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Original Article

The isoflavonoid tectorigenin has better antiplatelet potential than acetylsalicylic acid

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ABSTRACT

Background: One reason for the lower incidence of cardiovascular diseases in Asian countries may be the high intake of isoflavonoids and their antiplatelet effects may be an important factor. To date, there is limited comparison of a range of isoflavonoids and knowledge of their effects at different levels of platelet aggregation.
Purpose: To screen the antiplatelet effects of a number of isoflavonoids on the arachidonic acid based aggregation pathway and investigate how the antiplatelet activity might occur.
Methods: The antiplatelet effects were first screened in whole human blood where platelet aggregation was induced by arachidonic acid. Further analysis was targeted at search of the mechanism of action.
Results: Thirteen of the eighteen tested isoflavonoids had significant inhibitory effect on platelet aggregation in whole human blood. Genistein had the same potency as clinically used acetylsalicylic acid (ASA) while tectorigenin was clearly stronger than ASA. Further analyses showed that the effect of tectorigenin was not based on inhibition of cyclooxygenase-1 in contrast to ASA or thromboxane synthase but by competitive antagonism at thromboxane receptors.
Conclusion: Tectorigenin is a more potent antiplatelet compound than ASA and thus an interesting substance for further testing.

Introduction

Platelets are small anucleated cells and the second most abundant component of human blood (Harrison, 2005). Their main role is to stop bleeding from damaged blood vessels and hence prevent large blood losses. However, blood vessels significantly affected by atherosclerosis are very prone to enhanced platelet aggregation often leading to acute myocardial infarction or stroke.

Isoflavonoids naturally occur in many plant species, e.g., mung bean (*Vigna radiata* (L.) R. Wilczek) contains 4',6,7-trimethoxyisoflavone, daidzein, formononetin or genistein (Tang et al., 2014), red clover (*Trifolium pratense* L.) biochanin A, daidzein, genistein and formononetin (Boue et al., 2003; Liu et al., 2001), soybeans (*Glycine max* (L.) Merrill) genistein, daidzein and glycitein (Jian, 2009), *Belamcanda chinensis* (L.) DC. contains tectorigenin (Ha le et al., 2013) and *Ononis spinosa* L. ononin, formononetin, genistein and biochanin A (Yilmaz et al., 2006). A number of studies have suggested that consumption of isoflavonoid-rich food, especially those including daidzein and genistein, has beneficial effects on human health; anti-diabetes (Cornwell et al., 2004), anti-cancer (Cornwell et al., 2004; Jian, 2009) and anti-osteoporotic effects (the latter due to estrogenic activity) have been described (Boue et al., 2003; Cornwell et al., 2004; Liu et al., 2001). A lower risk of coronary artery disease has been reported too (Kurzer and Xu, 1997; Pase et al., 2011). The last mentioned has been described mainly in Asian countries where the dietary soy intake is higher than in Europe (Gottstein et al., 2003; Sagara et al., 2004). In addition to the commonly discussed antioxidant activity (Mladenka et al., 2010; Ruiz-Larrea et al., 1997), slight LDL-lowering (Cornwell et al., 2004) and blood pressure reducing effect (Sagara et al., 2004), antiplatelet activity may be one of the major causes of their

Abbreviations: AA, arachidonic acid; ASA, acetylsalicylic acid; COX-1, cyclooxygenase-1; EDTA, ethylendiamintetraacetic acid; PRP, platelet rich plasma; PPP, platelet poor plasma; TXA_2, thromboxane A_2

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claimed positive cardiovascular benefits.

The process of platelet aggregation is very complex and still not fully understood. Further, platelet sensitivity to proaggregatory factors varies greatly in populations. The key mediator appears to be thromboxane A_2 (TXA_2), which is synthetized in platelets on stimulation by various aggregation inducers including ADP, collagen and thrombin. TXA_2 formation is initiated by release of arachidonic acid (AA) from the cytoplasmatic membrane, followed by its conversion by platelet cyclooxygenase-1 (COX-1) into prostaglandin H_2 and into TXA_2 via platelet thromboxane A_2 synthase. TXA_2 is a potent inducer of platelet aggregation and a vasoconstrictor. Current clinical antiplatelet therapy is limited to acetylsalicylic acid (ASA), an irreversible inhibitor of COX-1, and antagonists of ADP receptors P2Y12 (clopidogrel, prasugrel, ticagrelor, elinogrel and cangrelor). Although the antiplatelet properties of ASA were described in 1950, this drug is still used and still has an important role in the secondary prevention of thrombus formation after stroke or in coronary artery disease (Luepker et al., 2015). A major problem with ASA is treatment failure in 5–45% of patients (Derle et al., 2016). ADP receptor antagonists have better platelet selectivity over ASA but unfortunately these drugs are associated with serious side-effects and potential pharmacokinetic interactions as is shown for ticlopidin or clopidogrel (Mugosa et al., 2015). These limitations can be surmounted by the latest representatives of this group which are not prodrugs and have less potential for pharmacokinetic interactions (ticagrelor, elinogrel, cangrelor). However, experience with them in clinical practice is brief and the side-effects have yet to be established. These findings are an impulse to search for new substances. In an ideal case, the mechanism of action should include another step(s) of platelet activation. Recently, some flavonoids have been shown to be able to block platelet aggregation at two levels of the AA-based pathway. The most active flavonoids, the isoflavonoids daidzein and genistein, appear to have an antiplatelet effect in micromolar concentrations and are thus feasible in clinical settings. The study described here is an extension of an earlier one in which we analysed a series of flavonoids including the isoflavonoids, genistein and daidzein (Karlickova et al., 2016). There are a number of studies suggesting the effect of some isoflavonoids on platelet aggregation (Bojic et al., 2011; Gottstein et al., 2003; Guerrero et al., 2005, 2007; Karlickova et al., 2016; Kim and Yun-Choi, 2008; Kim et al., 1999; Lo et al., 2003; Nakashima et al., 1991; Wang et al., 2000; You et al., 1999), but no comprehensive comparison of a larger number of isoflavonoids on different levels of platelet aggregation.

The main purpose of this study was to test the antiplatelet activities of a series of structurally related isoflavonoids (Fig. 1) on the arachidonic acid based aggregation pathway, to find ideal structural feature (s) for antiplatelet potential and to determine the mechanism of action of the most active compound.

Materials and methods

Tested compounds

Calycosin (99% purity), cladrin (98%), isoformononetin (99%) and tectorigenin (100%) were purchased from Phytolab (Vestenbergsgreuth, Germany), *R,S*-equol (98%) and *S*-equol (97%) from Toronto Research Chemicals (Toronto, Canada). Biochanin A (≥99%), daidzin (≥99%), formononetin (≥99%), genistin (≥99%), glycitein (≥95%), glycitin (≥95%), ononin (≥99%), prunetin (≥95%), puerarin (≥99%) were purchased from Extrasynthese (Lyon, France) and 4',6,7-trimethoxyisoflavone (≥99%), daidzein (≥98%), genistein (≥98%) and terutroban (99.9%) from Sigma Aldrich (Prague, Czech Republic). The purity of the isoflavonoids was checked by HPLC with the exception of *R,S*-equol and *S*-equol (TLC).

Chemicals

Arachidonic acid was purchased from Medista (Prague, Czech Republic) and heparin sodium from Zentiva (Prague, Czech Republic). Thromboxane B_2 EIA kit, prostaglandin H_2, U-46619 and COX inhibitor screening assay kit were purchased from Cayman Chemical Company (MI, USA). DMSO, EDTA, 1-benzylimidazole, kaempferol, epicatechin, indomethacin and ASA were purchased from Sigma Aldrich (Prague, Czech Republic). 96% ethanol was purchased from Penta (Prague, Czech Republic) and 0.9% sodium chloride from B. Braun (Prague, Czech Republic).

Blood volunteers

Blood samples from 25 non-smoking volunteers were collected by venipuncture into plastic disposable syringes containing heparin sodium (170 IU/10 ml). For specific experiments, the COX inhibitor, indomethacin, or the thromboxane synthase inhibitor, 1-benzylimidazole, were immediately added to the collected blood to a final concentration of 10 μM. No medication had been taken for least 14 days before the blood collection, written informed consent was obtained and the Ethics Committee of Charles University, Faculty of Pharmacy, Hradec Králové approved the study on November 12, 2012. It conformed to the latest Declaration of Helsinki. Whole blood was used in the screening experiments and in the experiments analysing the type of antagonism of tectorigenin at the level of thromboxane receptors. In others, platelet rich plasma was obtained as a supernatant by centrifuging the blood for 10 min at 500 g (centrifuge MPW-360, MPV Med. Instruments, Poland). Platelet poor plasma was prepared by centrifuging the remaining blood for 10 min at 2500 g. The platelet count was determined using a BD Accuri C6 flow cytometer (BD Accuri Cytometers Inc., USA) equipped with BD C6 flow software and adjusted to 2.5 or 3.5×10^8 platelets/ml according to the protocol using autologous plasma.

Screening of the antiplatelet activity

The aggregometer Multiplate (Roche, Switzerland) was used for screening antiplatelet activity. This uses two pairs of electrodes submerged in the stirred blood samples to measure the impedance between them (Toth et al., 2006). 300 μl of the whole blood was diluted by an equipotent volume of preheated 0.9% sodium chloride and incubated with 5 μl of a tested isoflavonoid (dissolved in DMSO) for 3 min at 37 °C. Platelet aggregation was then induced by addition of AA and the aggregation process was observed for 6 min and expressed as area under the curve. Since there is some variability in the general human population, the dose of AA was gradually increased in order to construct dose-response curves and to find the lowest dose to evoke maximal platelet aggregation. The average concentration of AA used for maximal aggregation was 140 μM. Acetylsalicylic acid and kaempferol were used as standards to ensure reliability. All test samples were compared to blank samples which contained 5 μl of DMSO instead of the tested compound. The concentration of DMSO was maintained in all samples at 0.8% (V/V).

Cyclooxygenase-1 inhibition

The method used for COX-1 inhibition was described previously (Karlickova et al., 2016). ASA was used as the standard.

Thromboxane A_2 synthase inhibition

This is also described (Karlickova et al., 2016). Results were compared to 1-benzylimidazol, a known blocker of thromboxane A_2 synthase.

Antagonism at thromboxane receptors

Antagonism at thromboxane A_2 receptors was analysed by turbidimetry using a Chrono-log 500-Ca aggregometer (Chrono-Log Co., USA)