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Dietary soy isoflavones inhibit activation of rat platelets

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Isoflavones (isoflavonoids) have been proposed to be the active compounds that contribute to decreased mortality from chronic diseases in populations that consume large amounts of soy products. Diets containing soy protein with and without isoflavones were fed to rats to determine if these compounds could exert in vivo effects on physiologic markers of platelet activation. Three methods were employed to monitor platelet activation: measurement of electronic mean platelet volume, which is an indicator of shape change; monitoring of collagen-induced production of reactive oxygen signals (hydrogen peroxide); and determination of increases in phosphorylation of protein tyrosine residues after collagen stimulation. Apparent volumes were significantly smaller for platelets from rats fed isoflavones, suggesting that these platelets were in a more disc-like, quiescent state compared with platelets from rats fed the isoflavone-reduced diet (means ± SEM, 5.37 ± 0.08 vs. 5.70 ± 0.06 fL, $n = 6$*/group,* $P < 0.008$*). Results from the other functional tests were consistent with this finding. Platelet production of hydrogen peroxide was found be significantly lower 1, 3, and 5 minutes after addition of collagen for rats fed isoflavones versus rats fed the isoflavone-reduced diet (*$n = 6$*/group,* $P < 0.004$*). Phosphorylated tyrosine residues in platelet proteins after stimulation also were shown to be significantly lower in the platelets exposed to dietary isoflavones (*$n = 5$*/group,* $P < 0.047$*). These combined results indicate that soy isoflavones can alter early-event signaling networks that result in less activated platelets and may partially explain the beneficial effects of dietary soy against human heart disease.* (J. Nutr. Biochem. 10:421–426, 1999) *Published by Elsevier Science Inc.*

Keywords: platelets; isoflavones; soy; protein tyrosine kinases; phosphatases; genistein

Introduction

Epidemiologic studies have shown that populations consuming diets high in soy have a lower risk for developing heart disease and cancer compared with populations eating only small amounts of soy.[1–4] The isoflavones or isoflavonoids found in soy (genistein and daidzein, predominantly) are thought to be the biologically active components that confer this beneficial effect against chronic diseases. Mechanisms to explain how these compounds slow disease processes have not been clearly established.

Genistein, which is the major isoflavone in soy, has been used extensively as an in vitro tool for studying signal transduction pathways in cells since its original characterization as an inhibitor of protein tyrosine kinases (PTK).[5] In the cell, an intricate balance is maintained between PTK and protein tyrosine phosphatases (PTP) such that in the resting state only a small percentage of the tyrosine residues on proteins is phosphorylated.[6,7] Activation of the cell via ligand-receptor interaction initiates a rapid increase in PTK activity, forming phosphorylated proteins that act as transducers of the signal that originated at the plasma membrane. Dietary isoflavones may produce their biological effects by inhibiting cellular signaling mechanisms that rely on sustained activity of still unidentified PTK.

There is a need for data showing that dietary isoflavones can indeed exert relevant physiologic effects on cellular function when fed in realistic amounts to humans or animals. In the study described here, we used platelet responsiveness as the paradigm for studying interactions of isoflavones with cellular signaling networks. Diets with (+IF) and without (−IF) isoflavones were fed to rats and the activation status of platelets was determined by three different methods. These methods were selected with the

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Received September 23, 1998; accepted March 26, 1999.

0955-2863/99/$–see front matter
PII S0955-2863(99)00023-6

purpose of providing multiple measures of early indicators of platelet activity.[8]

Materials and methods

Materials

The fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate and calibration beads (LinearFlow™ Green) for flow cytometric operational checks were purchased from Molecular Probes (Eugene, OR USA). Collagen reagent was purchased from Chrono-log Corp. (Havertown, PA USA). Quality control standards for whole blood counting procedures were obtained from Biochem Immunosystems, Inc. (Allentown, PA USA). The enhanced chemiluminescence (ECL) reagents, nitrocellulose membranes, and streptavidin-horseradish peroxidase (HRP) conjugate were obtained from Amersham Corp. (Arlington Heights, IL USA). Monoclonal antibody HRP-conjugated anti-phosphotyrosine (4G10) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY USA). Biotinylated molecular weight markers were from New England BioLabs Inc. (Beverly, MA USA) and the protein assay reagents were from Bio-Rad Laboratories (Hercules, CA USA). Tris/glycine/SDS buffer came from Amresco Inc. (Solon, OH USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO USA). Young adult male Wistar rats (200–250 g) were purchased from Hilltop Laboratories, Inc. (Scottdale, PA USA) and fed standard laboratory rodent rations (Diet 5001, PMI Feed, Inc., St. Louis, MO USA) or synthetic diets described below. Sources of ingredients for the purified diets are listed below. The animals were housed and cared for as prescribed in the *Guide for the Care and Use of Laboratory Animals* (1996 revision, National Research Council) in facilities accreditated by the American Association for the Accreditation of Laboratory Animal Care. The protocol for the use of the animals was reviewed and approved by the Beltsville Area Animal Care and Use Committee.

Animal diets and care

Experimental diets with and without isoflavones were fed to the rats for 12 to 14 days. The nutritionally adequate diets[9] contained the following materials in g/kg: soy protein [with isoflavones (+IF) and isoflavone reduced (−IF)], 200; tocopherol-stripped corn oil, 50; cornstarch, 550; sucrose, 99.5; mineral mix (AIN-93G-MX), 35; vitamin mix (AIN-93-VM), 10; cellulose, 50; DL-methionine, 3; and choline bitartrate, 2.5. The soy protein was provided by Protein Technologies International, Inc. (St. Louis, MO USA) along with the following analytical data. Total isoflavone content in the +IF soy protein was 2.39 mg/g protein (genistein, 1.45 mg/g). Total isoflavone content in the −IF soy protein was 0.11 mg/g protein (genistein, 0.08 mg/g). Corn oil was obtained from ICN, Inc. (Costa Mesa, CA USA) and the sucrose was purchased at a local market. All other ingredients were purchased from Harlan/Teklad (Madison, WI USA). Animals were maintained in rooms at 22°C, 55% humidity, and with settings for a 12-hour on/off light cycle and 15 air exchanges per hour.

Blood collection and platelet preparation

Blood samples were collected between 8:00 and 8:30 AM from hearts of anesthetized rats as previously described.[10] Sodium citrate (3.8%, 1:9 vol:vol) was the anti-coagulant used for preparation of platelet-rich plasma (PRP). PRP was prepared by centrifuging for 150 × *g* for 10 minutes. Ethylendaimine-tetraacetic acid (EDTA) (tri-potassium salt, 1 mg/mL blood) was used as anti-coagulant for samples analyzed by flow cytometry and for measurement of the electronic size [apparent mean platelet volumes (MPV)]. All procedures with blood and platelet samples were conducted at room temperature unless otherwise indicated.

Apparent mean platelet volumes

Cell counts and apparent MPV were determined with an electronic cell counter (System 9000, Serono-Baker, Allentown, PA USA). The counter is equipped with a veterinary software program that permits upper and lower thresholds to be set for proper detection of single platelets in samples from various species. This adjustment is necessary because of the smaller rat platelets compared with larger platelets from humans. MPV is derived by integrating the histogram of the platelet volume distribution and is an indicator of the extent of shape change (activation) that has occurred during sampling and preparative procedures.[11] Electronic sizing of platelets in EDTA was determined 15 minutes postsampling. The size of platelets collected with citrate as anti-coagulant was determined in PRP samples within 30 minutes of blood collection.

Flow cytometric analyses

This method was adapted for use in our laboratory from previously described procedures developed for analyses of leukocytes and washed platelets.[12,13] The probe for detection of hydrogen peroxide or reactive oxygen species (ROS) production is introduced into platelets by treating blood samples with 2′,7′-dichlorodihydrofluorescein diactetate. Intracellular esterases remove the acetyl groups, thus freeing the compound to react with hydrogen peroxide (ROS) to form the oxidized and fluorescent 2′,7′-dichlorofluorescein (DCF). A 50 μL aliquot of blood was diluted with 450 μL of a modified Tyrode's buffer (no calcium or magnesium[10]) and exposed to 5 μL of buffer, ethanol, or 2′,7′-dichlorofluorescin diacetate (10 μM, final concentration added in 5 μL ethanol) for 10 minutes. Prior to stimulation with collagen or addition of control reagents, 50 μL aliquots of the probe-loaded and control samples were removed and placed in 1 mL of phosphate buffered saline (PBS, pH 7.4, calcium and magnesium free). These PBS aliquots were then treated as follows: negative controls (vehicle solvents only); collagen (20 μg/mL); positive control (25 μM hydrogen peroxide in PBS). Tubes were gently agitated upon addition of collagen or control reagents and at 1-minute intervals over an 8-minute interval. The samples were monitored for fluorescence at 0, 1, 3, 5, and 8 minutes on a FACSCAN Flow Cytometer (Becton Dickinson, San Jose, CA USA) equipped with a 500 mW argon laser. Prior to analyses, the linear response of the cytometer was checked using polystyrene beads labeled with known intensities of fluorescein (LinearFlow™ Green, Molecular Probes). Platelets are easily distinguished from other blood cells because of their smaller size. Light scattering characteristics based on forward light scatter (FSC, a measure of cellular size) and side scatter (SSC, a measure of cellular granularity) characteristics were used to set the gate to collect data on fluorescent events that occurred in single platelets only. With the flow rate on low and amplification gain in the logarithmic mode, data for three parameters (FSC, SSC, and cellular fluorescence or FL1) were collected for 5,000 platelets per each tube analyzed. Data were stored as list mode files and positive FL1 events were determined using LYSYS II and PC-LYSYS software programs (Becton Dickinson). Positive fluorescent events produced by collagen stimulation were expressed as percentage of platelets demonstrating an increase in FL1 compared with controls not exposed to collagen.

Gel electrophoresis

To determine the extent of tyrosine phosphorylation in platelet proteins under basal and stimulated conditions, the experiments with PRP samples from individual animals were begun immedi-

ately after being prepared as described above. Modified Tyrode's buffer (calcium and magnesium free) was used to adjust platelet count to 200×10^6/mL. Siliconized tubes with Teflon stirring bars (1,200 rpm) were warmed to 37°C for 5 minutes prior to the addition of 500 μL of PRP. Samples were stirred for 1 minute (1,200 rpm) in the presence or absence of collagen reagent (20 μg) and immediately pelleted to remove plasma by centrifuging for $9,800 \times g$ for 1.5 minutes. Pellets were resuspended in 50 μL lysis buffer (2% Triton X-100; 100 mM Tris/HCl, pH 7.5; 50 mM NaCl; 5 mM EDTA; 4 mM sodium orthovanadate; 2 mM phenylmethylsulfonyl fluoride; 200 μg/mL leupeptin[14]), heated at 100°C for 5 minutes and then sonicated for 10 minutes at 4°C. Platelet proteins (either 15 or 30 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Blocking of nonspecific binding was accomplished by incubating the membranes with 2.5% low-fat dried milk at room temperature for 1 hour followed by incubation overnight at 4°C. Tyrosine-phosphorylated proteins were detected with the monoclonal anti-phosphotyrosine antibody and visualized by enhanced chemiluminescence according to instructions supplied by the manufacturer (Amersham). Prestained molecular weight protein markers were used as standards for the gel electrophoresis. Protein concentrations were determined according to the method of Bradford.[15] Densitometric analysis of the bands was performed using a scanner (DeskScan II, Hewlett-Packard Co., Palo Alto, CA USA) in combination with gel analysis software (SigmaGel 1.0, Jandel Corp., San Raphael, CA USA). Transfer of total proteins to membranes was checked by visualization with India ink according to the method described by Hancock and Tsang.[16]

Phosphatase assay

Phosphatase activity was measured using p-nitrophenylphosphate (pNPP) as substrate as previously described[17] except that another buffer was substituted for Tris/HCl buffer. Platelets were isolated rapidly as described above, resuspended in the modified Tyrode's buffer, and exposed to three cycles of freezing and thawing. Platelet protein was added to a N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES, 25 mM, pH 7.3) buffer containing 10 mM pNPP (final volume 200 μL) and the mixture was incubated with shaking at 37°C for 30 minutes. Reactions were terminated by the addition of 1 mL of 0.02M NaOH and absorbance was measured at 410 nm. The molar absorption coefficient 1.75×10^4 Mcm was used to calculate the concentration of the p-nitrophenolate anion. Protein concentration was determined as described above.

Statistical analyses

One-way analysis of variance (ANOVA) tests were used to compare means of normally distributed data (parametric). Pairwise multiple comparison procedures were used where noted (Student-Newman-Keuls Method). Data shown are expressed as means ± SEM. Differences were considered significant when P-values were less than 0.05.

Results

Apparent MPV

Apparent MPV are shown in *Table 1*. Platelets from rats eating the +IF soy protein diet had significantly smaller MPV than platelets from rats eating the −IF soy protein diet and was independent of the anti-coagulant used (n = 6/group; EDTA, $P < 0.008$; citrate, $P < 0.0005$). MPV determined in EDTA samples had the expected larger values compared with MPV measured in the citrated samples. It is well established that platelets collected in EDTA are larger than platelets collected in citrate.[18,19] Platelet counts in whole blood did not differ between the two dietary groups (data not shown).

Table 1 Effect of dietary isoflavones on apparent MPV of platelets in whole blood samples and in PRP

Diet	Apparent MPV (fL)*	
	EDTA†	Citrate‡
+IF	5.37 ± 0.08a	3.55 ± 0.05a
−IF	5.70 ± 0.06b	4.00 ± 0.07b

*Data are means ± SEM with n = 6. Different letter superscripts within a column denote significant differences between the means (analysis of variance).
†Apparent mean platelet volumes (MPV) of platelets in whole blood samples with ethylendaimine-tetraacetic acid (EDTA) as anti-coagulant ($P < 0.008$).
‡Apparent MPV of platelets in platelet-rich plasma (PRP) with citrate as anti-coagulant ($P < 0.0005$).

Collagen-induced production of DCF fluorescence

Platelets in whole blood samples from rats fed both diets were stimulated with collagen to activate a membrane oxidase that forms hydrogen peroxide. Increases in fluorescence due to oxidation of reduced DCF by hydrogen peroxide were monitored with a flow cytometer. *Figure 1* depicts the increase in the percentage of cells expressing positive fluorescence compared with controls. The fluorescence (FL1) after the addition of collagen or PBS was

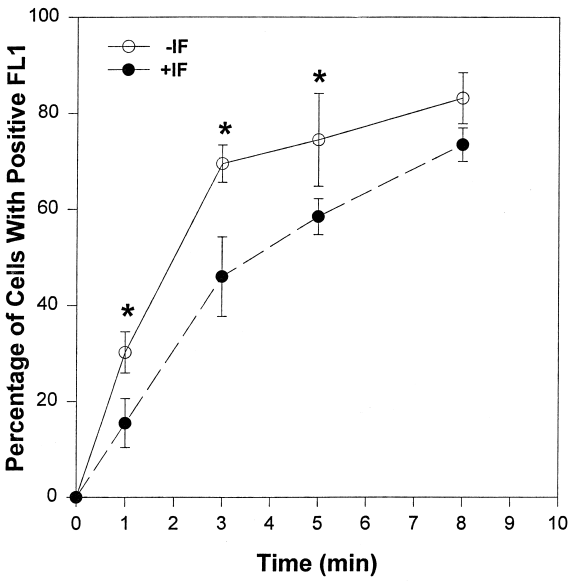

Figure 1 Increases in 2′,7′-dichlorofluorescein fluorescence (FL1) in collagen-stimulated platelets were determined on the flow cytometer for the times indicated. At 1, 3, and 5 minutes, platelets from rats fed the diet with isoflavones (+IF) showed significantly less percentage of positivity compared with the platelets from rats fed the isoflavone-reduced diet. (−IF) (*Denotes significantly different values, means ± SEM, n = 6/group, $P < 0.004$).

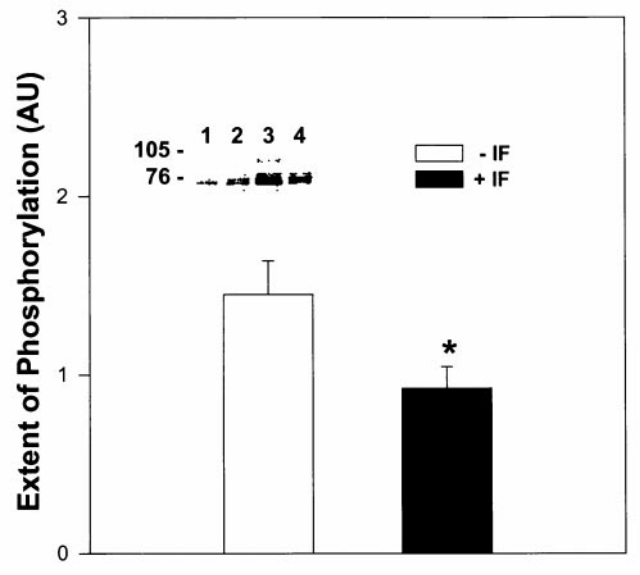

Figure 2 Bars represent values (means ± SEM) for phosphorylation of tyrosine residues in platelet proteins after stimulation with collagen for 1 minute. Total platelet proteins were separated by gel electrophoresis and phosphorylation was detected by immunoblotting with an antibody specific for phosphotyrosine in proteins (70–105 kDa; see Methods section and below for details on data depicted in inserted representative gel). Densitometry was used to measure phosphorylation and values are expressed in arbitrary units (AU). Tyrosine residues in platelet proteins from rats fed the diet with isoflavones (+IF) were significantly less phosphorylated than the tyrosine residues in platelet proteins from rats fed the isoflavone-reduced diet (−IF) ($n = 5$/group, $P < 0.047$). Gel insert: Molecular markers are denoted on the left. Lanes 1 and 2 are controls for the extent of phosphorylation in untreated platelets from −IF and +IF fed rats, respectively. Lanes 3 and 4 show phosphorylated proteins after stimulation with collagen, −IF, and +IF, respectively.

monitored for 8 minutes. Platelets from the rats eating the +IF diet expressed a significantly lower percentage of positivity at 1, 3, and 5 minutes compared with the platelets from rats eating the −IF diet ($n = 6$/group, $P < 0.004$).

Collagen-induced protein tyrosine phosphorylation

The phosphorylated tryosine residues of proteins with MW 70 to 90 kDa were determined by gel electrophoresis before and after stimulation with collagen for 1 minute. This region was selected because several platelet proteins are rapidly phosphorylated during platelet activation (e.g., the 72 kDa PTK syk and 77–80 kDa actin-binding cortactin.[7] Platelet proteins from rats eating the +IF diet were shown to have significantly less tyrosine phosphorylation than the platelet proteins from rats eating the −IF diet (*Figure 2*; means ± SEM; 0.9 ± 0.1; 1.4 ± 0.2; $n = 5$/group, $P < 0.047$, ANOVA with pairwise multiple comparison with Student-Newman-Keuls test). Overall, tyrosine residues on platelet proteins (70–90 kDa) from rats fed the +IF diet were 62% less phosphorylated than the tyrosine residues in platelet proteins from rats fed the −IF diet.

Basal phosphatase activity

Total phosphatase activity was measured in isolated platelets from rats fed +IF or −IF diets isoflavones as described in the Methods section. An ANOVA in conjunction with the Student-Newman-Keuls test for multiple pairwise comparisons was used to test for differences in means of the phosphatase specific activities (μM/min/mg protein) in platelets from the two groups. Mean values were found to be significantly different ($n = 6$/group; +IF, 22.6 ± 1.0; −IF, 19.6 ± 0.9; $P < 0.05$).

Discussion

Three independent methods were used to assess the effects of dietary soy isoflavones on indicators of platelet activation. In all three instances, platelets from rats fed the +IF diet demonstrated significant decreases in physiologic markers of activation or responsiveness compared with platelets from rats fed the −IF diet. There are numerous pitfalls associated with many of the in vitro tests of platelet aggregation used to study the effects of diet on platelet function in both humans and animals.[8] Our strategy of using two or more markers of platelet functional status and evaluating correlative associations among these tests was crucial to obtaining physiologically relevant data.

The amounts of isoflavones in the two diets represent realistic amounts in regard to human consumption. The +IF diet contained approximately two times the amount of isoflavones that would be found in a traditional diet in Japan whereas the −IF diet contained an amount of isoflavone equivalent to that found in a typical diet eaten in the United States.[20] The results reported here are among the first to provide direct evidence for the interactions of dietary isoflavones with signal transduction pathways affecting cellular responses.

The means of the apparent MPV in two different anticoagulants were significantly smaller for platelets exposed to the +IF diet compared with platelets exposed to the −IF diet. The apparent MPV reported by electronic cell counters can be used as indicators of the extent of shape change that has occurred in platelets during collecting and handling of blood samples.[11] Quiescent, nonactivated platelets are disc-shaped but assume a more spherical shape as they become activated during the initial stages of the multistep process of clot formation. The electronic volume of the disc-shaped platelets is smaller than the electronic volume of spherical-shaped platelets. Our data showing that the +IF diet resulted in less activated platelets in whole blood and PRP are consistent with reports showing that platelets in activating environments caused by heart disease, smoking, or lifestyle/diets of a population are apparently larger.[20–22] Platelets from smokers and from patients suffering from acute myocardial infarctions have been reported to have significantly larger MPV compared with MPV of platelets from nonsmoking and healthy controls, respectively.[21,22] With microscopic detection of the percentage of disc-like platelets in blood samples collected in a similar manner, investigators in Canada reported a value of approximately 65% disc-like platelets in samples from Canadian patients,[23] whereas Japanese investigators reported a value greater than 90% in samples from Japanese patients.[24] Many dietary and lifestyle differences exist between these two groups (ages were similar, approximately 40 years) that would explain these dissimilar observations, but it is well to remember that

the Japanese population consumes more than 10 times the amount of soy products eaten by North American populations.[20] Another interesting and pertinent difference in platelet status was noted between these two populations: Platelets from the females in the Canadian population were more disc-like (less activated) than the platelets from the males in this population. No gender differences were seen in the Japanese population, a population with a much lower incidence of coronary heart disease than the North American population. Estrogens protect premenopausal women from coronary heart disease that may involve both antiproliferative and antithrombotic mechanisms.[25] Isoflavones, acting as weak estrogens, may act similarly on prothrombotic signaling networks to reduce coronary heart disease.

Collagen stimulates platelets to aggregate by binding to a receptor in the plasma membrane. For this initial ligand-receptor interaction to end in aggregation, information must be transmitted from the plasma membrane through intricate intracellular networks. Transmission of these signals into the interior of the cell are aided and amplified by agents such as hydrogen peroxide, which is a ROS produced by a platelet plasma membrane oxidase during collagen stimulation.[13] This early event in collagen-mediated platelet aggregation can be monitored in a flow cytometer by following the oxidation of reduced DCF by hydrogen peroxide using procedures established for neutrophils[12] and adapted in our laboratory to study platelet hydrogen peroxide production in whole blood. The presence of isoflavones in the diet resulted in a diminished increase in fluorescence of DCF in platelets after collagen stimulation, which is indicative of less platelet activation. FL1 in platelets was followed for 8 minutes after introduction of the agonist, and at 1, 3, and 5 minutes the percentage of cells expressing positivity for FL1 was significantly less for the platelets from rats fed +IF than the platelets from rats fed −IF. Our results confirm and extend the findings reported by Wei et al.[26] on the inhibitory action of genistein on hydrogen peroxide production in tumor promoter-stimulated neutrophils, HL-60 cells, and mouse skin-punch biopsies. Our data also corroborate and expand on results from in vitro experiments with genistein and platelets performed in our laboratory[27] and others.[7,28,29] Moreover, they show, for the first time, direct evidence for the in vivo inhibitory effects of dietary isoflavones on amplification of platelet activation by hydrogen peroxide. Our strategy of monitoring early events in platelets may explain our success in observing this *in vivo* effect of the +IF diet in rats. Recently, Gooderham et al.,[30] using the traditional optical method, found no differences in platelet aggregation between subjects eating soy protein (+IF) compared with subjects eating casein supplements (−IF). Subtle effects on initial signaling steps can be missed when measuring aggregate formation, which is a very late stage in the activation process of platelets.

Genistein has been used by numerous investigators conducting in vitro studies with platelets to determine the participation of PTK in specific signal transduction pathways that depend on the balance between PTK and PTP.[7,28,29] Our results demonstrate that dietary isoflavones fed to rats in reasonable amounts can influence the extent of phosphorylation of tyrosine residues in platelet proteins. Platelet proteins with MW ranging from 70 to 105 kDa were analyzed for phosphorylated tyrosine residues before and after stimulation with collagen. This region contains proteins that undergo rapid phosphorylation on tyrosine residues upon stimulation of platelets with agonists. Syk is a 72 kDa PTK that is phosphorylated within seconds when platelets are activated by collagen.[31] Another candidate protein for phosphorylation in this region is cortactin, which is an actin binding protein (kDa 78−80).[7] Platelet proteins from rats fed the +IF diet showed 62% less phosphorylation of tyrosine residues on proteins in this region than the platelet proteins from rats fed the −IF diet. These results are consistent with reports on the effects of genistein on PTK and protein phosphorylation patterns during receptor-mediated stimulation of platelets.[7,14,31]

The extent of phosphorylation is a balance in the activities of PTK and PTP.[6,7] In the basal state, we showed that platelet total phosphatase activity was higher in the platelets from rats fed the +IF diet. Our observation of higher phosphatase activity in the +IF platelets in the basal (nonstimulated state) is in agreement with the apparent smaller MPV (more disc-like and quiescent) found for these platelets. Recently, Catalán et al.[32] reported that phosphatase activity was higher in rabbit platelets in the resting state than in platelets that had been stimulated.

In summary, our results demonstate that dietary isoflavones can inhibit platelet activation in rat platelets. These results are further strengthened by the consistent agreement on isoflavone inhibition among the three methods used to detect early signs of platelet activation. Dietary isoflavones may interact with cellular signaling networks that rely on kinases, phosphatases, and ROS signal generation with the end result of reducing over-responsive, dysfunctional signaling that produces chronic diseases.

Acknowledgments

We thank Protein Technology International, Inc. for supplying the soy proteins used in this experiment. We are also grateful to the Staff of the Animal Facility of the Beltsville Human Nutrition Research Center for their conscientious care and feeding of the animals.

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