trans-Resveratrol inhibits calcium influx in thrombin-stimulated human platelets

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1 The phytoestrogenic compound *trans*-resveratrol (*trans*-3,5,4′-trihydroxystilbene) is found in appreciable quantities in grape skins and wine. It has been shown that both products rich in *trans*-resveratrol and pure *trans*-resveratrol inhibit platelet aggregation both *in vivo* and *in vitro*. However the mechanism of this action still remains unknown.

2 An essential component of the aggregation process in platelets is an increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$). Ca^{2+} must enter the cell from the external media through specific and tightly regulated Ca^{2+} channels in the plasma membrane. The objective of this study was to characterize what effect *trans*-resveratrol had on the Ca^{2+} channels in thrombin stimulated platelets.

3 In this study we showed that *trans*-resveratrol immediately inhibited Ca^{2+} influx in thrombin-stimulated platelets with an IC_{50} of 0.5 μM. *trans*-Resveratrol at 0.1, 1.0 and 10.0 μM produced 20 ± 6, 37 ± 6 and $57 \pm 4\%$ inhibition respectively of the effect of thrombin (0.01 u ml^{-1}) to increase $[Ca^{2+}]_i$.

4 *trans*-Resveratrol also inhibited spontaneous Ba^{2+} entry into Fura-2 loaded platelets, with 0.1, 1.0 and 10.0 μM *trans*-resveratrol producing 10 ± 5, 30 ± 5 and $50 \pm 7\%$ inhibition respectively. This indicated that *trans*-resveratrol directly inhibited Ca^{2+} channel activity in the platelets in the absence of agonist stimulation.

5 *trans*-Resveratrol also inhibited thapsigargin-mediated Ca^{2+} influx into platelets. This suggests that the store-operated Ca^{2+} channels are one of the possible targets of *trans*-resveratrol. These channels rely on the emptying of the internal Ca^{2+} stores to initiate influx of Ca^{2+} into the cell.

6 The phytoestrogens genistein, daidzein, apigenin and genistein-glucoside (genistin) produced inhibitory effects against thrombin similar to those seen with *trans*-resveratrol.

7 We conclude that *trans*-resveratrol is an inhibitor of store-operated Ca^{2+} channels in human platelets. This accounts for the ability of *trans*-resveratrol to inhibit platelet aggregation induced by thrombin.

Keywords: *trans*-Resveratrol; platelets; aggregation; calcium; barium; channels; thapsigargin; phytoestrogen; thrombosis; thrombin

Abbreviations: ADP, adenosine diphosphate; $[Ca^{2+}]_i$, intracellular free calcium; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; IP_3, *myo*-inositol 1,4,5-trisphosphate; Me$_2$SO, dimethylsulphoxide; RMCE, receptor-mediated calcium entry; ROC, receptor-operated channel; SMOC, second messenger operated channel; SOCC, store operated calcium channel

Introduction

Platelets, the major hemostatic cell in blood, are formed in the bone marrow from the precursor cell megacaryocytes (Heyns, 1994). Structurally, platelets contain no nucleus and have a large number of granules (dense granules and α-granules) for the storage of biologically active compounds such as serotonin, ADP and platelet derived growth factor (White, 1994). The contents of the granules are released upon platelet activation. The intracellular dense tubular system (analogous to the endoplasmic reticulum) serves as a calcium storage site (Crawford & Scrutton, 1994).

Platelets are activated by multiple factors (agonists) produced at the site of vascular injury (Brass *et al.*, 1993; Hourani & Cusack, 1991). These factors include ADP, collagen, thromboxane A_2 and thrombin, the most potent platelet activator. Upon activation, platelets undergo shape change, express fibrinogen receptors, aggregate and release their granule contents. As a result a hemostatic plug is formed on a fibrin meshwork thus sealing the damaged blood vessel.

Excessive platelet aggregation however plays a role in the development of thrombosis and therefore it is important that this process is properly regulated for the efficient functioning of the cardiovascular system.

The major intracellular stimulus involved in platelet aggregation is an increase in free cytosolic calcium concentration ($[Ca^{2+}]_i$) (Sage & Rink, 1990). As in most non-excitable cells the increase in platelet $[Ca^{2+}]_i$ induced by various agonists involves both influx of extracellular calcium through plasma membrane calcium channels and mobilization of intracellular calcium from the dense tubular system (Berridge, 1997; Heemskerk & Sage, 1994; Sage, 1997).

The pathway(s) of the calcium entry in platelets share common properties with the calcium entry pathways found in other non-excitable cells. The mechanism of calcium release from the *myo*-inositol 1,4,5-P_3 (IP_3)-sensitive internal stores is well characterized. On the other hand, the mechanism of the calcium influx pathway in platelets is still not completely resolved (Sage, 1997). Calcium from the external medium enters the cell through specific ion channels of several different types. Platelets, according to Sage (1997), lack voltage-gated

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calcium channels, so the change in the membrane potential does not generate calcium influx, and platelets are reported not to bind verapamil and nifedipine. Instead, receptor-mediated calcium entry (RMCE) through the plasma membrane channel following binding of a ligand to its membrane receptor seems to be responsible for calcium influx into platelets. RMCE channels may be one of the following types: ROC (receptor-operated channel), SMOC (second messenger operated channel) and SOCC (store-operating calcium channel) (Sage, 1997). SOCC channels are activated to promote calcium influx upon the emptying of the internal calcium stores (dense tubular system). The mechanism which links the depletion of the IP_3-sensitive internal calcium stores to the SOCC have not yet been elucidated (Parekh & Penner, 1997; Clementi & Meldolesi, 1996). There are currently two models proposed for activating SOCC: (1) the two kinds of channels (plasma membrane calcium channel and the intracellular endoplasmic reticulum IP_3 sensitive calcium channel) are coupled to each other by physical proximity or (2) the emptying of the internal stores generates a calcium influx factor (CIF), which then acts on the plasma membrane calcium channels. The specific nature of CIF is poorly defined, however it may involve arachidonate metabolites and S-nitrosylation (e.g. Rzigalinski *et al.*, 1996; 1999; Favre *et al.*, 1998).

A very important tool used to study SOCC is a plant derived tumor promoter known as thapsigargin (Thastrup *et al.*, 1990). Thapsigargin effectively inhibits the calcium ATPase pump of the endoplasmic reticulum without increasing the level of IP_3. When the pump is inhibited, calcium is prevented from entering the intracellular stores. Stored calcium leaks into the cytoplasm, some of which is extruded from the cell by the plasma membrane Ca^{2+}-ATPase pump. Consequently, store emptying evokes calcium influx through SOCC in the plasma membrane (Parekh & Penner, 1997).

It has been reported numerous times that consumption of certain foods, grapes and red wine, serve as a protective factor against coronary heart disease (CHD). The 'French paradox' correlates the lower prevalence of CHD with the regular moderate consumption of red wine (Soleas *et al.*, 1997). The beneficial effects of this product have been attributed to their high antioxidant/phytoestrogenic content (e.g. Miksicek, 1995; Williams & Rutledge, 1998).

Phytoestrogens are naturally occurring antioxidant compounds, usually polyphenols, which are present in the variety of foods and beverages. Phytoestrogens derived their name for their ability to mimic the actions of oestrogens (female sex hormones). They can bind to the human oestrogen receptor and activate genomic oestrogen-response elements, although at a much higher concentration than the natural oestrogens (Miksicek, 1995; Gehm *et al.*, 1997). *trans*-Resveratrol (*trans*-3,5,4′-trihydroxystilbene) (Figure 1) is a phytoestrogenic compound found in grapes (Miksicek, 1995), it possesses antitumor, antioxidative and antifungal activities (Jang *et al.*, 1997; Tham *et al* 1998).

trans-Resveratrol inhibits human platelet aggregation both *in vitro* and *in vivo* (Pace-Asciak *et al.*, 1995; 1996; Bertelli *et al.*, 1995). This has important implications in ascribing how the intake of phytoestrogens in food can help prevent thrombosis, essentially serving as a blood thinner, having potentially beneficial effects on the cardiovascular system. However, the mechanism by which *trans*-resveratrol inhibits platelet aggregation has not been elucidated. Since aggregation can not occur without a sufficient increase in the $[Ca^{2+}]_i$, the goal of the present study was to establish if dietary phytoestrogens, such as *trans*-resveratrol, could have an effect on the calcium channels in stimulated human platelets.

Phytoestrogenic isoflavonoids genistein and daidzein and the flavonoid apigenin (Figure 1) are also encountered in foods such as soy beans and were also examined briefly in this study. Genistein is also known as a potent tyrosine kinase inhibitor (Jayatilake *et al.*, 1993), and is also known to inhibit certain tumor cell growth (e.g. Fioravanti *et al.*, 1998). Previous studies have shown that 100 μM genistein but not daidzein was able to inhibit ADP and thrombin induced calcium influx in human platelets (Sargeant *et al.*, 1993a,b), it was concluded that tyrosine phosphorylation controlled calcium entry. Our findings are more consistent with genistein and daidzein having a more direct effect on calcium channels.

Methods

Reagents and material sources

The following were from Sigma Chemical Co: *trans*-resveratrol, apigenin (4′,5,7-trihydroxyflavone), genistein (4′,5,7-trihydroxyisoflavone), genistein-7-O-D-glucoside (genistin), daidzein (4′,7-dihydroxyisoflavone), thrombin, Me₂SO (dimethylsulphoxide) and EGTA [ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid]. The following were from Calbiochem: thapsigargin and ionomycin. Fura-2 A/M was from Molecular Probes. Other chemicals were from Fisher Scientific and Sigma Chemical Co.
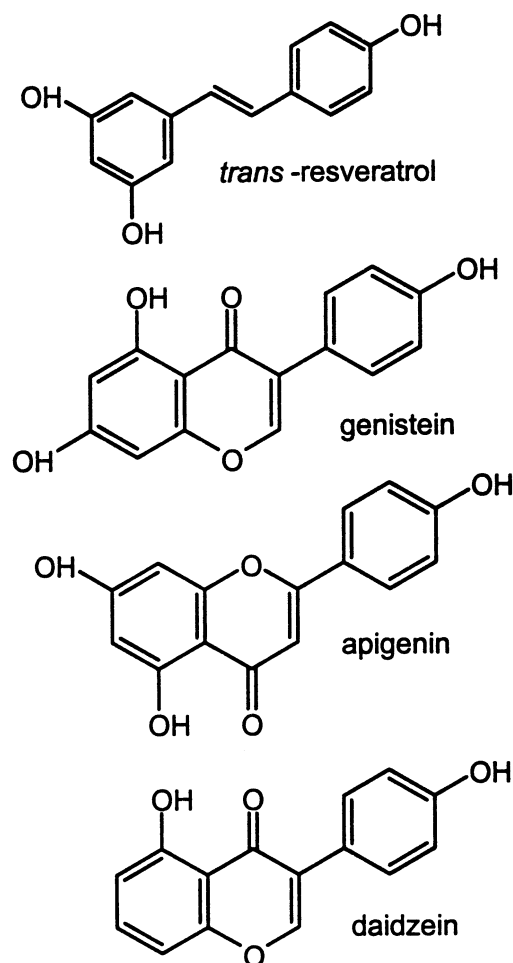

Figure 1 Structures of the phytoestrogens *trans*-resveratrol, genistein, apigenin and daidzein.

Blood donors and platelet preparation

All donors were healthy volunteers (aged 20 – 40 years) who had not consumed any medication known to affect platelet function (e.g. calcium channel blockers and aspirin) for at least 10 days prior to the study. Venous blood was collected into 1/10 volume of ACD (74.8 mM sodium citrate, 38.1 mM citric acid and 123 mM dextrose pH 6.4) (Baxter Healthcare Corp.). The blood was centrifuged at $250 \times g$ for 10 min at room temperature to obtain platelet rich plasma (PRP). The PRP was centrifuged at $550 \times g$ for 12 min to sediment the platelets. The platelets were then re-suspended in a modified Tyrodes physiological salt solution (in mM): NaCl 145, KCl 4, $MgSO_4$ 1, Na_2HPO_4 0.5, Na/HEPES 10, glucose 6, pH 7.4, containing 1.0 mM EGTA which acted to prevent spontaneous aggregation during the various experimental manipulations by binding extracellular Ca^{2+} (Nolan & Lapetina, 1990). The platelets were washed once ($500 \times g$ for 15 min) and finally re-suspended at a count of approximately 3×10^8 platelets ml^{-1}.

Phytoestrogen solution preparations

Stock solutions of the phytoestrogens (*trans*-resveratrol, genistein, daidzein, apigenin) in Me_2SO (3 mg ml^{-1}) were prepared and stored at $-20^{\circ}C$. Just before each experiment aliquots were thawed and diluted with Me_2SO up to a desirable concentration (see specific figure legends). Stock solutions of phytoestrogens were diluted 200 fold into the spectrofluorometric cuvette to give the desired final concentration.
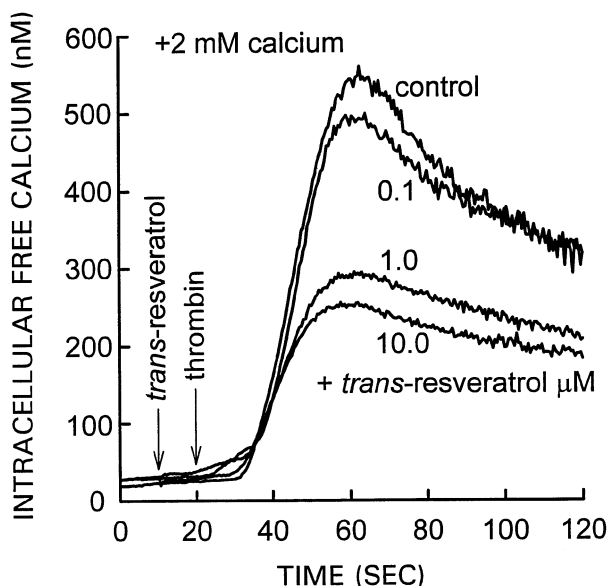
Platelet loading with fura-2 and measurement of $[Ca^{2+}]_i$

Intracellular calcium measurements $[Ca^{2+}]_i$ employed the fluorescent dye fura-2, which involved incubating the platelets with the cell permeant acetoxymethyl ester (fura-2/AM) (e.g. Sargeant *et al.*, 1992). A suspension of human platelets (isolated as described above) was incubated with 2 μM fura-2/AM for 1 h at room temperature (on a rocking platform). Excess fura-2/AM was removed by centrifugation ($500 \times g$ for 10 min) and the platelets suspended in fresh buffer, without added EGTA. Aliquots of platelet suspension (0.5 ml) were added to 1.0 ml cuvettes containing a Teflon coated stirrer bar (Chrono-log, Havertown, PA, U.S.A.). Just before $[Ca^{2+}]_i$ measurements were performed, Ca^{2+} was added back to the buffer to a final concentration of 2 mM, then phytoestrogens (various concentrations in 2.5 μl) and thrombin 0.01 u ml^{-1} were added (see individual Figure legends for specific details). The effects of *trans*-resveratrol and other phytoestrogens to inhibit the actions of thrombin (see Results) were immediate. In preliminary experiments we added *trans*-resveratrol together with thrombin and the inhibitory effect was the same as if we preincubated the *trans*-resveratrol for 5 – 10 s or 120 min before thrombin. *trans*-Resveratrol therefore produced an immediate blockade of calcium influx channel. Also when *trans*-resveratrol was added 60 s after thrombin, the $[Ca^{2+}]_i$ declined immediately to a value approaching the inhibitory effect observed when *trans*-resveratrol was added just prior to the addition of thrombin. The measurements of $[Ca^{2+}]_i$ were performed at room temperature in a SPEX
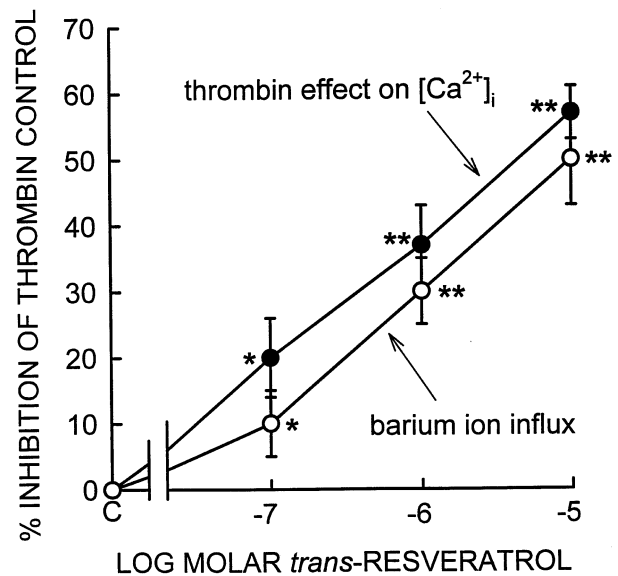

Figure 2 Effect of *trans*-resveratrol on $[Ca^{2+}]_i$ in thrombin-stimulated platelets. Calcium 2 mM was added to the platelet suspension 20 s before data collection started (zero time). Ten seconds later, *trans*-resveratrol solution was added to yield a final concentration in the platelet suspension of either 0.1, 1.0 or 10 μM. Thrombin (0.01 u ml^{-1}) was added 10 s later. Thrombin caused a large increase in $[Ca^{2+}]_i$ in the control condition, platelets started to aggregate at 60 – 80 s. All three concentrations of *trans*-resveratrol inhibited the thrombin-induced rise in $[Ca^{2+}]_i$ in a dose-dependent manner. The trace shown is from a representative experiment; similar results were obtained from six other platelet preparations and are presented in Figure 3.

Figure 3 Dose-response of *trans*-resveratrol to inhibit thrombin-induced elevation of $[Ca^{2+}]_i$ and Ba^{2+} ion influx. The % inhibitory effect of *trans*-resveratrol was calculated by subtracting the basal or pre-stimulated value from the peak effect with *trans*-resveratrol present and comparing it to the effect observed in the control with thrombin and without *trans*-resveratrol. The IC_{50} was 0.5 μM for both parameters, and was calculated by estimating the concentration of *trans*-resveratrol that produced 50% inhibition of the maximum inhibitory effect which was 57% inhibition of the $[Ca^{2+}]_i$ response, and 50% inhibition of the barium ion influx response. These values were 28.5% for $[Ca^{2+}]_i$ (i.e. 1/2 of 57%) and 25% for barium ion influx (i.e. 1/2 of 50%). The data represent mean $\pm$ s.e.mean from six separate experiments with different platelet donors, typical experiments are shown in Figures 2 and 7. Statistical significance of *trans*-resveratrol effect when compared to thrombin control: *$P < 0.05$, **$P < 0.01$.

ARCM spectrofluorometer using excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Calibration was performed as previously described for human sperm (Blackmore *et al.*, 1990). [Ca^{2+}]$_i$ was calculated by using the SPEX dM3000 software package.

Measurement of [Ba^{2+}]$_i$

To assess basal Ca^{2+} channel activity without agonist stimulation, Ba^{2+} was added to platelets in Ca^{2+}-free medium to act as a calcium surrogate. Ba^{2+} enters the cell through Ca^{2+} channels, but unlike Ca^{2+}, it cannot be extruded from the cell by plasma membrane Ca^{2+}-ATPase pump therefore it accumulates in the cell (Ozaki *et al.*, 1992; Zschauer *et al.*, 1988; Blache & Clavatti, 1987; Blache *et al.*, 1887). The removal of Ba^{2+} from the cytoplasm by the endoplasmic reticulum may be limited when compared to Ca^{2+} and Sr^{2+} (Ozaki *et al.*, 1992). When Ca^{2+} enters the cell through plasma membrane calcium channels it can be either extruded from the cell by the Ca^{2+}-ATPase pump or sequestered within organelles such as the endoplasmic reticulum and mitochondria Therefore when Ca^{2+} is added to platelets suspended in low Ca^{2+} containing medium the [Ca^{2+}]$_i$ changes very little. Once inside the cell, Ba^{2+} forms a fluorescent complex with the dye fura-2, in a similar manner to Ca^{2+}, but with a different affinity (K_D = 1360 nM for Ba^{2+} and K_D = 227 nM for Ca^{2+}, Ozaki *et al.*, 1992). The intensity of the fluorescence is directly proportional to the [Ba^{2+}]$_i$. BaCl$_2$ (5 mM) was added to the fura-2 loaded platelets in the absence of Ca^{2+} and in the absence of agonist. The fura-2/Ba^{2+} fluorescent complex was determined by measuring the 340/380 nm fluorescence ratio. The addition of thrombin with Ba^{2+} causes a large increase in 340/380 nm ratio, consistent with thrombin stimulating Ba^{2+} influx through SOCC. However it was not possible to determine how much of the fluorescence increase was due to
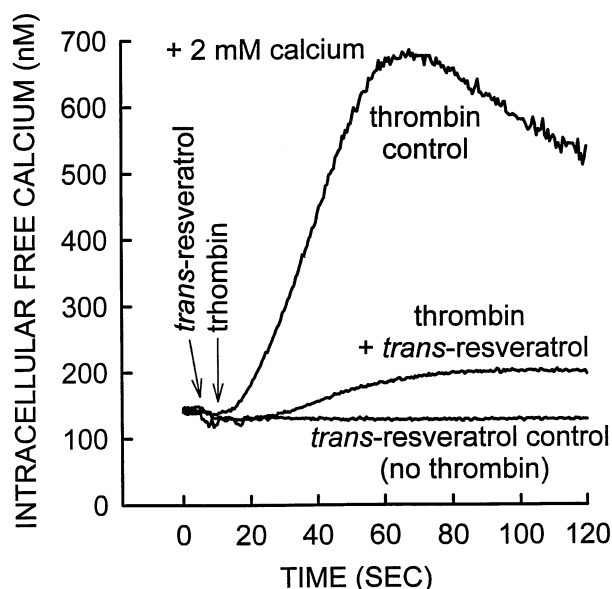
Ba^{2+} and how much was due to Ca^{2+} being mobilized from intracellular stores, although mobilization of Ca^{2+} from intracellular Ca^{2+} stores only produces a small increase in [Ca^{2+}]$_i$ (compare Figures 4 and 5).

Thapsigargin-induced [Ca^{2+}]$_i$ entry

After Ca^{2+} was added back to the platelet suspensions, thapsigargin (dissolved in Me$_2$SO) was added to the platelet suspension to a final concentration of 10^{-7} M and the fluorescence was monitored as described previously.

Statistical analysis

Data are reported as mean ± s.e.mean for the number of individual experiments specified in the Figure legends. Comparisons were made using Student's *t*-test, with a *P* value < 0.05 considered significant. Different platelet donors were used for each experiment.

Results

Effects of trans-*resveratrol on thrombin-induced [Ca^{2+}]$_i$ increase in human platelets*

Treatment of the platelet suspension with *trans*-resveratrol markedly reduced the elevation of the [Ca^{2+}]$_i$ in response to the thrombin. The data in Figure 2 shows a representative experiment in which three different concentrations of *trans*-

Figure 4 *trans*-Resveratrol does not affect [Ca^{2+}]$_i$ level in platelets in the absence of thrombin stimulation. All experiments were performed in the presence of 2.0 mM external calcium. Thrombin control experiment represents [Ca^{2+}]$_i$ elevation in platelets, stimulated with thrombin alone (0.01 u ml^{-1}). *trans*-Resveratrol 10 μM significantly inhibited thrombin response, similar to data shown in Figure 2. *trans*-Resveratrol 10 μM, by itself did not influence [Ca^{2+}]$_i$. The trace shown is from a representative experiment; similar results were obtained from six other platelet preparations.
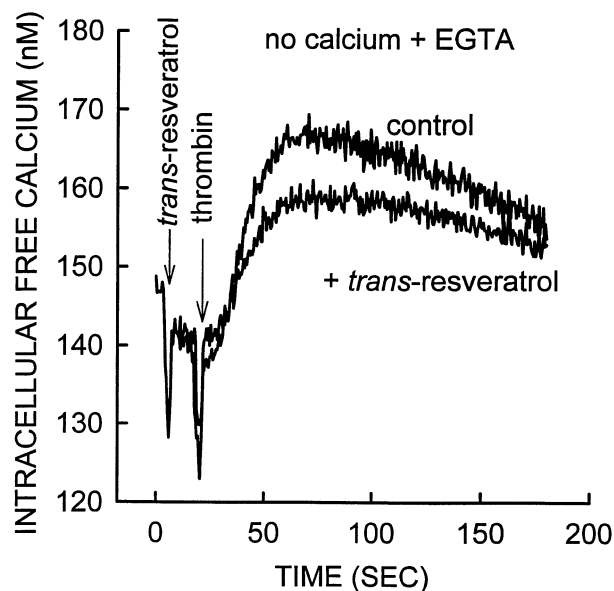

Figure 5 Effect of *trans*-resveratrol on the release of the internally stored calcium in thrombin stimulated platelets. External calcium was not added to the platelet suspension, 5 mM EGTA was also added 20 s prior to data collection (zero time). Thrombin (0.01 u ml^{-1}) caused a very small increase in [Ca^{2+}]$_i$ when calcium was removed from the external medium, compare to data in Figure 4. *trans*-Resveratrol 10 μM produced a small inhibition of thrombin to mobilize internal calcium, although the inhibitory effect was not statistically significant (see text for actual values). The data in Figure 5 (no calcium plus EGTA) is directly comparable to the data shown in Figure 4 (with 2.0 mM calcium) since the same platelet preparation was used in both experiments. The trace shown is from a representative experiment; similar results were obtained from three other platelet preparations.

resveratrol were added to platelets. The effect of *trans*-resveratrol was observed after a short 5–10 s incubation with *trans*-resveratrol before adding thrombin. It takes 5–10 s for complete mixing of the 2.5 μl aliquot of the *trans*-resveratrol solution in the 500 μl aliquot of platelet suspension in the spectrofluorometric cuvette using a magnetic stirrer bar. *trans*-Resveratrol reduced both the rate of $[Ca^{2+}]_i$ rise together with the peak level of $[Ca^{2+}]_i$ induced by thrombin. The data in Figure 3 is an average of six experiments, similar to that shown in Figure 1, and shows that *trans*-resveratrol produced a dose-dependent inhibition of the $[Ca^{2+}]_i$ increase with an IC_{50} of 0.5 μM, see Figure 3 legend for details. The data shows that 0.1, 1.0 and 10.0 μM *trans*-resveratrol produced statistically significant inhibitory effects of $20 \pm 6\%$ ($P < 0.01$), $37 \pm 6\%$ ($P < 0.001$) and $57 \pm 4\%$ ($P < 0.001$). The maximum degree of inhibition ($57 \pm 4\%$) was observed with 10 μM *trans*-resveratrol, higher concentrations of *trans*-resveratrol were not used because they caused large changes in fluorescence that interfered with the fura-2 fluorescence measurements (data not shown). Cholesterol (negative control steroid) at a concentration of 10 μM, and the Me_2SO solvent (0.5% v v⁻¹) used to dissolve *trans*-resveratrol did not influence $[Ca^{2+}]_i$ levels (data are not shown). *trans*-Resveratrol did not interfere with the calcium ionophore ionomycin (10.0 μM) or digitonin (0.01%) induced $[Ca^{2+}]_i$ elevations in human platelets, thereby demonstrating that *trans*-resveratrol does not influence fura-2 fluorescence and therefore rules out any non-specific effects of the phytoestrogen on the $[Ca^{2+}]_i$ measurements. Both these agents increase $[Ca^{2+}]_i$ by mechanisms independent of plasma membrane calcium channels.

The data in Figure 4 depicts a representative experiment that indicates *trans*-resveratrol alone did not have an effect on $[Ca^{2+}]_i$ level in the absence of thrombin stimulation. In the same experiment *trans*-resveratrol blocked the thrombin effect similar to that shown in Figure 2, although in this particular experiment the inhibitory effect was larger. The elevation of
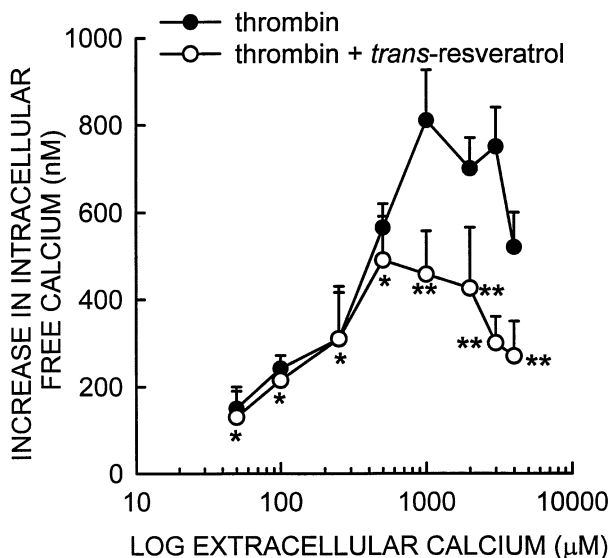
$[Ca^{2+}]_i$ in thrombin stimulated platelets, without the addition of Ca^{2+} to the medium together with 5.0 mM EGTA added to the buffer, was substantially smaller (25 nM increase, Figure 5), compared with $[Ca^{2+}]_i$ elevation in the presence of the external Ca^{2+} (500 nM increase, Figure 4). The elevation of $[Ca^{2+}]_i$ induced by thrombin in EGTA containing media was $7 \pm 2\%$ of that seen in the presence of 2.0 mM calcium (average from three separate experiments). This suggested that the majority of the $[Ca^{2+}]_i$ increase, due to thrombin (0.01 u ml⁻¹) stimulation, was primarily due to Ca^{2+} influx, and not the result of the emptying of the internal Ca^{2+} stores. The data in Figure 5 showed that 10 μM *trans*-resveratrol produced a very small inhibition of the thrombin-induced internal mobilization of calcium. The average of four separate experiments showed that 10.0, 1.0 and 0.1 μM *trans*-resveratrol produced an 8 ± 10, 2 ± 11 and $12 \pm 11\%$ inhibition respectively of thrombin, these small inhibitory effects were not statistically significant ($P > 0.05$).

Extracellular calcium in the concentration range of 50–4000 μM did not overcome the *trans*-resveratrol inhibition of Ca^{2+} influx into the thrombin-stimulated platelets (Figure 6). The effect of *trans*-resveratrol to inhibit thrombin-induced increases in $[Ca^{2+}]_i$ was very small (not statistically significant, $P > 0.05$) when the extracellular calcium was in the 50–250 μM range. This is consistent with the data shown in Figure 5 in which *trans*-resveratrol produced a small but not statistically significant inhibition (see above) of thrombin in the presence of extracellular EGTA and no added calcium. When the extracellular calcium was increased to 500–4000 μM, thrombin was then able to promote a much larger increase in $[Ca^{2+}]_i$ due to a greater calcium influx and the effect of *trans*-

Figure 6 Dose response of extracellular calcium on the ability of thrombin to increase $[Ca^{2+}]_i$ in the presence and absence of *trans*-resveratrol. Various concentrations of calcium were added to the medium 20 s before data collection was started (zero time). Thrombin 0.01 u ml⁻¹ was then added to the platelets in the presence and absence of 10 μM *trans*-resveratrol as described in the legend to Figure 2. The data shown is the mean $\pm$ s.e.mean from three separate experiments.*P > 0.05, **P < 0.05.
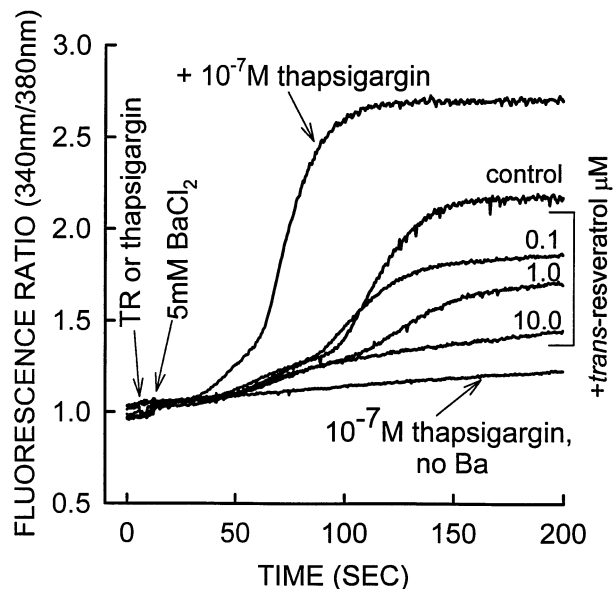

Figure 7 Effect of thapsigargin and *trans*-resveratrol on Ba^{2+} ion influx into platelets in calcium-free medium. Control represents the experiment where 5.0 mM Ba^{2+} was added alone to the platelet suspension. *trans*-Resveratrol concentrations 10, 1.0 and 0.1 μM inhibited basal Ba^{2+} influx into platelets. Results are expressed as 340/380 fluorescence ratios. Thapsigargin 10^{-7} M stimulated Ba^{2+} ion influx over control levels. When no calcium or Ba^{2+} was added, thapsigargin 10^{-7} M produced a small elevation in $[Ca^{2+}]_i$ as indicated by a small increase in the 340/380 nm ratio. The trace shown is from a representative experiment, similar results were obtained from six other platelet preparations. The dose response of *trans*-resveratrol to inhibit Ba^{2+} influx into platelets is shown in Figure 3.

resveratrol was much more pronounced (Figures 4 and 6). The degree of inhibition induced by *trans*-resveratrol remained constant as the Ca^{2+} concentration increased in the 1000–4000 μM range (Figure 6).

Effect of trans-*resveratrol on the basal Ca^{2+} channel activity measured by monitoring Ba^{2+} uptake*

Non-stimulated (control) platelets exhibit a biphasic (an early and a late component) rise in Ba^{2+} possibly due to at least two different populations of Ca^{2+} channels, which are accessible for Ba^{2+} entry (Figure 7). In most of the experiments the influx of Ba^{2+} began to induce platelet aggregation after 2 min. The rational for examining basal Ba^{2+} influx in the absence of thrombin was that the influence of various second messenger pathways (e.g. tyrosine phosphorylation, Ca^{2+} store depletion, cyclic nucleotides) would not be involved and hence the effect of the various phytoestrogens would only be on the Ca^{2+} influx channels and not on any agonist stimulated signal transduction pathway(s). *trans*-Resveratrol produced a large inhibition of Ba^{2+} influx into the platelets, affecting mostly the later component of entry. Three different concentrations (0.1, 1.0 and 1.0 μM) of *trans*-resveratrol are shown in this representative experiment (Figure 7). The dose-response of *trans*-resveratrol to inhibit Ba^{2+} influx from six different experiments is shown in Figure 3, with 0.1, 1.0 and 10.0 μM *trans*-resveratrol producing 10 ± 5 ($P < 0.05$), 30 ± 5 ($P < 0.01$) and $50 \pm 7\%$ ($P < 0.01$) inhibition respectively. Thapsigargin stimulated Ba^{2+} influx, both the rate of influx and the magnitude of the influx (Figure 7). This result shows that at least some of the Ba^{2+} that enters the platelet is through SOCC (see also Figure 8). When thapsigargin was added to platelets

in the absence of both calcium and Ba^{2+} there was a small increase in the 340/380 nm ratio (Figure 7). This result demonstrates that thapsigargin was able to mobilize intracellular calcium stores and that these stores were relatively small in platelets. This result is consistent with the data in Figures 4 and 5 in which chelation of extracellular calcium with EGTA reduced the ability of thrombin to increase [Ca^{2+}]$_i$ by 93%. Even though thapsigargin only produced a small mobilization of intracellular calcium, it was sufficient to promote a large Ba^{2+} ion influx through SOCC (Figure 7).

Effect of trans-*resveratrol on thapsigargin-induced Ca^{2+} entry*

trans-Resveratrol markedly decreased, in a dose-dependent manner, thapsigargin-induced Ca^{2+} influx, which suggested that SOCC are one of the possible targets for *trans*-resveratrol in the platelets (Figure 8). This result is consistent with the data on Ba^{2+} influx shown in Figure 7. Thapsigargin produced a delayed response to increase [Ca^{2+}]$_i$, compared to that seen with thrombin (also see Figure 2), with [Ca^{2+}]$_i$ values reaching 1 μM, approximately 100 s after stimulation. In most of the experiments, even though the *trans*-resveratrol inhibited the rate of increase in [Ca^{2+}]$_i$ and delayed the onset of the increase in [Ca^{2+}]$_i$, the increase in [Ca^{2+}]$_i$ eventually approached that obtained in the control. We therefore expressed the inhibition data as the time to reach the half maximum [Ca^{2+}]$_i$ value (EC$_{50}$) for the thapsigargin control. The basal [Ca^{2+}]$_i$ value was subtracted from the maximum [Ca^{2+}]$_i$ value obtained in each run, this number was divided by 2, then this number was added to the basal value. The time it takes to reach this 50%
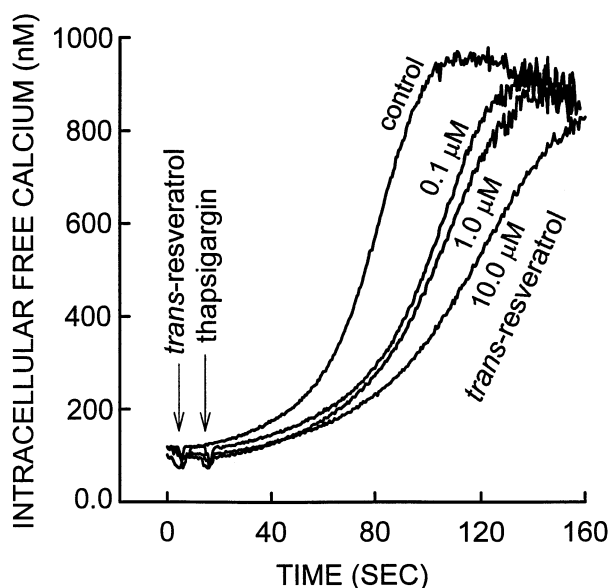

Figure 8 Effect of *trans*-resveratrol on thapsigargin-induced [Ca^{2+}]$_i$ in platelets. Experiments were conducted in the presence of 2.0 mM external calcium. The control trace represents the [Ca^{2+}]$_i$ increase in platelets stimulated with thapsigargin 10^{-7} M alone. *trans*-Resveratrol concentrations of 0.1, 1.0, and 10 μM dramatically decreased the rate of [Ca^{2+}]$_i$ increase in thapsigargin-exposed platelets. The trace shown is from a representative experiment, similar results were obtained in seven other platelet preparations. *trans*-Resveratrol, 0.1, 1.0 and 10.0 μM, increased the time to reach the EC$_{50}$ for maximum [Ca^{2+}]$_i$ elevation by 20 ± 6, 30 ± 7 and $94 \pm 10\%$ respectively.
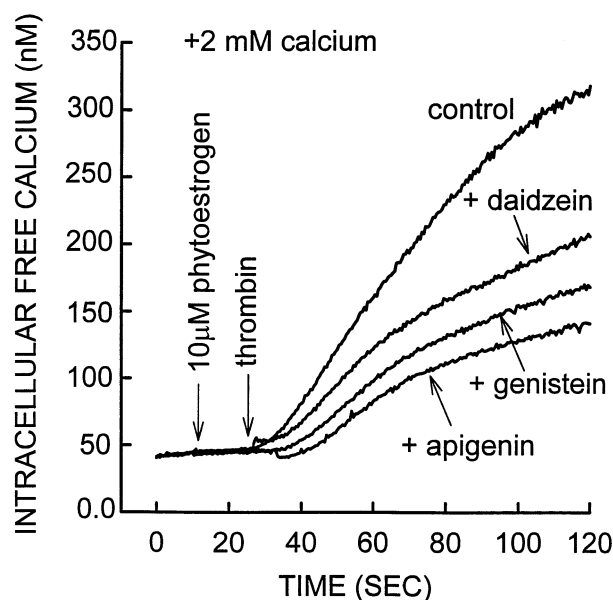

Figure 9 Effect of phytoestrogens genistein, daidzein and apigenin on [Ca^{2+}]$_i$ level in thrombin-stimulated platelets. Calcium was added to the platelet suspension to a concentration of 2.0 mM 20 s before data collection was started (zero time). Approximately 10 s later, either genistein, daidzein or apigenin was added to yield final concentrations in the platelet suspension of 10 μM. Thrombin (0.01 u ml^{-1}) was added approximately 10 s later. All three phytoestrogenic compounds inhibited the thrombin-induced rise in [Ca^{2+}]$_i$ in a similar manner to that seen with *trans*-resveratrol (Figures 2, 3 and 4). The trace shown is from a representative experiment; similar results were obtained from four other platelet preparations.

value, after thapsigargin addition with various concentrations of *trans*-resveratrol, was the time to reach the EC$_{50}$. To normalize each experiment we expressed the data as % increase in time over the control. Therefore when 0.1, 1.0 and 10.0 μM *trans*-resveratrol was added the time for thapsigargin to reach the EC$_{50}$ was 20 ± 6 ($P<0.01$), 30 ± 7 ($P<0.01$) and $94\pm10\%$ ($P<0.001$), data from seven separate experiments. This data shows that the greater the inhibition by *trans*-resveratrol the greater the % increase in time to reach the EC$_{50}$.

Effect of other phytoestrogens on the thrombin-induced $[Ca^{2+}]_i$ increase in human platelets

The phytoestrogens apigenin, daidzein, genistein, (Figure 1) as well as genistein-glycoside did not cause a change in the $[Ca^{2+}]_i$ when added to non-stimulated platelet suspension, similar to that observed with *trans*-resveratrol (Figure 4). All the above compounds caused a marked decrease in $[Ca^{2+}]_i$, in thrombin-stimulated platelets (Figure 9). Daidzein (10 μM) produced a $42\pm4\%$ inhibition of thrombin, average from four experiments, genistein (10 μM) produced a $50\pm5\%$ inhibition of thrombin, average from seven experiments and in three separate experiments 10 μM apigenin produced a $50\pm8\%$ inhibition of thrombin. The degree of the inhibition produced by each phytoestrogen was similar to the inhibition caused by 10 μM *trans*-resveratrol, which was $57\pm4\%$ (see Figures 2 and 3). The IC$_{50}$ was 1 μM for each phytoestrogen tested.

The very rapid inhibitory action of apigenin, daidzein, and genistein (immediately upon contact with platelets) suggested that these compounds produced their effects on the platelet surface. To confirm this suggestion, the addition of hydrophilic

conjugates of these compounds should produce a similar inhibitory effect, since they are unlikely to be able to traverse the hydrophobic plasma membrane and hence will be confined to the extracellular space. One such analogue was commercially available, namely genistein-7-O-D-glucoside (4',5,7-trihydroxyisoflavone-7-glucoside). The data in Figure 10 shows that genistein-7-O-D-glucoside produced an inhibitory effect similar to that of genistein, which implied that the effect was mediated by genistein-7-O-D-glucoside acting on the platelet surface and not on some intracellular target. Genistein-glucoside (10 μM) produced a $37\pm6\%$ inhibition of thrombin in three separate experiments, while genistein (10 μM) produced a $50\pm5\%$ inhibition of thrombin in seven separate experiments. The inhibitory effects of both agents were not significantly different from each other.

Discussion

The inhibitory effect of *trans*-resveratrol on platelet aggregation has been described (Pace-Asciak *et al.*, 1995; 1996; Bertelli *et al.*, 1995). However, little is known about the actual mechanism of this phenomena. The present study demonstrates for the first time that *trans*-resveratrol significantly inhibited Ca^{2+} influx into thrombin-stimulated platelets. Since a rise in the $[Ca^{2+}]_i$ is a necessary step for aggregation, we suggest that the Ca^{2+} blocking action of *trans*-resveratrol was responsible for the observed inhibition of thrombin induced platelet aggregation in platelet rich plasma (Pace-Asciak *et al.*, 1995; 1996; Bertelli *et al.*, 1995), which we have also confirmed using washed human platelets. The mode of inhibition was very rapid (several seconds preincubation), essentially at the moment of contact with the platelets. Incubating the platelets for longer periods (up to 120 min) with *trans*-resveratrol did not produce any further inhibitory effect (data not shown). Studies performed in polymorphonuclear leukocytes (PMN) demonstrated that *trans*-resveratrol was able to inhibit formyl methionyl leucyl phenylalanine (fMLP) induced elevations of $[Ca^{2+}]_i$ (Rotondo *et al.*, 1998). The PMN's do not appear to be as responsive to *trans*-resveratrol as the platelets are in our present study. The rise in $[Ca^{2+}]_i$ in the PMN's was inhibited slightly by 22 μM *trans*-resveratrol, with near maximum inhibition seen with 110 μM *trans*-resveratrol (Rotondo *et al.*, 1998). The elevation of $[Ca^{2+}]_i$ in platelets induced by thrombin was seen with as little as 0.1 μM *trans*-resveratrol, with a maximum $57\pm4\%$ inhibition seen with 10 μM *trans*-resveratrol (Figure 3). We did not use amounts of *trans*-resveratrol higher than 10 μM due to its insolubility at higher concentrations, and the quenching of the fura-2 fluorescence.

The question remains whether *trans*-resveratrol acts immediately on the platelet Ca^{2+} channels or whether inhibition of Ca^{2+} influx is a secondary phenomenon to some other unknown primary effect of *trans*-resveratrol. It is possible that *trans*-resveratrol and other phytoestrogens generate an inhibitory second messenger to block calcium influx, such as cyclic AMP or cyclic GMP (Sage, 1997). However, the finding that *trans*-resveratrol inhibited the basal activity of Ca^{2+} channels, measured by measuring Ba^{2+} ion influx in non-stimulated platelets, suggested that *trans*-resveratrol may have a binding site on the cell surface, thus directly affecting the channel itself. The first component of Ba^{2+} entry seems to remain unaffected by *trans*-resveratrol, however the second component was inhibited dramatically. An IC$_{50}$ of 0.5 μM was estimated for *trans*-resveratrol to block Ba^{2+} influx (Figure 3). The phenomena, where *trans*-resveratrol immediately inhibited Ba^{2+} influx into platelets,
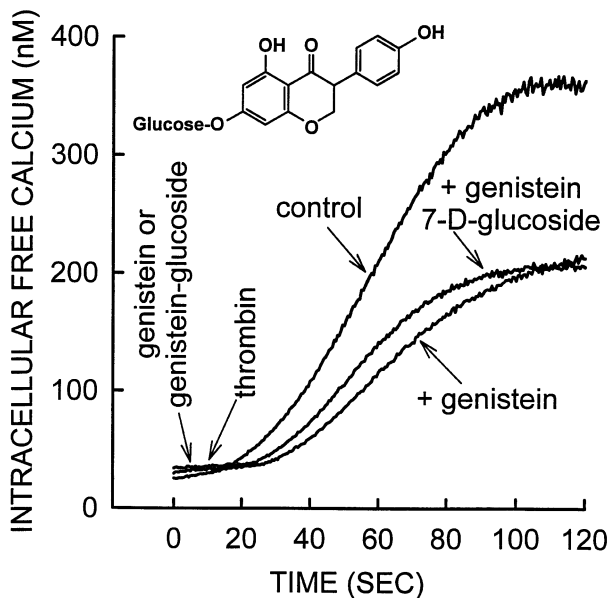

Figure 10 Genistein-7-D-glucoside inhibited calcium influx into thrombin-stimulated platelets in a manner similar to that of genistein. Calcium was added to the platelet suspension to a concentration of 2.0 mM 20 s before data collection starts (zero time). Approximately 5 s later, either genistein or genistein-7-D-glucoside was added to yield final concentration in the platelet suspension of 10 μM. Thrombin (0.01 u ml^{-1}) was added at approximately 10 s after data collection started. No phytoestrogens were added to the platelets in the thrombin control experiment. Both compounds inhibited the thrombin-induced rise in $[Ca^{2+}]_i$ in a similar manner. The trace shown is from a representative experiment; similar results were obtained from three other platelet preparations.

supports the hypothesis that ionic channels are involved. Since basal Ba^{2+} influx is independent from the generation of second messengers (no agonist used), *trans*-resveratrol probably acts on the channel directly.

The apparent target for *trans*-resveratrol action seems to be the SOCC, since thapsigargin mediated increases in $[Ca^{2+}]_i$ are also inhibited by *trans*-resveratrol (Figure 8). This is consistent with the fact that thrombin stimulated Ca^{2+} influx is primarily through the SOCC. Experiments also suggested that *trans*-resveratrol was unable to inhibit thrombin induced release of intracellular stored Ca^{2+} (Figure 5), although a very small, not statistically significant, inhibition was observed. Though it appears that *trans*-resveratrol acts on the platelet surface, there is still a possibility that a very small inhibition of internal Ca^{2+} release may translate into an inhibition of the plasma membrane Ca^{2+} channels, since the Ca^{2+} influx process through SOCC depends on the degree of filling of the internal Ca^{2+} stores (Parekh & Penner, 1997). The data in Figure 6 suggests that *trans*-resveratrol and Ca^{2+} are not competing for the same site on the calcium channel, since the *trans*-resveratrol inhibition of thrombin induced elevation of $[Ca^{2+}]_i$ could not be overcome by increasing the extracellular Ca^{2+}.

Other types of phytoestrogens (apigenin, daidzein, genistein, genistein-glucoside) showed a similar mode of action to *trans*-resveratrol on thrombin-stimulated platelets, by inhibiting Ca^{2+} influx. Genistein-glucoside, being a very hydrophilic compound, is a tool to explore indirectly if the phytoestrogens have their primary targets on the platelet surface rather than on an intracellular target. Genistein and genistein-glucoside have a very similar time course of action, inhibiting Ca^{2+} influx apparently at the moment of their contact with the platelet (Figure 10). This evidence also indirectly supports the hypothesis that phytoestrogens act upon the platelet surface, directly or indirectly changing Ca^{2+} channel activity.

The key point to resolve is whether *trans*-resveratrol and other phytoestrogens act directly on the cell surface or must penetrate the inside of the platelet. This could be clarified by a direct radiolabelled ligand binding and affinity labelling experiment to platelet membranes. It would be of interest to explore what intracellular signal transduction pathways are involved in the *trans*-resveratrol action. Examining whether *trans*-resveratrol and other phytoestrogens change the phosphorylation state of the intracellular targets would be important since several phytoestrogens have been shown to act as tyrosine kinase inhibitors, although high concentrations are required to inhibit a variety of kinases. For genistein the IC_{50} for the epidermal growth factor receptor tyrosine kinase is 2.6 μM, for $p60^{v\text{-}src}$ the $IC_{50} = 25$ μM and for protein kinase C and protein kinase A the $IC_{50} > 100$ μM (Akiyama *et al.*, 1987).

In our experiments we observed inhibition of thrombin effects by genistein and the other phytoestrogens daidzein, apigenin and *trans*-resveratrol in the $0.1 - 10$ μM concentration range (Figure 9 and data not shown). Since daidzein is used as an inactive analogue of genistein in tyrosine kinase experiments (Akiyama *et al.*, 1987), and since both daidzein and genistein produced the same degree of inhibition of thrombin (Figure 9) this would argue against these compounds producing the inhibition of Ca^{2+} influx in platelets by inhibiting a tyrosine kinase.

The levels of *trans*-resveratrol in red wine are typically in the $10 - 100$ μM ($0.4 - 4$ mg l^{-1}) range (Soleas *et al.*, 1997), with wine from Cabernet Savignon having levels as high as 7 mg l^{-1}. Also the presence of *trans*-resveratrol-glucoside (piceid) in wine may also contribute to the levels of *trans*-resveratrol in the blood. Even though much experimental data supports the biological activity of *trans*-resveratrol *in vivo*, following regular wine consumption in man (e.g. Tham *et al.*, 1998), no serum levels have been reported. Also there is no data on clearance from the blood and first-pass effects in the portal circulation of the liver (e.g. Gehm *et al.*, 1997). The data presented by Pace-Asciak *et al.* (1996), in which humans had consumed 2 mg of *trans*-resveratrol per day, showing decreased platelet activity was very convincing. This amount of *trans*-resveratrol is equivalent to consuming approximately 1 L of a typical red wine per day (Soleas *et al.*, 1997). We observed that genistein and genistein-glucoside (Figures 9 and 10) were able to inhibit thrombin induced elevations of $[Ca^{2+}]_i$ similar to that seen with *trans*-resveratrol (Figures 2 and 3). After consuming a soybean flour-based meal on two occasions, 6 days apart, the levels of genistein and daidzein reached peak plasma levels of 4 and 3 μM respectively, with elimination half-lives of 5.7 and 4.7 h, respectively (King & Bursill, 1998). These levels of genistein and daidzein were able to inhibit the ability of thrombin to elevate $[Ca^{2+}]_i$ in platelets *in vitro* (Figure 9). In another study involving 14 Japanese men, average age 55 years on an uncontrolled diet, the plasma phytoestrogen levels were approximately 0.4 μM (Adlercreutz *et al.*, 1993).

In conclusion, since phytoestrogenic compounds are present in a variety of foods (Mikscicek, 1995), their Ca^{2+}-channels blocking action may be potentially beneficial in the prevention and management of cardiovascular disease and other cardiovascular dysfunctions in which thrombosis plays a role.

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