

# *In Vitro* Effect of Soy Isoflavone and Equol on Soluble CD40L Release Stimulated by Ristocetin in Platelets from Postmenopause Women

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## Abstract

The inhibition of specific flavonoid on the *in vitro* platelet aggregation induced by collagen, arachidonic acid and thromboxane A<sub>2</sub> (TxA<sub>2</sub>) agonist, seems to be related mostly to their ability to compete for binding to the TxA<sub>2</sub> receptor (TP). The aim of this study was to analyze the effect of soy isoflavone and equol in terms of inhibiting the platelet aggregation and sCD40L release stimulated by ristocetin, an *in vitro*-activator of glycoprotein Ib/IX/V, in platelets from postmenopausal women. When platelets were stimulated by 0.75 mg/ml ristocetin, equol (10 μM) exhibited a greater inhibitory activity on platelet aggregation (~68%) than genistein or daidzein. The effect of equol was dependent on the concentration of platelet aggregation agonist. In the presence of ristocetin (0.75 mg/ml, 1.125 mg/ml and 1.5 mg/ml), the inhibitory effect of 10 μM equol was 68% ± 5%, 54% ± 4% and 31% ± 5%, respectively. Equol (10 μM) was a potent inhibitor (~35%) of sCD40L release when stimulated with 1.5 mg/ml ristocetin. However, no significant differences were noted in platelets incubated in the presence of genistein or daidzein and stimulated by ristocetin. On the other hand, SQ29548, a high TP antagonist, also inhibited the sCD40L release stimulated by ristocetin. Finally, 10 μM of genistein, daidzein or equol did not significantly affect the thromboxane B<sub>2</sub> production when platelets were incubated with 1.5 mg/ml ristocetin. The relevance of this study was to find that equol exhibits a potent activity by inhibiting ristocetin-induced sCD40L release, suggesting that soy isoflavone has important biological effects on the hemostatic system. However, clinical trials will be necessary to assess the effect of equol on platelet and their impact on inflammatory markers.

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## Keywords

**Platelet, sCD40L, Phyoestrogen, Isoflavone, Equol**

## 1. Introduction

The bean of soy produces isoflavones, principally, genistein, daidzein and glycitein. Also, daidzein is metabolized by intestinal microbiota and transformed into equol. Isoflavones have been suggested as the principal chemical constituents responsible for the potential preventive effect of soy bean against risk factor of hormone-related diseases and cardiovascular disease (CVD) [1] [2]. Different mechanisms have been proposed for the activity of isoflavones against risk factor of CVD, including estrogen-like effects, prevention of oxidative LDL and anti-platelets aggregation [3] [4].

Platelets initially interact with the sub endothelium via adhesive receptors such as glycoprotein Ib/IX/V receptors, which mediate rolling and tethering of the platelets to von Willebrand factor on the sites of vascular injury and induce glycoprotein IIb/IIIa activation, resulting in platelet aggregation [5]. Platelet adhesion and aggregation at the site of plaque rupture is a crucial event in thrombus formation and in the development of acute coronary syndrome [6]. Thus, platelets play a key role in the process of athero-thrombosis via release of inflammatory and pro-thrombotic molecules such as reactive oxidant species, P-selectin and soluble CD40 ligand (sCD40L) [7]. The interaction of von Willebrand factor-glycoprotein Ib/IX/V is known to be induced by ristocetin, an *in vitro* activator of glycoprotein Ib/IX/V [8]. Recently, Enomoto *et al.* demonstrated that ristocetin stimulate the release of sCD40L from human platelets through TXA<sub>2</sub>-mediated activation of TP [9]. On the other side, we and other groups have shown that soy isoflavone compete for binding to TP in human platelets [10] [11].

In the present study, we analyzed the effect of genistein, daidzein and equol in the action of ristocetin release of sCD40L from platelets of postmenopausal women. In addition, the effect of isoflavone and equol on ristocetin-stimulated platelets aggregation was examined to provide the antiplatelet effect.

## 2. Materials and Methods

The study protocol was reviewed and approved by the Institutional Review Board for Research on Human Subjects of the Institute of Nutrition and Food Technology, University of Chile.

### 2.1. Subjects

Twenty-five women, aged 40 - 60 years, were recruited from Santiago Metropolitan Area, and gave informed consent according to the Declaration of Helsinki. To be eligible for this study, women had to be in menopause at least 6 months, have FSH levels over 20 IU/L, without any type of hormonal treatment during previous 6 months, and not currently using soybean-derived products, or herbal supplement diets. Exclusion criteria included: cigarettes smoking within the last 5 years, diabetes, heavy alcohol consumption (more 30 g/day), hypertension and coexistent major illnesses.

### 2.2. Materials

Genistein, daizein and ristocetin were purchased from Sigma-Aldrich Co. (St. Louis, MA, USA). Equol was obtained from Cayman Chemical, Co. (Ann Arbor, MI, USA). The other materials and chemicals were obtained from commercial sources.

### 2.3. Preparation of Platelets and Aggregation Assay

Human platelets were prepared as previously described [12]. In brief, blood from donors was collected by venipuncture into sodium citrate (9:1) and centrifuge at  $200 \times g$  for 15 min at 25°C to prepare platelets-rich plasma (PRP). Platelets-poor plasma (PPP) was prepared from blood by centrifugation at 2500 g for 5 min.

A turbidimetric method was applied to measure platelet aggregation, using a Lumi-aggregometer (Chrono-log

Corp, Havertown, PA, USA). Platelet suspensions (0.25 ml) were preincubated with various concentrations of genistein, daidzein, equol or an isovolumetric solvent control (0.5% DMSO) for 15 min before the addition of ristocetin. The reaction was allowed to proceed for 6 min, and the extent of aggregation was expressed in light-transmission units. The percentage of transmittance of the isolated platelets was recorded as 0%, and platelet-poor plasma (blank) was recorded as 100%.

## 2.4. Release of sCD40L

The measurement of sCD40L was performed as described by Enomoto *et al.* [8]. In brief, PRP ( $3.0 \times 10^8$  cells/ml) were preincubated with different concentration of genistein, daidzein, equol or a solvent control (0.5% DMSO) for 15 min at 37°C and then stimulated by vehicle (0.5% DMSO) or 1.5 mg/ml of ristocetin for 30 min with constant stirring of 800 rpm. Platelet incubation was ended by the addition of ice-cold 10 mM EDTA. The PRP mixture was centrifuged at  $20,000 \times g$  for 2 min at 4°C. Supernatants were removed and store at -80°C. Soluble CD40L levels were determined using a Human sCD40-Ligand Quantikine kit (R & D systems, Minneapolis, MN).

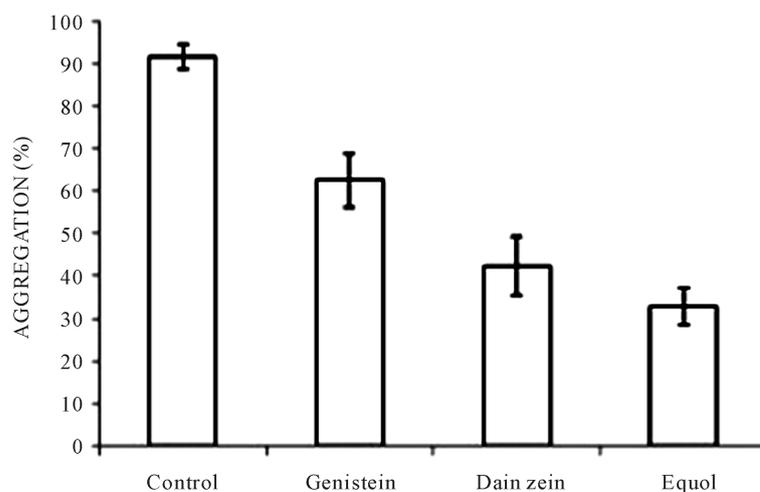
## 2.5. Statistical Analysis

Values shown in the text, tables, and figures are means  $\pm$  SD. One way ANOVA test was applied for comparisons of means, and then Student's t test was performed.  $p < 0.05$  was considered significant.

## 3. Results

### 3.1. Effect of Isoflavone on Ristocetin Induced Platelet Aggregation

In a preliminary assay, we first tested the inhibitory capacity of genistein, daidzein and equol (10  $\mu$ M) on platelets aggregation induced by 0.75 mg/ml ristocetin. As observed in **Figure 1**, the three isoflavones inhibited platelet aggregation, but equol exhibited the most potent inhibitory activity of platelet aggregation. The half maximal inhibitory concentration of equol for platelet aggregation stimulated by 0.75 mg/ml ristocetin, was approximately 8  $\mu$ M (**Figure 2**). The solvent control (DMSO, 0.5%) did not significantly affect platelet aggregation stimulated by the agonist. The inhibitory effect of equol was dependent on the concentration of platelet aggregation agonist. In presence of ristocetin (0.75 mg/ml, 1.125 mg/ml and 1.5 mg/ml), the inhibitory effect of 10  $\mu$ M equol was  $68\% \pm 5\%$  ( $p < 0.01$ ),  $54\% \pm 4\%$  ( $p < 0.02$ ) and  $31\% \pm 5\%$  ( $p < 0.03$ ) respectively.



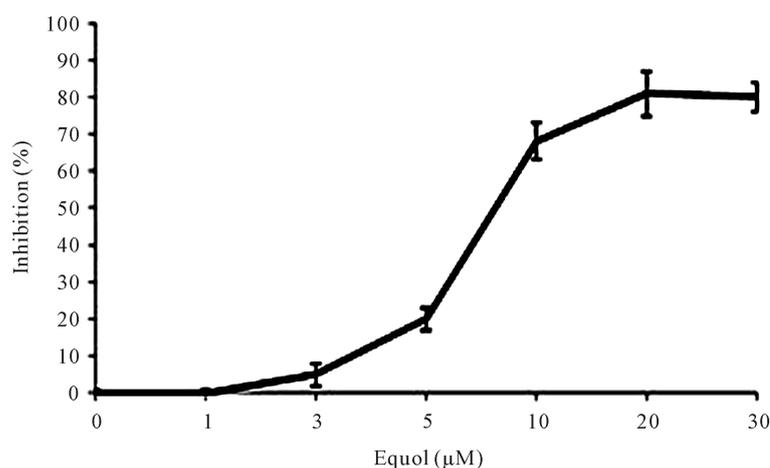
**Figure 1.** Effect of soy isoflavone and equol on platelet aggregation induced by ristocetin. Platelet ( $3 \times 10^8$  platelets/ml) from postmenopause women were preincubated (15 min, 37°C) with 10  $\mu$ M of genistein, daidzein, equol or the solvent control (0.5% DMSO) followed by the addition of ristocetin (0.75 mg/ml) to trigger platelet aggregation. Data are present as the mean  $\pm$  SD (n = 8). Mean values were significantly different from control value:  $p < 0.001$ .

### 3.2. Effect of Genistein, Daidzein, Equol and SQ29548 on Ristocetin Induced sCD40L Release from Platelets

When was analyzed the effect of 10  $\mu\text{M}$  isoflavone on sCD40L release reaction stimulated by 1.5 mg/mL of ristocetin (**Table 1**), equol exhibited a potent inhibitory activity (~35%). Ristocetin-stimulated sCD40L release from platelets was clearly inhibited by 10  $\mu\text{M}$  SQ29548 (~79%) a TP antagonist. However, no significant differences were noted in platelet incubated in the presence of genistein or daidzein and stimulated by ristocetin. Also, when platelets were incubated with a higher concentration of equol (30  $\mu\text{M}$ ) and stimulated by ristocetin (1.5 mg/mL), no significant differences were found between the inhibition observed with 10  $\mu\text{M}$  and 30  $\mu\text{M}$  equol. Preincubation of platelets with 10  $\mu\text{M}$  of genistein, daidzein or equol, did not significantly modified basal sCD40L release reaction (data not shown).

### 3.3. Effects of Isoflavone and Equol on Ristocetin-Induced TXA<sub>2</sub> Formation from Platelets

Ristocetin (1.5 mg/mL) induces a marked increase in TXB<sub>2</sub> compared to control ( $0.80 \pm 0.21$  vs.  $2.79 \pm 0.61$  ng/ml n = 12). However, 10  $\mu\text{M}$  genistein, daidzein or equol did not affect significantly TxB<sub>2</sub> stimulated by



**Figure 2.** Inhibitory effect of equol on platelet stimulated by ristocetin. Platelets ( $3 \times 10^8$  platelets/ml) were preincubated (15 min,  $37^\circ\text{C}$ ) with equol (1 - 10  $\mu\text{M}$ ) or the solvent control (0.5% DMSO) followed by the addition of ristocetin (0.75 - 1.5 mg/ml) to trigger platelets aggregation. Data are presented as the mean  $\pm$  SD (n = 6).

**Table 1.** Effect of isoflavone and SQ29548 on ristocetin induced sCD40L release from platelets ( $3 \times 10^8$  per ml). Platelets were preincubated (15 min,  $37^\circ\text{C}$ ) with 10  $\mu\text{M}$  of genistein, daidzein, equol, SQ29548 (TP antagonist) or the solvent control (0.5% DMSO) followed by the addition of 1.5 mg/ml of ristocetin for 30 min to trigger sCD40L.

Condition	sCD40L, ng/mL Mean $\pm$ SD, N= 6
Basal	$0.38 \pm 0.13$
Ristocetin	$4.86 \pm 0.63$
Ristocetin + Genistein	$4.71 \pm 0.57$
Ristocetin + Daidzein	$4.81 \pm 0.51$
Ristocetin + Equol	$3.20 \pm 0.32^*$
Ristocetin + SQ29548	$0.95 \pm 0.21^*$

\*  $p < 0.001$  compared with ristocetin (control) condition.

ristocetin;  $2.85 \pm 0.42$  ng/ml,  $2.93 \pm 0.43$  ng/ml and  $2.76 \pm 0.31$  ng/ml, respectively.

#### 4. Discussion

Besides the classic inflammatory markers, CD40L has been introduced as a new inflammatory marker. Studies on the cellular distribution of CD40L indicate that more than 95% of the circulating CD40L exist in platelets. Platelets express CD40L on their surface upon stimulation, and CD40L is then cleaved and circulates as sCD40L. When expressed on the surface of platelets and exposed to CD40-bearing vascular cells, platelet-associated CD40L is capable of initiating various inflammatory responses [7] [13]. Increasing evidence shows that CD40-CD40L interaction plays a crucial role in the pathogenesis of atherosclerosis and CVD [14]. Moreover, there is evidence that the sCD40L level is a strong predictor of cardiovascular risk [15].

The main interest of the present study was to investigate the effect of soy isoflavones and equol in terms of inhibiting the aggregation and sCD40L release stimulated by ristocetin on platelets from postmenopausal women. The results of these studies confirm that GPIb/IX/V activity induces sCD40L release from human platelets [9]. Additionally, our data demonstrated that equol exhibited a more potent activity at inhibiting ristocetin-induced platelet aggregation and sCD40L release than genistein and daidzein.

Under normal physiological conditions, platelets play a primary role in hemostasis through their aggregation. However, alteration of platelets activity regulation is an important factor in CVD and thrombosis. Evidence of platelets involvement in these pathologies includes increased TxA<sub>2</sub> production and increased TP density [16] [17]. Interestingly, dietary flavonoids have a potential to affect platelet aggregation and function, although specific mechanisms by which this activation occurs has not been fully established [18].

Several studies, including ours, have demonstrated that inhibition of specific flavonoid of *in vitro* platelet aggregation induced by collagen, arachidonic acid and thromboxane A<sub>2</sub> agonist, seems to be related, to a great extent, to their ability to compete for binding to the TP [10] [11] [19]. Moreover, we have demonstrated that equol is more active than genistein and daidzein to compete for binding to TP [10]. Interestingly, Enomoto *et al.* [9] showed that ristocetin induces TxA<sub>2</sub> production, which leads to stimulation of the release of sCD40L from human platelets. Therefore, when studied the effect of soy isoflavone and equol on ristocetin-induced sCD40 ligand release via TxA<sub>2</sub>, the ristocetin-stimulated sCD40 ligand release from postmenopause women platelets was significantly inhibited when the platelets were pretreated with equol, but not with genistein or daidzein. Moreover, SQ29548, a highly TP antagonist, also inhibited the sCD40L release stimulated by ristocetin. On the other hand, equol did not affect TxB<sub>2</sub> stimulated by ristocetin. Therefore, it is probable that equol inhibits sCD40L release through the competition for the binding to TP.

Soy-derived isoflavone have received wide attention because of its potential beneficial effects on chronic diseases, such as CVD [20] [21]. However, the results of the *in vitro* studies of these compounds and their relevance on human health can be controversial. The concentrations of soy isoflavones and equol necessary to elicit inhibitory *in vitro* activities observed in the present study (10 - 30  $\mu$ M) may reflect a pharmacological rather than physiological action. Clinical studies showed, after soy supplementation, that plasma genistein, daidzein and equol levels can reach 0.6  $\mu$ M, 0.5  $\mu$ M and 0.2  $\mu$ M, respectively [22]. However, isoflavone and equol may accumulate in tissues, reaching concentrations much higher than those found in serum [23]. So, the potential health effect of soy and its isoflavones cannot be completely understood until their bioactivity has been fully established [24].

#### 5. Conclusion

The present study shows new experimental evidence to the biological plausibility of the epidemiological finding that soy-isoflavones decreases CVD morbidity and mortality. The present finding, although preliminary, show that equol exhibits a potent activity by inhibiting ristocetin-induced sCD40L release, suggesting that soy isoflavone have biologically significant effects on the hemostatic system. However, clinical trials will be necessary to assess the effect of equol on platelet and their impact on inflammatory markers.

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