on ice with constant agitation to a "Wotan ultra-vitalux" sunlamp with a lower wavelength cut-off of 290 nm. The UVA emission was 0.36 $J/cm^2/min$ and the UVB was 0.12 $J/cm^2/min$. Genistein was added to the aqueous phase at the desired concentrations. Liposomal suspensions were incubated on ice with constant agitation during the course of irradiation. Peroxyl radicals were generated by the thermal decomposition of AAPH at 37°C. Liposomal samples (0.1 mL) were removed at various times and boiled for 20 min with 1 mL of 0.25% thiobarbituric acid in 15% trichloroacetic acid.[14,15] After cooling and centrifugation the absorbances of the thiobarbituric acid reaction products (TBARS) were read at 532 nm.

Conjugated dienes

Formation of conjugated dienes from linoleic acid was carried out as described by Pryor et al.[16] Linoleic acid micelles (2.5 mM) in sodium dodecyl sulphate (0.1 M) were exposed to peroxyl radicals generated by AAPH (4 mM), and the production of conjugated dienes was monitored spectrophotometrically at 234 nm. After 8 min the antioxidants (Trolox 10^{-7} M) or genistein (5×10^{-5} to 5×10^{-4} M) were added. Higher concentrations of genistein could not be used due to the high absorbance of the compound at 234 nm.

ABTS and metmyoglobin

Metmyoglobin peroxidase activity was monitored as described[17] by its reaction with ABTS (150 μM) and hydrogen peroxide (75 μM), except that the temperature of the reaction was maintained at 26.5°C.

Iron chelation

The comparative ability of genistein to chelate iron was determined by the decolorization of a $FeCl_3$/catechol solution (0.25 mM each).[18] Initial attempts to estimate the binding of iron by catechol were hampered by the dependence of the color on the pH of the solution. Neutralization of an unbuffered solution as suggested in the original method proved difficult and unreliable, thus the analysis was carried out in 0.05 M barbiturate buffer (to avoid phosphate precipitation) at pH 7.4.

Hydrogen peroxide measurement

Peroxides were measured using the xylenol orange method,[19] in which the peroxide sample is added to a solution containing 100 μM xylenol orange, 250 μM Fe^{2+}, 25 mM sulphuric acid, and 4 mM butylated hydroxytoluene in 90% methanol or hydrogen peroxide using horseradish peroxidase (40 μg/mL) and phenol red (100 μg/mL)[19] or o-dianisidine (0.05 mg/mL).[20]

Results

Liposomes, AAPH, and UV

Significant protection was observed on incubation of liposomes containing genistein in the presence of the peroxyl radical generating agent, AAPH, although the level of inhibition reached a maximum of about 50% at a concentration of 50 nmol/mg of PC (*Figure 1*). Liposomes exposed to UV light or hydroxyl radicals generated by Fe/ascorbic acid/H_2O_2 were afforded significant protection by genistein with 50% protection at about 25 nmol genistein/mg of PC (*Figure 1*).

Conjugated dienes

Linoleic acid micelles were exposed to AAPH, and the production of conjugated dienes was monitored. Under these conditions genistein had no observable antioxidant activity up to a level of 5×10^{-4} M. Alpha tocopherol on the other hand inhibited the production of diene conjugates for approximately 20 min at 5×10^{-7} M (*Figure 2*). Higher genistein levels could not be used due to the absorbance of the compound at the wavelength used for detection of conjugated dienes (234 nM) which increased the background absorbance.

ABTS and metmyoglobin

Genistein was able to reduce metmyoglobin-induced oxidation of ABTS (*Figure 3*), but kinetic analysis showed that added genistein had no initial effect on the rate of formation of the reaction product, until about 100 sec when the reaction was slowed in a dose-dependent manner. Trolox however inhibited production of $ABTS^+$ until the antioxidant was consumed.

Iron chelation

There was no evidence of chelation of iron by the catechol binding assay up to a concentration of 2×10^{-4} M, compared with desferrioximine which showed a maximum chelation between 6×10^{-5} M and 1×10^{-4} M (*Figure 4*). The effects of higher concentrations of genistein could not be assessed because precipitation occurred in the reaction vessel. On reduction of the concentrations of catechol and iron to 100 μM, it was possible to increase the genistein concentration to 200 μM. At this level there was no evidence that the isoflavonoid had any substantial chelating

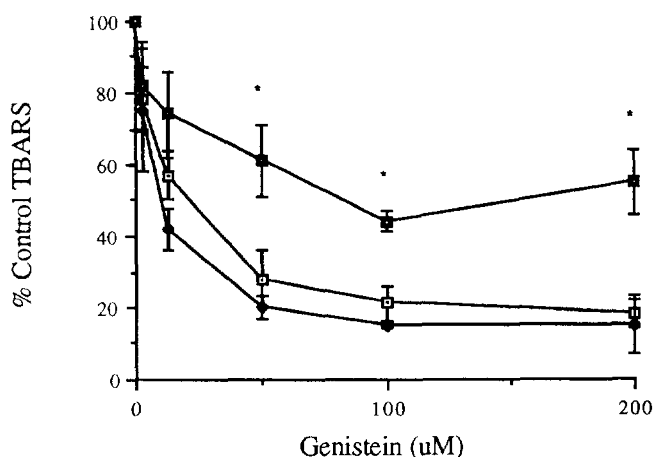

Figure 1 Inhibition of AAPH (■), UV (□), and Fe^{2+}/Ascorbate/H_2O_2 (◆)-induced liposomal lipid peroxidation by genistein. Liposomes (n = 3) were peroxidized for 1 hr prior to determination of TBARS as described in Methods and materials. *Indicated significantly less inhibition than in the other two assays. Values are means ± SD.

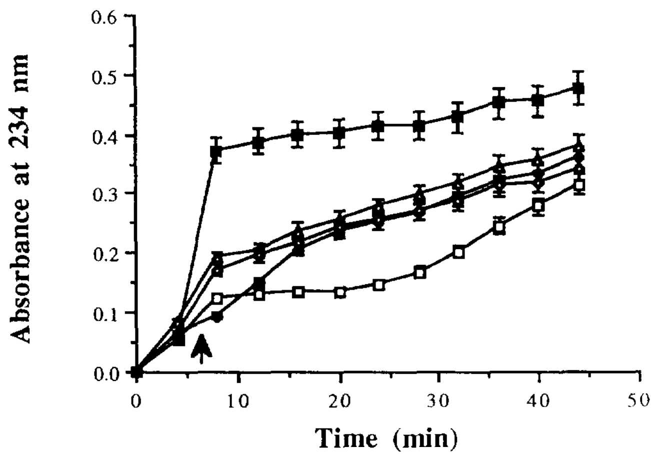

Figure 2 Inhibition of AAPH-induced conjugated diene production from linoleic acid by 5×10^{-7} M α-tocopherol (□), genistein at 5×10^{-5} M (▲); 10^{-5} M (◇); 5×10^{-4} M (■), or no addition (♦) ↑ indicates addition of antioxidant. Values are means ± SD of three readings.

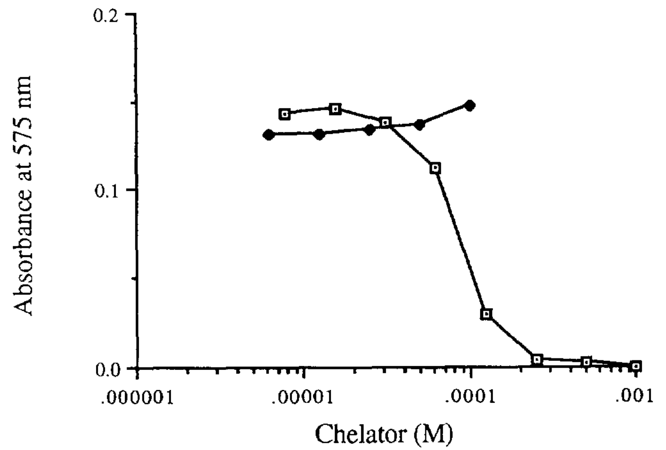

Figure 4 Decolorization of a catechol-Fe^{2+} complex by different concentrations of desferrioximine (□) and genistein (♦). Standard deviations were less than 2% of the plotted values (n = 3).

ability. Lower levels of catechol and iron could not be used due to the lack of sensitivity of the assay.

Hydrogen peroxide

Genistein and hydrogen peroxide were mixed and allowed to stand at room temperature for 30 min prior to determination of hydrogen peroxide using horseradish peroxidase coupled with o-dianisidine. Under these conditions there appeared to be no loss of hydrogen peroxide when genistein was present at twice the concentration of hydrogen peroxide (10^{-4} versus 5×10^{-5} M). No loss of hydrogen peroxide was observed when iron and xylenol orange were used to measure peroxides. When horseradish peroxidase and phenol red were used as the detection system for hydrogen peroxide, genistein did appear to remove hydrogen peroxide (*Figure 5*). Genistein on its own showed no interference with any of these assays.

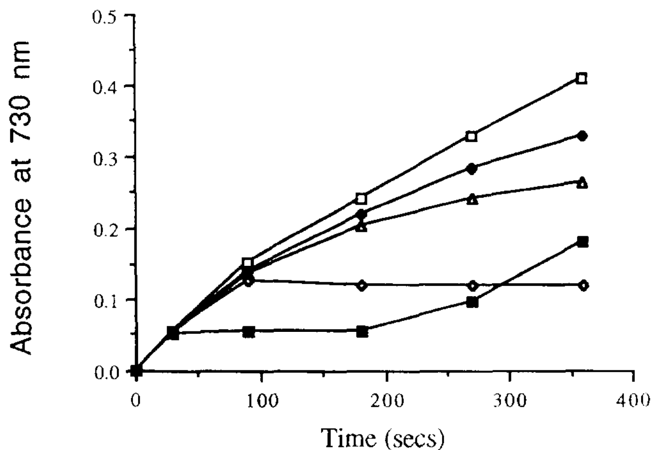

Figure 3 Inhibition of metmyoglobin peroxidase activity by Trolox (8.4 μM, ■) and genistein (0.84 μM ♦; 4.2 μM, △; 42 μM ◇, no addition, □). Standard deviations were less than 2.5% of the plotted values (n = 3).

Discussion

Genistein, the major isoflavone in soybeans, has recently attracted a great deal of attention as an anticancer agent. A number of workers have implicated the antioxidant activity of genistein as one of the major anticarcinogenic influences.[9–12] In this study we have attempted to characterize some of the antioxidant properties of genistein in order to obtain further insight into the possible protective nature of the compound.

Genistein was only capable of reducing AAPH-induced TBARS formation by a maximum of 50% and had little effect on conjugated diene formation from linoleic acid. However, in each of the systems examined where either hydrogen peroxide or iron ions were involved as the oxidative stimulus, genistein behaved as an effective antioxidant. This is true even in the case of UV exposure where superoxide radicals are generated from dissolved oxygen and then spontaneously dismute to form hydrogen peroxide.[21] These observations led us to suppose that genistein was either chelating iron or scavenging hydrogen peroxide. By using the catechol binding method,[18] we could not demonstrate that genistein, within the limits of its solubility, was

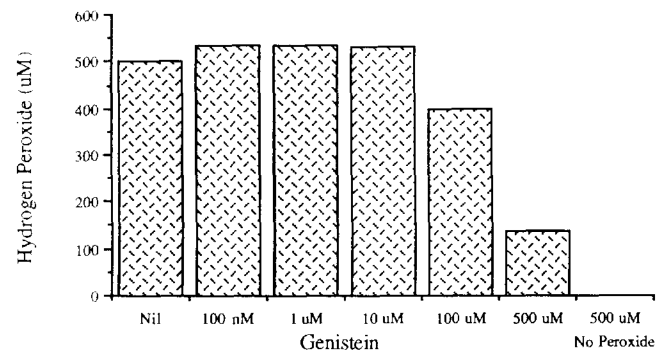

Figure 5 Removal of hydrogen peroxide by genistein. Peroxide was determined by measuring the oxidation of phenol red by horseradish peroxidase. Standard deviations were less than 2% of the plotted values (n = 3).

capable of chelating iron. This is perhaps not surprising considering that genistein is phenolic in structure and not a catechol or resorcinol, although there is evidence that the keto group at position 4 and the hydroxy group at position 5 could afford some degree of chelation.[22] This leads to the conclusion that in vivo genistein would be unlikely to be sufficiently potent a chelator to compete with endogenous iron binding ligands.

Wei et al.[9] have reported recently that genistein is an effective scavenger of hydrogen peroxide. Using their method of assessment (horseradish peroxidase coupled with phenol red) we also found genistein to apparently remove hydrogen peroxide. In two other systems, however, one using horseradish peroxidase coupled with o-dianisidine and the other using $Fe^{2+/3+}$ complexation with xylenol orange, we were unable to detect any change in the apparent hydrogen peroxide concentrations.

It would seem from these results that, in the peroxidase-based assays for hydrogen peroxide, addition of o-dianisidine along with the enzyme actually reduces the oxidized form of genistein, thus becoming oxidized itself, and giving a false positive indication of the presence of hydrogen peroxide. Phenol red does not appear to be a sufficiently powerful antioxidant to be able to produce this effect.

In the xylenol orange-based assay, which is not specific for hydrogen peroxide, it is possible that, as appears to be the case with o-dianisidine, genistein once oxidized by hydrogen peroxide could be reduced by Fe^{2+}, yielding Fe^{3+} which would complex with xylenol orange and again give a false positive reaction for hydrogen peroxide. It is also possible that genistein could be peroxidized by hydrogen peroxide, and this peroxide then reacts with the Fe^{2+}/xylenol orange reagent. The fact that color is developed in the presence of genistein again supports the contention that the material does not exert its antioxidant capabilities through chelation.

The ability of genistein to scavenge hydrogen peroxide explains its antioxidant role in the inhibition of metmyoglobin-induced peroxidation of ABTS. The observations that it protects well against UV-induced peroxidation, less so against AAPH (peroxyl radical) TBARS production, and not at all against AAPH-mediated conjugated diene production suggests that it is not a chain-breaking antioxidant, such as α-tocopherol, but acts as a scavenger, possibly of lipid and other peroxide residues.

Although there is little in the scientific literature regarding levels of the isoflavanoids in genistein and daidzein in humans Xu et al.[23] have reported levels of up to 2.15 μM in women fed 2 mg/kg of isoflaones in a test meal. Adlercreutz et al.[24] have reported that in Finnish men the levels of genistein averaged 6.3 nM, but in Japanese men the levels averaged 276 nM with a maximum of approximately 650 nM. In these current in vitro studies antioxidant activity was observed in the micromolar range. It would seem therefore that genistein could be present in humans at sufficient levels to have beneficial effects with respect to removal of lipid hydroperoxides.

Acknowledgments

The authors would like to thank Ms. Joanne Lane for excellent technical assistance.

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