Antiplatelet Effects of Flavonoids Mediated by Inhibition of Arachidonic Acid Based Pathway

Abstract

Flavonoids, important components of human diet, have been claimed to possess a significant antiplatelet potential, in particular due to their effects on the arachidonic acid cascade. Due to variable and incomplete results, this study was aimed at delivering a detailed analysis of the effects of 29 structurally relevant, mainly natural flavonoids on three consecutive steps of the arachidonic acid cascade. Only the isoflavonoids genistein and daidzein were shown to possess a marked cyclooxygenase-1 inhibitory activity, which was higher than that of acetylsalicylic acid using the isolated ovine enzyme, and physiologically relevant, although lower than acetylsalicylic acid in human platelets. None of the tested flavonoids possesses an effect on thromboxane synthase in a clinically achievable concentration. Contrarily, many flavonoids, particularly those possessing an isolated 7-hydroxyl group and/or a 4′-hydroxyl group, acted as antagonists on thromboxane receptors. Interestingly, the substitution of the free 7-hydroxyl group by glucose might not abolish the activity. In conclusion, the consumption of few flavonoids in a diet, particularly of the isoflavonoids genistein and daidzein, may positively influence platelet aggregation.

Introduction

Platelets are essential components of the human blood responsible for rapid blood coagulation during injuries. On the other hand, excessive platelet aggregation is associated with cardiovascular diseases, in particular with the risk of serious or fatal coronary heart disease. Because both decreased and increased platelet aggregation are associated with pathological states, the process of aggregation has to be tightly controlled. The homeostasis is very complex and scientists have not been able to precisely define the process in all aspects up to date [1]. There are several proaggregatory factors which enhance thrombus formation. The central role in this cascade appears to be associated with the release of AA from the cytoplasmatic membrane and its transformation into prostaglandin H2 by platelet COX-1, with further metabolism into thromboxane A2 by platelet thromboxane synthase. This process is stimulated in particular by collagen, but other inducers like ADP and thrombin play a role as well [2, 3]. Despite this fact, most of the current clinically used drugs are irreversible inhibitors of COX-1, like ASA, and antagonists on ADP receptors (clopidogrel, ticagrelor, prasugrel) [4]. There are no other drugs acting on other levels of the AA cascade in clinical settings. Such drugs blocking thromboxane synthase or acting as antagonists on thromboxane receptors [5] might enrich the palette of clinically useful drugs in the future. Since they act specifically on the described steps of platelet aggregation, they may have fewer side effects in comparison to inhibitors of COX-1, which is an enzyme with many essential physiological roles. Flavonoids are promising candidates to be both, natural modulators of the disruption of platelet aggregation, and antiplatelet drugs. There is a vast amount of literature on the subject showing that many flavonoids are potent inhibitors of platelet aggregation induced by molecules such as colla-
gen, AA, thromboxane receptor agonist U-46619, ADP, and epinephrine [6–10]. The data on thrombin-induced aggregation are controversial, some claim that flavonoids have no effect [9, 11], other report positive effects [12]. Additionally, the inhibition of platelet aggregation by flavonoids is a reversible process [9], which is another important factor when considering possible side effects of the current antiplatelet therapy. Although many mechanisms responsible for antiplatelet effects have been proposed [13], only a few are documented by published studies. In particular, their effects on the AA-based aggregation cascade are well documented [10, 14, 15] but there are several discrepancies ensuing from the use of different protocols (e.g., animal or human platelets, different analytical procedures, use of washed platelets, platelets in plasma, or in the whole blood). Nevertheless, flavonoids may potentiate the effects of ASA via different mechanisms of action [15].

A comprehensive investigation between the flavonoid structure and the effects on the AA cascade including COX-1, thromboxane synthase and thromboxane receptors is still missing. In addition, the effects on thromboxane synthase have been analyzed only indirectly so far. Therefore, this study was aimed at delivering a detailed analysis of 29 flavonoids (Fig. 1), representing the most commonly found natural flavonoids, and some relevant synthetic congeners in order to establish structure-activity relationship on three consecutive steps of the AA-based platelet aggregation using human platelets.

**Results**

Initially, all flavonoids were tested for their possible effects on the inhibition of ovine COX-1 in a concentration of 100 µM and compared to ASA. ASA was moderately active at this concentration, the isoflavones genistein and daidzein were more potent inhibitors (p < 0.01), while all other flavonoids were essentially inactive (Fig. 2A). Considering these surprising results, we retested ASA and the active isoflavonoids for their concentration-dependent effects. Herein, genistein was more potent at lower concentration again but comparable to ASA in higher concentrations. In the case of daidzein, a threshold effect at about 40% was found (Fig. 2B). To ascertain if this could be valid for humans, human platelet suspension in plasma was used. In these physiological conditions, ASA showed an excellent effect in units of µM and completely inhibited COX-1 in higher concentrations. Both isoflavonoids were significantly less active, they did not reach full inhibition even at high, pharmacologically unachievable, concentrations but their activity in units of µM could have a real clinical relevance (Fig. 2C). The activities of daidzein and genistein were similar in this set of experiments.

The second step of the AA cascade is the transformation of prostaglandin H₂ into thromboxane A₂ via thromboxane synthase. All flavonoids were tested for their effect on this enzyme and compared to the known inhibitor 1-benzylimidazol. At the concentration of 100 µM, only three flavonoids showed more than 25% inhibition, but all of them were clearly less potent than 25 µM of 1-benzylidimazole (Fig. 3A). These partly active flavonoids, 7-

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**Fig. 1** Chemical structure of flavonoids tested in this study. Glc: glucose, Rha: rhamnose, Glu: glucuronic acid.
hydroxyflavone, apigenin and epicatechin, were additionally tested for a concentration-dependent effect. From these data (Fig. 3 B) it was clear that all three flavonoids were similarly active, and were able to markedly block the enzyme at concentrations which are pharmacologically not relevant, and were more than about 1 order less efficient (IC50 in the range of 151 to 226 µM) compared to 1-benzylimidazol with an IC50 of 8 ± 1 µM. The last well-known part of the aggregation cascade is the activation of thromboxane A2 receptors. In this assay, we used the stable thromboxane A2 receptor agonist U-46619. Again, all flavonoids were tested. Notwithstanding the equal platelet concentration used in this study, the effect of U-46619 on platelet aggregation was highly variable, and therefore, we used a two-step calibration in order to ascertain reproducibility of the results: first, the used concentration of U-46619 in the range of 0.75–1.5 µM had to produce more than 90% aggregation, and second, epicatechin at the concentration of 300 µM had to decrease the aggregation to 15–30%. The need of the final concentration of U-46619 in the mentioned range suggests that this analogue has a lower affinity to thromboxane receptors in comparison to the endogenous substrate thromboxane A2, but it is well known that it has a much better stability. Due to these reasons, it is not easy to determine the clinical relevance of the inhibitory concentrations. Herein, we tested all compounds up to a concentration of 300 µM. If no inhibition of platelet aggregation was achieved at this high concentration, the compounds were considered inactive. According to their activity, we divided the flavonoids in 3 classes (Table 1). The active flavonoids were then compared according to two criteria, i.e., IC50 and the effect at a concentration of 100 µM (Fig. 4 B, C), because their anti-aggregatory curves had different shapes (Fig. 4 A). Although there were some differences between active flavonoids in terms of IC50 (Fig. 4 B, daidzein and apigenin-7-glucoside were the most potent), there were insignificant differences among all five active flavonoids concerning their effect at a concentration of 100 µM.
Discussion

Several epidemiological studies have suggested protective effects of flavonoids against cardiovascular diseases. These effects may be linked to their influence on platelets as can be implied from the lower incidence of ischaemic stroke observed with increasing flavonoid consumption [16–21]. Although positive effects of flavonoids in experimental models of thrombosis have been documented in animal studies [22,23], clinical data are still missing and the few available small human studies have reported no effect of flavonoids on platelet aggregation in healthy volunteers [12, 24,25]. Thus the current knowledge does not enable an unambiguous conclusion. In particular, the following issues should be considered: 1) epidemiological data are based on a food questionnaire, such estimates of flavonoid consumption represent a very imprecise indicator of flavonoid plasma levels and in addition, patient medication cannot be analyzed in detail, 2) small clinical evaluations have been performed on healthy volunteers and the fact that flavonoid consumption has not modified normal human platelet aggregation can be considered as a positive fact, 3) there is high variability in flavonoids pharmacokinetics and, as well, pharmacodynamic effects [3]. The variation in response to the thromboxane receptor agonist was observed in our study too, notwithstanding PRP from different donors was normalized to the same platelet concentration. Moreover, artificial animal models of platelet thrombus injuries may not sufficiently mimic the clinical situation.

Data from ex vivo and in vitro studies are much more convincing. A number of known flavonoids has been shown to possess antiplatelet effects [3, 6,8,9]. Although many mechanisms, including inhibition of phospholipase A2, phosphodiesterases and/or protein kinases, have been reported [12,26–29], the mechanism of action, which seems to be common for the majority of flavonoids and in some of them within the range of achievable plasma concentrations, appears to be the inhibition of the AA-based pathway of platelet aggregation [10,14]. Indeed, flavonoids are able to decrease aggregation stimulated by different inducers. Particularly, flavonoids seem to be potent inhibitors of aggregation caused not only by AA, but also by collagen, which is known to play the key role in phase 1 of (patho)physiological platelet aggregation [30]. In addition, certain flavonoids, rather at higher concentrations, may block aggregation induced by ADP or thrombin [8], because the AA-based pathway is only one part of the complex mechanism of platelet activation evoked by endogenous signal molecules mentioned above. However, as described earlier, it is reasonable to think that several flavonoids may possess additional effects on platelet aggregation beyond their influence on the AA-based pathway due to the structural diversity of this group.

<table>
<thead>
<tr>
<th>Class</th>
<th>Effect range</th>
<th>Flavonoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inefficient</td>
<td>no effect at 300 µM</td>
<td>baicalein, baicalin, flavone, hesperidin, hesperetin, 3-hydroxyflavone, 5-hydroxyflavone, mosloflavone, negeleitin, luteolin, rutin, naringin, taxifolin and traxerutin</td>
</tr>
<tr>
<td>Slightly efficient</td>
<td>effect at 300 µM but no effect at 150 µM</td>
<td>kaempferol, myricetin, naringenin and quercetin</td>
</tr>
<tr>
<td>Moderately active</td>
<td>effect at 150 µM but no effect at 100 µM</td>
<td>catechin, diosmin, epicatechin, chrysin, genistin* and morin</td>
</tr>
<tr>
<td>Active</td>
<td>effect below 100 µM</td>
<td>apigenin, apigenin-7-glucoside, daidzein, genistein and 7-hydroxyflavone</td>
</tr>
</tbody>
</table>

* genistin activity was on the border between active and moderately active
In this study we concentrated on the AA-based pathway which plays likely the key role in the effect of a majority of flavonoids. AA is, through a two-enzyme mediated reaction (COX-1 and thromboxane A₂ synthase), transformed into a potent platelet aggregation inducer, thromboxane A₂, which stimulates its own receptors, resulting in platelet aggregation. We have critically assessed the effects of flavonoids on all three levels of the AA-based aggregation cascade. Similarly to our recent study, where we have shown that some coumarins may affect two steps in this cascade [31], we documented that the isoflavonoids genistein and daidzein acted both as inhibitors of COX-1 and as functional antagonists at thromboxane A₂ receptors.

In addition, a number of flavonoids are able to act as thromboxane receptor antagonists. In the literature, there are few reports showing that flavonoids and isoflavonoids in microsomal suspension of COX-1, COX-1 inhibition are more in accordance with this study and with previous reports. In a study comparing the effect of different flavonoids, COX-1 inhibition was achieved in lower concentrations than in PRP [11] and interindividual differences cannot be excluded either. The latter can be supported by this study where genistein and daidzein reached partial inhibition of human platelet COX-1 at lower concentrations than in the case of the recombinant ovine COX-1 enzyme, and also by another study, where quercetin blocked COX-1 from bovine platelets with an IC₅₀ of 8 µM [33]. It should also be mentioned that the activation of isolated rat peritoneal leucocytes with ionophore A23187 leads to thromboxane A₂ formation, which may be inhibited by several flavonoids at very low concentrations as well [34,35], but here, flavonoids seemed to act upstream of the AA metabolism.

As far as we know, no results on the direct inhibition of thromboxane A₂ synthase by flavonoids have been published. One possible reason is the lack of an effect and ensuing difficulties in the publication of negative data. Older reports suggested that flavonoids decrease thromboxane A₂ levels indirectly mainly by inhibition of COX-1 in line with our data [32]. The available data on COX-1 inhibition are more in accordance with this study and with previous reports. In a study comparing the effect of different flavonoids and isoflavonoids in microsomal suspension of COX-1, genistein was the most active. Interestingly, daidzein was markedly less active, but its IC₅₀ was still lower than that of ASA [36]. The essential structural features for both activities are reported here for the first time. For COX-1 inhibition, an isoflavone ring with a 7-hydroxyl group was necessary, while for being an antagonist at thromboxane A₂ receptors, the free 7-hydroxyl group is not absolutely needed, since its blockade in apigenin–7-glucoside did not abolish the effect. On the other hand, the presence of glucose at position 7 in isoflavones (genistin vs. genistein) decreased the effect. The position of ring B (isoflavones vs. corresponding flavones) is important for inhibition of COX-1 but not for antagonism at thromboxane receptors, where there was insignificant difference between isoflavone genistein and the corresponding flavone apigenin. The catechol ring B, absence of the 4-keto group or the 2,3-double bond and the presence of the 3-

![Fig. 5](attachment://AA-based_platelet_aggregation_andSites.png)

**Fig. 5** AA-based platelet aggregation and sites of effects of active flavonoids. Thromboxane A₂ formed by this AA-based pathway may stimulate its receptors both on the same platelet or of other platelets. Proposed mechanisms of action of isoflavonoids are shown. PGH₂: prostaglandin H₂; TxA₂: thromboxane A₂. (The figure was redrawn from our previous article [31].) (Color figure available online only.)
or 6-hydroxyl group were factors decreasing the activity. It is not known if effects on both levels of the AA-aggregation pathway might be an advantage, because apigenin and genistein were similar.

In conclusion, this study confirmed the previous finding that flavonoids, e.g. luteolin and apigenin, may have a positive impact on human platelet aggregation and thus on cardiovascular diseases associated with enhanced platelet activity.

Materials and Methods

Materials

AA was purchased from Chrono-Log Co. and sodium citrate solution from Biotika. Thromboxane B2 EIA kit, prostaglandin H2, U-46619 and the COX inhibitor screening assay kit were purchased from Cayman Chemical Company. Genistin and apigenin-7-glucoside were purchased from Extrasynthese. Mosloflavone and negletein were synthesized by a convergent synthesis starting from chrysin according to the previous report [40] at the Sapienza University of Rome. All other flavonoids (minimal purity of 95%, Fig. 1), DMSO, EDTA, 1-benzylimidazole (99% purity), indomethacin (>99% purity) and ASA (>99% purity) were purchased from Sigma-Aldrich. 96% ethanol was purchased from Penta.

Blood samples from 31 healthy non-smoking volunteers were collected by venipuncture into plastic disposable syringes containing 3.8% sodium citrate (1:9, v/v). For mechanistic experiments, the COX inhibitor indomethacin, or the thromboxane synthase inhibitor 1-benzylimidazol, were immediately added to the collected blood at a final concentration of 10 µM. All volunteers were health-workers who had not taken any drug for at least 14 days before the blood collection and who had given informed consent for the study. The study was performed under the supervision of the Ethical Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové (approval date: November 12, 2012) and conforms to the Declaration of Helsinki.

PRP was obtained as a supernatant by centrifugation of the collected blood for 10 min at 500 g (centrifuge MPW-360, MPW Med. Instruments). Platelet poor plasma was prepared by centrifugation of the remaining blood for 10 min at 2,500 g. The platelet count was determined using a BD Accuri C6 flow cytometer equipped with BD CFlow Software and adjusted to 2.5 or 3.5 × 10^9 platelets/mL according to the planned protocol with the use of autologous plasma.

Cyclooxygenase-1 inhibition

A commercial set from Cayman Chemicals [41], which does not give false positive results for antioxidants, was used for the evaluation of COX-1 inhibition. Shortly, ASA or flavonoids dissolved in DMSO (final concentration of DMSO was 2% v/v) were incubated with ovine COX-1 at 37 °C and AA (final concentration of 100 µM) was added to the mixture to start the reaction. The formed prostaglandin H2 was measured following its reduction to prostaglandin F2α by stannous chloride and assessed by enzyme immunoassay. The percentage of inhibition was related to the positive control with DMSO. Analogously, in additional experiments, PRP with a platelet concentration of 3.5 × 10^9 per mL pretreated with 1-benzylimidazol, to block further metabolism of prostaglandin H2, was used instead of ovine COX-1 for testing of inhibition of human COX-1.
Thromboxane A₂ synthase inhibition

Thromboxane A₂ synthase inhibition was evaluated according to the method of Chang et al. [42], with minor modifications. PRP containing indomethacin with a platelet concentration of 3.5 × 10⁸ per ml was incubated with the tested compounds for 3 min at 37°C. After addition of prostaglandin H₂ (50 ng), the mixture was incubated for 5 min. The incubation was immediately terminated by addition of chilled EDTA (2 mM) and the solution was centrifuged at 10,500 g for 2 min (centrifuge MPW-52, MPW Med. Instruments). The thromboxane B₂ levels in the supernatant were measured using a thromboxane B₂ EIA kit according to the instructions of the manufacturer.

Antagonism at the thromboxane A₂ receptors

Antagonism at the thromboxane A₂ receptors was performed by turbidimetry using a Chrono-log 500-CA aggregometer connected to a computer (Aggro/Link software, Chrono-Log Co) according to a previously reported method [31]. The turbidities of PRP and platelet poor plasma were measured as the controls. In brief, PRP (500 µl, 2.5 × 10⁸ per mL) was pipetted into a siliconized tube and platelet aggregation was induced by the addition of U-46619, a stable agonist of thromboxane A₂ receptors. The aggregation was monitored for 5 min.

Statistical analysis

The differences between compounds were assessed by one-way ANOVA followed by Tukey multiple comparison test. The differences between concentration-effect curves were analyzed by use of 95% confidence intervals. The percent inhibition of aggregation at a concentration of 100 µM (Y100µM) was calculated according to the following formula:

\[
Y_{100\mu M} = \frac{\max + (100 - \max)}{1 + 10^{(C_{50} - \log_{10}(100)) \cdot \text{slope}}
\]

where max is the maximal inhibition of platelet aggregation expressed as per cent of platelet aggregation, slope is the slope of the curve, and C₅₀ has the common meaning.

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Conflict of Interest

Authors declare no conflict of interest.

References

24. Conquer JA, Maiani C, Azinu E, Raguzzini A, Holub BJ. Supplementation with quercetin markedly increases plasma quercetin concentration...
27 Guerrero JA, Navarro-Nunez I, Lozano MI, Martinez C, Vicente V, Gibbins JM, Rivera J. Flavonoids inhibit the platelet TxA(2) signalling pathway and antagonize TxA(2) receptors (TP) in platelets and smooth muscle cells. Br J Clin Pharmacol 2007; 64: 133–144
28 Lindahl M, Tagesson C. Flavonoids as phospholipase A2 inhibitors: importance of their structure for selective inhibition of group II phospholipase A2. Inflammation 1997; 21: 347–356
38 Williamson G. The use of flavonoid aglycones in *in vitro* systems to test biological activities: based on bioavailability data, is this a valid approach? Phytochem Rev 2002; 1: 215–222