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The phytoestrogen 8-prenylnaringenin inhibits agonist-dependent activation of human platelets

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ABSTRACT

Background: Phytoestrogens are plant-derived polyphenolic compounds that exert beneficial effects on human health, mostly related to their estrogen mimetic activity. In particular a strong correlation between phytoestrogens intake and a lower risk of cardiovascular diseases has been reported. The flavanone 8-prenylnaringenin, extracted from hop flowers, has been identified as a novel phytoestrogen, unique with respect to estrogen receptors specificity and potency. However, to date no investigations on the 8-prenylnaringenin role in modulating platelet function have been undertaken.
Methods: We evaluated the effect of 8-prenylnaringenin on platelet aggregation, intracellular calcium mobilization and protein phosphorylation triggered by thrombin and collagen, and platelet adhesion and dense granule secretion triggered by collagen.
Results: 8-Prenylnaringenin inhibited platelet aggregation induced by different agonists and platelet adhesion to collagen matrix. 8-Prenylnaringenin directly increased intracellular cAMP and cGMP levels and thus promoted VASP phosphorylation. However, these molecular events were not responsible for the inhibitory action of 8-prenylnaringenin on platelets. Moreover, 8-prenylnaringenin inhibited the phosphorylation of Pyk2, Akt, and ERK1/2. Finally, 8-prenylnaringenin suppressed the mobilization of calcium and the secretion of dense granules. All these effects were independent of estrogen receptors recruitment.
Conclusions: 8-Prenylnaringenin exerted anti-aggregatory and anti-adhesive effects on human platelets, independently of estrogen receptors, acting as an inhibitor of multiple proteins essential for the morphological and biochemical transformations that occur during platelet activation and aggregation.
General significance: 8-Prenylnaringenin may represent a useful tool in the therapy and prevention of vascular diseases associated with platelet aggregation, such as atherosclerosis, myocardial infarction, coronary artery disease, and thrombosis.

1. Introduction

Phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogen molecules [1]. Similar with estrogens, phytoestrogens exert beneficial effects on human health, especially against hormone-dependent tumors [2], osteoporosis, irregular menopause syndrome, and cardiovascular, and neurodegenerative diseases [3].

The biological effects of a phytoestrogen compound depend on its peculiar structure and the target cell type, and now it is generally accepted that these molecules function through the engagement of estrogen receptors.

Although phytoestrogens do not have a steroid structure, they can interact with the estrogen receptors on target cells triggering signals that mimic mammalian estrogens [1]. Among all, the cardiovascular system, which undergoes aging- and inflammation-dependent injury resulting in atherosclerotic disease, seems to be one of the most promising targets for phytoestrogen-based therapies. Interestingly, some dietary phytoestrogens are able to inhibit the function of platelets, anucleated cell fragments known to play an important role in the development of atherosclerotic disease [4].

Studies in rats have demonstrated that the phytoestrogens genistein and raloxifene might inhibit platelet aggregation by increasing

Abbreviations: 8-PN, 8-prenylnaringenin; BSA, bovine serum, albumin; ERK1/2, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PKA, protein kinase A; PKG, protein kinase G; sGC, soluble guanilyl cyclase; SNP, sodium NitroPrusside; VASP, vasodilator-stimulated phosphoprotein

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nitric oxide (NO) synthesis and enhancing phospholipase A_2 activity and prostacyclin release in an estrogen receptor-dependent manner [4]. By contrast, previous studies in our laboratory have reported that 17β-estradiol causes a strong potentiation of integrin αIIbβ3 activation and aggregation in platelets stimulated with low concentrations of thrombin in an estrogen receptor β-dependent manner [5] with a mechanism coordinated by membrane lipid rafts. Indeed, in platelets the estrogen receptor β reversibly translocated to the lipid raft fractions in a hormone-dependent manner and promoted the rapid and transient recruitment and activation of the tyrosine kinases Src and Pyk2 within the membrane raft domains [6].

It has been reported that a considerable number of phytoestrogens and flavonoids are able to impair the activity of enzymes involved in cell signaling such as cycloxygenases and lypoxygenases [7], phosphodiesterases [8], tyrosine kinases [9], and phospholipases [10,11] and to inhibit calcium mobilization and subsequent platelet secretion [12].

8-Prenylnaringenin (8-PN) is a prenylated flavonone discovered in 1992 by Rosenblum and colleagues [13], which displays an estrogen-like activity, stronger than that of genistein or coumestrol [14–16]. 8-PN, extracted from glands of the hop flowers, is present in beer in a concentration of 0.24 mg/l [17,18] and in an increasing number of dietary supplements containing hop extracts used to reduce the discomforts associated to menopause [19] and to induce "breast enhancement" [20]. Studies of molecular modeling underline that 8-PN and 17β-estradiol display similar structural features, mainly due to the presence of the prenyl-side chain at the C-8 position in 8-PN. Indeed, 8-PN binds to estrogen receptors with higher affinity than naringenin, which exhibits only weak estrogenic properties [20–23].

Despite this evidence, until now no investigations on the possible role of 8-PN in modulating platelet function have been undertaken. This paper focuses on the effects of 8-PN on platelet function, demonstrating that this phytoestrogen behaves in vitro as a powerful inhibitor of agonist-induced platelet aggregation. The molecular mechanism of this inhibitory effect was also investigated. Our data have demonstrated that 8-PN interacts with human platelets through a complex mechanism, not mediated by platelet estrogen receptors. We found that 8-PN prevents agonist-dependent activation of Akt, ERK1/2, and Pyk2, key modulators of platelet activation, calcium mobilization, and dense granule secretion and increases cyclic nucleotides within the platelets.

2. Methods

2.1. Materials

Collagen used in the aggregation studies was from Mascia Brunelli (Milano, Italy), while the collagen used in the adhesion assays was provided by Prof. M. E. Tira (University of Pavia, Italy). Thrombin, U46619, and sodium nitroprusside (SNP) were from Alexis Biochemicals (Lausen, Switzerland). ICI 182,780 and ODQ (1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one) were from Tocris Bioscience (Bristol, UK). Monoclonal antibody anti-phosphotyrosine (4G10) was from Millipore (Billerica, MA); monoclonal antibodies against vasodilator-stimulated phosphoprotein VASP (phospho-Ser 239 and phospho-Ser 157) were both from Calbiochem (San Diego, CA, USA); antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 MAPK and phospho-Pyk2 were all from Cell Signaling Technology (Beverly, MA, USA); phosphospecific polyclonal antibody against Src kinases (Tyr418), FURA 2-AM, and PGE_1 were from Sigma-Aldrich (St. Louis, MO, USA); polyclonal antibody against phospho-Akt and Alexa488-phalloidin were from Invitrogen (Milano, Italy); polyclonal antibody against integrin β3 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary anti-rabbit and anti-mouse antibodies, Hybond-P polyvinylidene difluoride (PVDF) membranes, and cAMP Biotrak Enzymeimmunoassay (EIA) system were from GE Healthcare (Milan, Italy); cyclic GMP Complete was from Assay Designs (Vinci, FI, Italy). Western Lightning Plus-ECL was from Perkin Elmer (Boston, MA, USA); ATP Determination Kit was from Biaffin GmbH & Co KG (Kassel, Germany). 8-Prenylnaringenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2enyl)chroman-4-one) was synthesized by Prof. G. Appendino (University of Piemonte Orientale A. Avogadro, Italy) according to literature methods, and 8-PN was identified by spectroscopic comparison (^{1}H- and ^{13}C-NMR, MS, IR) with an authentic sample available from previous work [24]. Purity, when evaluated by HPLC, was 96%.

2.2. Platelet preparation

Blood was withdrawn, according to the Declaration of Helsinki and after informed consent was obtained, from healthy volunteers who had not taken any drugs for at least 10 days before venipuncture, using ACD (130 mM citric acid, 152 mM sodium citrate, 112 mM glucose) or 0.32% citrate as anti-coagulant. The blood was centrifuged at 120 ×*g* for 10 min at room temperature. The platelet rich plasma (PRP) was collected. To prepare gel-filtered platelets PRP was centrifuged at 300 ×*g* for 10 min. Platelets were then purified by gel filtration on Sepharose CL-2B and eluted with Tyrode buffer (10 mM Hepes, 137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.40). Platelets were counted, and their concentration was adjusted in Tyrode buffer containing 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 5.5 mM glucose.

2.3. Measurement of platelet aggregation

Samples of PRP or gel-filtered platelets (3×10^8 cells/ml) were prewarmed at 37 °C under constant stirring (1000 rpm), incubated with different concentrations of 8-PN or vehicle (0.125% methanol) for 5 min and then induced to aggregate with collagen type I (0.25 or 5 μg/ml), thrombin (0.05 U/ml) or U46619 (0.25 or 2 μM). Transmittance of platelet suspension was monitored continuously for at least 5 min in a Chronolog aggregometer (Mascia Brunelli). In some experiments platelets were incubated with 2 μM ODQ, 0.1-150 μM ICI 182,780, or vehicle (0.1% ethanol) for 5 min or with 1 μM SNP for 1 min prior to stimulation with 8-PN. For wash-out experiments, gel-filtered platelets (3×10^8 cells/ml) were incubated for 5 min with 30 μM 8-PN or vehicle, centrifuged at 300 ×*g* for 10 min, resuspended in Tyrode buffer at 3×10^8 cells/ml and induced to aggregate with 5 μg/ml collagen.

2.4. Adhesion assay

Glass coverslips were coated for 2 h at 37 °C with 100 μg/ml of type I collagen or 0.5% BSA/PBS and then blocked with 5% BSA in PBS for 2 h at 37 °C. Human gel-filtered platelets (3×10^7/ml, 0.2 ml) were incubated with 30 μM 8-PN or vehicle (0.125% methanol) for 5 min and added to the coated coverslips for 15, 30 or 60 min at 37 °C. Non-adherent cells were removed and coverslips were washed with PBS. Adherent platelets were fixed with 3% paraformaldehyde-4% sucrose in PBS for 10 min at room temperature, permeabilized by 0.2% Triton-X100 for 5 min, and stained with Alexa488-phalloidin. Platelets were viewed on a confocal microscope (Leica TCS SP2) and digital images (63×) were acquired. The number of adherent cells and the average cell area (index of platelet spreading) were determined using the Image-Pro Plus software, version 7.0. For each specimen, at least 20 different fields were analyzed.

2.5. Electrophoresis and immunoblotting analysis

Samples of gel-filtered platelets (5×10^8 cells/ml) were incubated with 30 μM 8-PN or vehicle (0.125% methanol) from 30 s to 15 min at 37 °C and then stimulated with 5 μg/ml collagen or 0.05 U/ml thrombin for 5 min, as indicated. In some experiments platelets were incubated with 2 μM ODQ, ICI 182,780 or vehicle (0.1% ethanol) for 5 min