Soy-Derived Isoflavones Exert Opposing Actions on Guinea Pig Ventricular Myocytes

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ABSTRACT

Soy-derived isoflavones appear to possess cardioprotective properties, although the precise nature of this protection and the particular isoflavones responsible remain unclear. We hypothesized that isoflavones may differ in their cardiac actions in view of their varying affinities for the estrogen receptor and differences in ability to inhibit tyrosine kinase. We investigated the direct effects of three closely related isoflavones, genistein, daidzein, and equol (a metabolite of daidzein formed by gut microflora), on the contractile function of isolated guinea pig ventricular myocytes. Genistein (10 and 40 μM) significantly increased cell shortening and the Ca\(^{2+}\) transient (measured using indo-1). In contrast, equivalent concentrations of equol produced the opposite effect, decreasing cell shortening and the Ca\(^{2+}\) transient, whereas daidzein was without effect. The opposing actions of genistein and equol were still observed in the presence of the specific estrogen receptor antagonist ICI 182,780 (10 μM). However, the stimulatory actions of genistein were markedly reduced in the presence of the potent phosphotyrosine phosphatase inhibitor, bpV(phen). Both genistein and equol significantly inhibited the peak L-type Ca\(^{2+}\) current. We conclude that genistein and equol affect the contractile function of ventricular myocytes in opposing ways despite a common initial action of Ca\(^{2+}\) current antagonism. These differences occur independently of the estrogen receptor but may be partly related to the unique actions of genistein as a tyrosine kinase inhibitor. Furthermore, isoflavone metabolites, such as equol, may be more biologically active than their precursors and have a greater role in cardioprotection than previously realized.

Epidemiological evidence suggests that dietary isoflavones, which are major constituents of soy products, may possess cardioprotective properties (Adlercreutz, 1990; Adlercreutz et al., 1993; Artaud-Wild et al., 1993). This may in part be due to favorable effects on the lipid profile, as isoflavones increase high-density lipoprotein and lower low-density lipoprotein cholesterol levels (Cassidy et al., 1995; Anthony et al., 1998). However, isoflavones have also been shown to produce direct actions on the cardiovascular system, which may contribute toward cardioprotection (Fitgreet et al., 2000; Chin-Dusting et al., 2001). The two main isoflavones found in soy products are genistein and daidzein (Tham et al., 1998), both of which undergo further metabolism by gut microflora to produce additional compounds (Chang and Nair, 1995; Chang et al., 1995). Equol, the isoflavone metabolite produced from the hydrogenation of daidzein, is of particular interest, as in vitro experiments suggest that it may be more potent than other isoflavones, including the parent compound (Chang et al., 1995; Sathyamoorthy and Wang, 1997; Morito et al., 2001). All three isoflavones are known to interact with both estrogen receptor (ER) α and β, although they have a greater affinity for the latter (Kuiper et al., 1998). Moreover, they differ with respect to their binding affinities for the ERs. For example, both genistein and equol have a greater affinity for the ER than daidzein (Sathyamoorthy and Wang, 1997; Kuiper et al., 1998). In addition, genistein differs from the other two isoflavones in its ability to inhibit tyrosine kinase (TK) (Akiyama et al., 1987; Schultz-Mosgau et al., 1998). Therefore, important differences exist between these three isoflavones despite close structural similarities (Tham et al., 1998). All three isoflavones directly relax arterial ring preparations in vitro (Fitgreet et al., 2000; Chin-Dusting et al., 2001), and genistein and daidzein have been reported to inhibit the L-type Ca\(^{2+}\) current, \(I_{Ca,L}\), in isolated cardiac myocytes (Yokoshiki et al., 1996; Ogura et al., 1999). It has been assumed that this results in an overall inhibition of cell contraction, although this has not been well explored. Major unresolved issues include whether the cardioprotective effects of isoflavones are largely attributable to a group effect or to a particular isoflavone and whether the cardiovascular actions of...
isoflavones are all beneficial. These questions are particularly relevant in view of the increasing availability of isoflavone supplements, many of which contain genistein and daidzein (Djuric et al., 2001; Lewis et al., 2002).

The aim of the present study was to investigate the hypothesis that soy-derived isoflavones exert different actions on isolated cardiac myocytes. We studied the direct effects of genistein, daidzein, and equol on cell contraction and further investigated whether any observed differences could be attributed to their varying affinities for the ER or the unique properties of genistein as a TK inhibitor. We report here the novel finding that genistein and equol produce directly opposing cardiac actions and discuss the possible mechanisms involved.

Materials and Methods

Cardiomyocyte Isolation. Left ventricular myocytes were isolated from adult male guinea pigs (weighing 350–550 g) using a Langendorff apparatus and enzymatic digestion as previously described (MacLeod and Harding, 1991). All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Myocytes were stored in Dulbecco’s modified Eagle’s medium solution at room temperature and used within 6 to 8 h of isolation.

Cell Loading. Cells were incubated at room temperature with 10 μM of the acetoxyethyl ester form of the Ca2+-sensitive fluorescent dye, indo-1 (Molecular Probes, Eugene, OR). After 25 min, the supernatant was removed and replaced with fresh Dulbecco’s modified Eagle’s medium. Cells were not used for at least another 30 min to allow the intracellular indo-1 to be de-esterified.

Effect of Isoflavones on In Vivo Calibration of Indo-1. To use indo-1 to measure the Ca2+ transient, we first established whether the isoflavones studied affected the indo-1 signal other than via changes in intracellular Ca2+. Calibration experiments were performed to determine the in vitro indo-1 dissociation constant (Kd) and in vivo minimum and maximum fluorescence ratios (Rmin and Rmax) (Bassani et al., 1993; Kao, 1994) in the presence and absence of 40 μM of each isoflavone. Genistein (Sigma Chemical, Poole, Dorset, UK) and equol (Indofine Chemical Company, Inc., Somerville, NJ) did not significantly affect the above parameters (n = 5). However, daidzein (Sigma Chemical) significantly decreased the in vitro Kd and in vivo Rmin of indo-1 as well as unpredictably decreasing the Rmax in some cells, although this was not significant (n = 5). Therefore, we were unable to reliably measure the Ca2+ transient using indo-1 in the presence of daidzein. As each cell acted as its own control, the indo-1 ratios measured in the presence of genistein and equol could be expressed as a percentage of that measured in control solution, without the need to calculate the absolute intracellular Ca2+ concentration.

Cell Shortening and Intracellular Ca2+ Transients. Cells were placed on the coverslip base of a superfusion chamber mounted on the stage of an inverted microscope. They were superfused with normal Tyrode’s (NT) solution containing 140 mM NaCl, 6 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 using NaOH delivered at a rate of 2 ml/min. Cells were field stimulated at 1 Hz by a pair of platinum electrodes placed in the bath, and cell shortening was followed at one end with a video edge-detector system. Ultraviolet light from a 100-W xenon arc lamp (Nikon, Tokyo, Japan) was used to excite the fluorescent dye in the cells. Light emitted at 405 and 485 nm was measured, allowing the indo-1 ratio (405/485 signals) to be calculated. Cell shortening and the indo-1 ratio were recorded during steady-state contractions in NT and in the presence of 10 and 40 μM isoflavone. The change in indo-1 ratio, ∆indo-1 ratio (i.e., the difference between systolic and diastolic values), was used as a measure of the Ca2+ transient.

Effect of ICI 182,780 on Isoflavone Action. The specific ER antagonist ICI 182,780 (Toznic Cookson Inc., Bristol, UK) was used to determine whether the actions of genistein and equol on cell shortening and the Ca2+ transient were mediated via the ER. A steady-state level of cell shortening was attained in NT during field stimulation before 10 μM ICI 182,780 was added for 2 min. Previous studies have demonstrated that ICI 182,780 is able to block the ER acutely within minutes (Prakash et al., 1999). The effects of 40 μM isoflavone in the continuing presence of ICI 182,780 were then determined. This was followed by a further period of superfusion in NT, allowing parameters to return to baseline values, before the effects of 40 μM isoflavone alone were measured in the same cell.

Relation of Isoflavone Action to TK Inhibition. The potent and specific phosphotyrosine phosphatase (PTP) inhibitor, bpV(phen) (Calbiochem, San Diego, CA) (Posner et al., 1994), was used to investigate whether any differences between genistein and equol actions could be attributed to differences in TK inhibition. Myocytes were superfused with 1 μM bpV(phen) for 5 min before the effects of 40 μM genistein or equol in the continuing presence of 1 μM bpV(phen) were determined. One hundred micromolar bpV(phen) has been reported to have no significant effect on ICa-L in guinea pig ventricular myocytes (Sims et al., 2000), although we found that this concentration of bpV(phen) markedly decreased cell shortening. We therefore chose to use a concentration of 1 μM bpV(phen), which has been shown to be effective in inhibiting PTP in cultured hepatoma cells (Posner et al., 1994).

Voltage-Clamp Experiments. Cells were impaled with high-resistance borosilicate glass microelectrodes and electrophysiological recordings made with an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA). ICa-L was measured in switch-clamp mode (discontinuous single-electrode voltage clamp, 5–6 kHz) and taken as the difference between the peak inward current in the presence and absence of 200 μM cadmium. Since equal tended to shift the end of pulse current in the outward direction, in these experiments we measured ICa-L by subtracting the current at the end of the pulse from the peak current in the presence and absence of the isoflavone (Ogura et al., 1999). The effect of 40 μM genistein and equol on ICa-L were compared with the control values in NT.

Only cells that were rod shaped with clear striations and good contractions were used in the studies. All solutions contained 0.1% dimethyl sulfoxide carrier, and all experiments were performed at 37°C.

Statistical Analysis. Results are expressed as mean ± S.E.M. The repeated-measures analysis of variance with Bonferroni’s post hoc test and the paired Student’s t test were used to analyze the data as appropriate. A value of p < 0.05 was considered significant.

Results

Cell Shortening and Ca2+ Transients. Genistein, 10 and 40 μM, increased baseline shortening in NT (taken as 100%) to 133 ± 7% (p < 0.05, n = 13) and 189 ± 18% (p < 0.001), respectively (Fig. 1A). In contrast, 10 and 40 μM equol markedly decreased cell shortening to 57 ± 6% (p < 0.001, n = 9) and 40 ± 5% (p < 0.001) of control values, respectively. Daidzein had no significant effect on cell shortening at equivalent concentrations (n = 11). The opposing actions of genistein and equol occurred over different timescales, as can be seen in Fig. 1B. The stimulatory action of genistein took over a minute to reach a new maximum steady-state level, whereas the inhibitory action of equol occurred very rapidly within several seconds. In some cells, it was noted that the initial washout period in NT after genistein application produced a further increase in cell contraction before a gradual return back to baseline levels (Fig. 1B). Genistein and equol similarly affected the Ca2+ transients
in opposing ways (Fig. 2). Genistein, 10 and 40 μM, increased the percentage Δ indo-1 ratio to 114 ± 2% (p < 0.001, n = 13) and 119 ± 4% (p < 0.001), respectively, whereas equivalent concentrations of equol decreased the Δ indo-1 ratio to 87 ± 2% (p < 0.05, n = 9) and 75 ± 4% (p < 0.001) of control values.

**Twitch Characteristics.** The time-to-peak (TTP) contraction and time-to-half-relaxation (TTR50) were measured from the averaged trace of eight twitches during steady-state conditions (Fig. 3). TTP decreased from 230 ± 12 ms (in NT) to 210 ± 10 ms (p < 0.05, n = 13) and 190 ± 6 ms (p < 0.001) in the presence of 10 and 40 μM genistein, respectively. Similarly, 10 and 40 μM genistein decreased TTR50 from 110 ± 9 ms to 90 ± 7 ms (p < 0.001) and 80 ± 5 ms (p < 0.001), respectively. Daidzein only affected twitch kinetics at the higher concentration; TTP was decreased from 290 ± 20 ms to 250 ± 15 ms (p < 0.001, n = 10), and TTR50 decreased from 175 ± 24 ms to 150 ± 20 ms (p < 0.05) in the presence of 40 μM daidzein. Equol did not significantly alter twitch kinetics of cell shortening (n = 9).

The time-to-peak Ca\(^{2+}\) transient and time-to-50% decay were also measured for genistein and equol. Forty micromolar genistein decreased the time-to-peak Ca\(^{2+}\) transient from 220 ± 8 ms to 190 ± 7 ms (p < 0.001, n = 12) and the
time-to-50% decay from 240 ± 12 ms to 190 ± 12 ms (p < 0.01), although 10 μM had no significant effect. Figure 4 shows sample traces of the effect of 40 μM genistein on twitch characteristics. Equol also did not significantly affect the time parameters of the Ca²⁺ transient.

**Effect of ICI 182,780 on Genistein and Equol Actions.** The stimulatory effects of genistein and inhibitory effects of equol on cell shortening and the Ca²⁺ transient were still seen in the presence of 10 μM ICI 182,780 (Fig. 5). ICI 182,780 alone had no significant effects on cell shortening or the Δ indo-1 ratio (n = 9 cells; data not shown). There was no statistical difference in the extent of genistein or equol actions in the presence and absence of ICI 182,780.

**Effect of bpV(phen) on Genistein and Equol Actions.** Genistein (40 μM) increased cell shortening to 128 ± 11% (p < 0.05, n = 17) in the presence of 1 μM bpV(phen) compared with cell shortening in bpV(phen) alone (Fig. 6A). However, in contrast to the previous effects of genistein on the Ca²⁺ transient, there was no associated increase in the Ca²⁺ transient when genistein was applied in the presence of bpV(phen) (Δ indo-1 ratio after genistein application was 91 ± 4% of control values in bpV(phen), n = 17, p = nonsignificant). Equol (40 μM) continued to significantly decrease cell shortening and the Ca²⁺ transient in the presence of bpV(phen) (n = 14).

In some cells, genistein increased cell shortening while simultaneously decreasing the Δ indo-1 ratio in the presence of bpV(phen). A sample continuous recording of cell shortening and the indo-1 ratio from the same cell is shown in Fig. 6B, in which genistein, in the presence of bpV(phen), produced clearly divergent actions on the two parameters. Superfusion of cells with bpV(phen) also altered the effects of genistein on twitch kinetics. Although 40 μM genistein continued to shorten TTP contraction in the presence of bpV(phen), TTR50 was no longer affected (Fig. 7). Similarly, bpV(phen) abolished the effects of genistein on the Ca²⁺ transient kinetics.

**Effect of Genistein and Equol on I_{Ca,L}.** Sample recordings showing the inhibition of I_{Ca,L} by genistein and equol (40 μM) are shown in Fig. 8A. Genistein and equol decreased peak I_{Ca,L} by 71 ± 7% (p < 0.001, n = 12) and 55 ± 6% (p < 0.05, n = 10), respectively (Fig. 8B). Inhibition was consistent throughout the voltage range tested and occurred rapidly within several seconds. There was no evidence of a stimulatory effect on I_{Ca,L} in any of the cells studied. The

![Fig. 3. Effect of isoflavones on cell contraction and relaxation times. A, genistein (Gen) significantly decreased the TTP contraction (closed circles) and TTR50 (open circles) at both 10 and 40 μM. B, daidzein (Daid) decreased TTP and TTR50 only at 40 μM. C, equol did not significantly affect either time. Values are mean ± S.E.M. (n = 9–13 cells from three hearts for each isoflavone; *, p < 0.05, ***, p < 0.001).](image)

![Fig. 4. Sample traces showing effect of 40 μM genistein on cell shortening (A) and Ca²⁺ transient (B) morphologies. The traces are the average of eight twitches during steady state and have been normalized to the same amplitude to facilitate comparison. Genistein (Gen) decreased the time taken to reach peak cell contraction and the peak Ca²⁺ transient compared with control times in normal Tyrode, NT (lighter traces). Genistein also decreased the time-to-half-relaxation of cell shortening and the time-to-50% decay of the Ca²⁺ transient.](image)
isoflavones did not appear to decrease the reversal potential of $I_{Ca,L}$.

**Discussion**

We have reported here the novel finding that soy-derived isoflavones exert directly opposing actions on ventricular myocytes, suggesting that they possess distinctly different mechanisms of actions. Consequently, the cardiovascular protective effects attributed to isoflavones may not be due to a group effect but rather may be exerted via specific and dominating actions of individual isoflavones. Furthermore, we have shown that the isoflavone metabolite, equol, is biologically active and exerts greater cardiac effects than those of its precursor, daidzein.

We have demonstrated here that genistein and equol both significantly inhibit $I_{Ca,L}$. It therefore seems likely that this is a common mechanism of action shared by the isoflavones, particularly in view of their close structural similarities (Murkies et al., 1998; Tham et al., 1998). The equol-induced inhibition of $I_{Ca,L}$ resulted in an expected decrease in the $Ca^{2+}$ transient and inhibition of cell contraction. However, this was not the case with genistein, and, in fact, the opposite effect was observed. This implies that genistein exerts additional cellular actions distinct from its effect on $I_{Ca,L}$. This is supported by the finding that genistein inhibited $I_{Ca,L}$ within several seconds but took over a minute to produce maximal levels of cell stimulation and the observation of a temporary increase in cell contraction upon washout of genistein in NT before a return to baseline levels. Our results suggest that genistein produces at least two competing actions on cardiac myocytes, an initial inhibitory component mediated via $I_{Ca,L}$ (which has a rapid onset and removal) and a subsequently slower, stimulatory one. The latter action appears to dominate and mask the inhibitory component, since the overall effect in most cells was that of stimulation. However, in a minority of cells (less than 5%), genistein completely inhibited contraction. This was probably due to the complete block of $I_{Ca,L}$ by genistein in these cells, preventing the initial stimulus to excitation-contraction coupling and therefore preventing the manifestation of any subsequent stimulatory effect.

**Fig. 5.** Effect of ICI 182,780 on genistein (A) and equol (B) actions. The stimulatory actions of 40 μM genistein (Gen) on cell shortening and the $Ca^{2+}$ transient were still seen in the presence of 10 μM ICI 182,780 (ICI) (n = 15 cells from three hearts). Similarly, the inhibitory actions of 40 μM equol (Eq) were still seen in the presence of ICI 182,780 (n = 12 cells from three hearts). Results are expressed as a percentage (mean ± S.E.M.) relative to initial baseline values in normal Tyrode, NT1. NT2 represents steady-state levels after washout of Gen/ICI or Eq/ICI in normal Tyrode. There was no statistical difference between the extent of genistein and equol actions in the presence and absence of ICI 182,780 (**, p < 0.01, ***, p < 0.001 relative to NT1).
Although genistein increased both cell shortening and the Ca\(^{2+}\) transient, the effect on cell shortening was disproportionately greater. Moreover, in some cells, where both parameters were simultaneously measured, genistein markedly increased cell shortening without affecting the indo-1 ratio. In addition, 10 \(\mu\)M genistein significantly decreased the time-to-peak contraction and time-to-half-relaxation without affecting the corresponding times for the Ca\(^{2+}\) transient (40 \(\mu\)M genistein decreased both times for cell shortening and the Ca\(^{2+}\) transient). These observations suggest that genistein may increase myofilament Ca\(^{2+}\) sensitivity in addition to increasing the Ca\(^{2+}\) transient.

Our findings of a lack of effect of daidzein on cell shortening initially appear to be at odds with previous studies show-
Cardiac myocytes have been shown to express functional ERs (Grohe et al., 1997). It has been suggested that differences in isoflavone action may be attributed to their differing affinities for the two ERs (Cassidy, 1999). However, our study demonstrates that the acute cardiac actions of genistein and equol are unlikely to be mediated through the ER, as the effects were still observed in the presence of the specific ER antagonist ICI 182,780. Although ICI 182,780 was originally developed as a pure antagonist to the classical ER, ERα (Wakeling, 1995), there is evidence that it can also block some ERβ-mediated effects (Sun et al., 1999; Hodges et al., 2000). Despite this, it is still possible that the isoflavones are acting through an as yet undetermined membrane-associated ERβ that is not completely blocked by ICI 182,780. However, there is no evidence from the present study that the functional differences observed between the isoflavones on cardiac myocytes are related to differences in ER affinity.

We further investigated whether the differences in cardiac actions of genistein and equol could be explained by the unique properties of genistein as a TK inhibitor. Although genistein continued to stimulate cell contraction in the presence of the potent PTP inhibitor, bpV(phen), the increase was less pronounced compared with the increase in cell contraction observed with genistein alone (cell shortening increased to $128 \pm 11$ and $189 \pm 18\%$ of control values, respectively). Moreover, bpV(phen) completely abolished the genistein-induced increase in the Ca$^{2+}$ transient. These findings suggest that the stimulatory actions of genistein on cell shortening occur via at least two distinct mechanisms. One mechanism appears to be independent of TK inhibition and results in increased myofilament Ca$^{2+}$ sensitivity. In contrast, the second mechanism appears to be related to the TK-inhibitory action of genistein and results in an increased Ca$^{2+}$ transient. These inferences are further supported by the findings that 40 μM genistein continued to accelerate the TTP shortening, consistent with a sensitizing effect, but no longer affected the TTP Ca$^{2+}$ transient in the presence of bpV(phen). Furthermore, the possibility that daidzein (a structural analog of genistein but lacking TK-inhibitory activity) also increases myofilament Ca$^{2+}$ sensitivity is in agreement with a TK-independent sensitizing mechanism of action of genistein.

The concentrations of isoflavones used in this study (10 and 40 μM) were greater than those generally found in the plasma of humans who consume soy products. The maximum plasma concentration of isoflavones after a soy-rich meal is usually of the order of 2 μM (Figtree et al., 2000), although this may be higher in some Asian populations that consume a greater amount of soy-based products (Adlercreutz et al., 1993). However, many factors influence the plasma concentrations and bioavailability of isoflavones (Setchell et al., 2001). The tissue distribution and concentrations of isoflavones have not been well described, although there is evidence that genistein can rapidly accumulate in brain tissue after intraperitoneal injection (Setchell, 1998). It is possible that certain tissues might be exposed to higher levels of isoflavones than the mean plasma levels indicate, due to tissue accumulation.

We have shown that genistein and equol produce opposing effects at the level of single ventricular myocytes, although it remains to be seen whether and how this translates into any clinically beneficial or detrimental effects in humans. If we consider isoflavones as naturally occurring Ca$^{2+}$ channel antagonists, then the benefits of equol become clear, since the expected effects of suppression of myocardial contractility and arterial relaxation are observed (Chin-Dusting et al., 2001). In contrast, our study suggests that the beneficial effects of genistein are not as obvious as previously thought. Genistein also appears to be a naturally occurring Ca$^{2+}$ channel antagonist and produces arterial relaxation (Figtree et al., 2000) in a similar way to equol. However, our observations of stimulatory effects of genistein on isolated ventricular myocytes suggest that genistein may in fact increase cardiac contraction. Theoretically, this may be a detrimental effect, especially in patients with established cardiac disease and little functional reserve.

The net cardiac effect of the isoflavones is unknown, although the marked and consistent decrease in cell contraction produced by equol suggests that this may be significant.
There is wide interindividual variation in isoflavone metabolism and excretion (Wiseman, 1999), and synthesis of equol is dependent on gut microflora (Rowland et al., 2000). As a result, about one-third of the population are classed as "good" equol excretors (Kelly et al., 1993), and so only this subpopulation may potentially benefit from any advantageous cardiovascular effects of equol.

In conclusion, we have shown that soy-derived isoflavones directly affect ventricular myocytes in opposing ways, with genistein increasing and equol decreasing contraction de-
Opposing Cardiac Actions of Soy-Derived Isoflavones


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