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European Journal of Pharmacology 481 (2003) 227–232
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The phytoestrogen genistein enhances endothelium-independent relaxation in the porcine coronary artery

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Received 29 July 2003; received in revised form 4 September 2003; accepted 10 September 2003

Abstract

Genistein, a phytoestrogen, possesses cardioprotective effects. Responses to genistein (0.1–100 μM) were assessed in 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619)-contracted porcine coronary arterial rings, with significant relaxations at high concentrations. At concentrations with little relaxation, genistein (0.3–3 μM) did not affect relaxation produced by bradykinin and the calcium ionophore, A23187. In contrast, sodium nitroprusside- and cromakalim-induced relaxations were enhanced by genistein (3 μM). N^{ω}-nitro-L-arginine methyl ester (L-NAME) (300 μM) or Triton X-100 (0.5%) did not affect the enhancement of relaxation by genistein. The tyrosine kinase inhibitor, tyrphostin 23 (30 μM), had no effect on sodium nitroprusside-elicited relaxation. In summary, genistein relaxed porcine coronary artery at relatively high concentrations. At a physiologically relevant concentration (3 μM), it is devoid of significant vascular effect, but enhanced endothelium-independent relaxations. This effect of genistein does not involve the nitric oxide synthase (NOS) pathway and the endothelium, and is mediated through a mechanism different from tyrosine kinase inhibition.

Keywords: Phytoestrogen; Genistein; Vasodilatation; Smooth muscle

1. Introduction

Asian people have a lower incidence of cardiovascular diseases as well as various cancers when compared to Western countries. This might be due to, in part, the soy-rich diet consumed by the Asian people (Messina et al., 1994; Setchell, 1998). One of the known effects of soybean products is to reduce the low-density lipoprotein cholesterol while raising high-density lipoprotein cholesterol (Anderson et al., 1995; Tikkanen et al., 1998; Wagner et al., 1997). Apart from changing serum lipid profiles, improvement of vascular function may also play an important role in lowering the mortality and morbidity of cardiovascular diseases by the phytoestrogens found in soybean. Genistein, a major phytoestrogen, produced direct vasodilatation, an effect comparable to that of 17β-estradiol (Nevala et al., 1998). At relatively high concentration (>30 μM), genistein potentiated endothelium-independent relaxations by isoproterenol in rat aortic rings (Satake and Shibata, 1999). Inhibition of tyrosine kinase attenuated the agonist-induced vascular smooth muscle contraction and hence favored relaxation (Di Salvo et al., 1993). Genistein and tyrphostin 23, at pharmacological effective doses as tyrosine kinase inhibitors, inhibited the contractile responses to noradrenaline in porcine coronary arteries (Saifeddine et al., 1992) and in rat aortae without endothelium (Abebe and Agrawal, 1995). In the present study, the most commonly used phytoestrogen, genistein, was used to investigate the vascular effects in the porcine coronary artery. Furthermore, the ability of physiological concentrations of genistein to modulate relaxation to various relaxant agents was examined. The possible involvement of nitric oxide synthase and tyrosine kinase inhibition in the action of genistein was also studied.

2. Methods

2.1. Drugs and chemicals

9,11-Dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619) was obtained from Biomol (Plymouth Meeting, PA, USA) and cromakalim was a gift from Beecham Pharmaceuticals. Triton X-100 was bought from Pharmacia

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doi:10.1016/j.ejphar.2003.09.028

Biotech (Uppsala, Sweden). All other drugs and chemicals were purchased from Sigma (St. Louis, MO, USA). Stock solutions of bradykinin, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and sodium nitroprusside were dissolved in distilled water. For cromakalim and U46619, ethanol was used as solvent and the final concentration of ethanol in each bath was always $\leq 0.1\%$. Calcium ionophore A23187, genistein and tyrphostin 23 were dissolved in dimethyl sulphoxide (DMSO) and indomethacin in 1 mM sodium carbonate solution. All stock solutions were kept refrigerated and used within 1 week of preparation except bradykinin and indomethacin, which were more stable and so were used within 1 month. Triton X-100 at 0.5% was freshly prepared and diluted by warm modified Krebs-Henseleit Solution (KHS). The final organ bath concentrations of 0.1% ethanol and 0.03% DMSO used had no significant effect on the results obtained when compared to the corresponding control (without any treatment). KHS was used to make up the final working solutions. KHS had a composition of the followings in mM: 120 NaCl, 4.76 KCl, 1.18 $MgSO_4$, 1.25 $CaCl_2$, 25 $NaHCO_3$, 1.18 NaH_2PO_4 and 5.5 glucose.

2.2. Coronary artery ring preparation

Pig hearts of either sex were collected from local abattoir after the pigs (50–70 kg) were killed in the early morning. Pigs were processed according to the regulations laid down by Urban Services Department of the Government of Hong Kong. Hearts were then immersed in cold KHS at 4 °C and transported to the research laboratory within 90 min. The left anterior descending and the right coronary arteries were used. Excess fat and connective tissue were removed and the arteries were cut into 3-mm rings. The rings were then immediately mounted on two stainless steel hooks under a resting tension of 2 g in 5 ml KHS-filled organ baths. One of these hooks was anchored and the other was attached to a force transducer for isometric tension measurement. The preparations were allowed to equilibrate for 120 min in oxygenated condition (95% O_2: 5% CO_2) at 37 °C. KHS was changed every 15 min. Basal tension of 2 g was maintained continuously throughout the equilibration period. In experiments requiring disruption of endothelium, rings were perfused with 0.5% Triton X-100 at a rate of 1 ml/min for 30 s. For the corresponding control, perfusion was carried out using normal KHS.

The use of animals for this study had been approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong following guidelines as recommended by the Helsinki Declarations for use of experimental animals.

2.3. Experimental protocols

After the equilibration period, a viability test was carried out. Rings were first incubated with 10 μM indomethacin for 15 min (to block prostanoids production) and remained exposed to the drug for the rest of the experiment. Rings were contracted with U46619 (30 nM, a thromboxane A_2 analog) and then relaxed with bradykinin (1 μM). Only rings that contracted more than 4 g and relaxed more than 80% were regarded as viable (Teoh et al., 1999). In case of the experiments requiring disrupted endothelium, rings producing less than 10% relaxation to 1 μM bradykinin after Triton X-100 treatment were used. After the viability test, the rings were allowed to return to the basal tension by changing the KHS every 10 min before further experimentation. U46619 (30 nM) was then added until a stable and sustained contraction was achieved. Concentration–relaxation curves to genistein were obtained. In subsequent studies, 30-min incubation with genistein, in the presence of DMSO or L-NAME (300 μM) was performed. After the incubation period, concentration–relaxation curves to different vasodilators (bradykinin, the calcium ionophore A23187, sodium nitroprusside and cromakalim) were carried out at half-log basis. Except where noted, the genistein concentration in the bath used for incubation was 3 μM, and the corresponding vehicle concentration of DMSO was 0.03%.

2.4. Data and statistical analysis

Rings from the same heart were used for one treatment only. Results are expressed as the mean ± S.E.M. and n represents the number of rings used in the experiments. Relaxation was expressed as percentages of the U46619-induced contraction. EC_{50} values were determined using a curve-fitting program (SigmaPlot, SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and Bonferroni's test were used to determine the significant differences between treatments (SPSS). A P-value less than 0.05 was considered as statistically significant.

3. Results

3.1. Direct relaxing effect of genistein on contracted porcine coronary artery rings

Genistein (0.1–100 μM) and the vehicle (DMSO, 0.001%–1%) were added cumulatively to U46619 contracted rings. Genistein produced significant direct relaxation at 3 μM, and higher concentrations produced full relaxation (Fig. 1). High concentrations of DMSO in the bath also produced a small relaxation ($P > 0.05$) when compared to rings without any treatment (control). The maximal relaxation by 1% DMSO averaged about 30%.

3.2. Acute effects of physiologically relevant concentration of genistein on U46619-contracted porcine coronary artery with intact endothelium

With a soy-rich diet, plasma concentrations of genistein were estimated to be in the low μM range (King and Bursill,

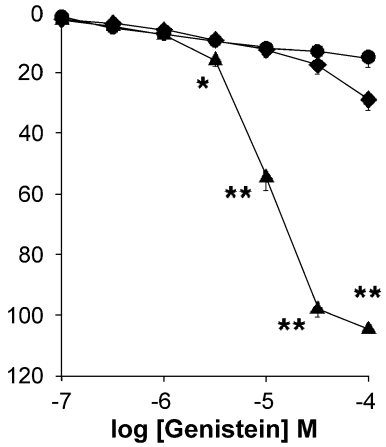

Fig. 1. Vasodilatory ability of genistein (▲, 0.1–100 μM) or the equivalent volume of the vehicle, DMSO (♦), in porcine coronary arterial rings contracted with 30 nM U46619. Arterial rings without any addition were used as control (●). Values are expressed as mean ± S.E.M. ($n=7$). *$P<0.05$ and **$P<0.001$ when compared to the corresponding data with DMSO.

1997). Incubation with 0.3, 1 and 3 μM genistein for 30 min were therefore used. Relaxation to the endothelium-dependent vasodilators, bradykinin (0.1 nM–1 μM) and A23187 (0.1 nM–1 μM) were not altered significantly (Fig. 2A and B). Relaxations to the endothelium-independent vasodilators, sodium nitroprusside (1 nM–100 μM) and cromakalim (0.1 nM–100 μM) were also examined. The relaxation to sodium nitroprusside was shifted significantly to the left with 3 μM genistein (Fig. 2C). For cromakalim, both 1 and 3 μM genistein significantly shifted the concentration–relaxation curve of cromakalim to the left (Fig. 2D). The shift in EC_{50} values of sodium nitroprusside and cromakalim by 3 μM genistein is shown in Table 1.

A lower peak tension was associated with a shift of EC_{50}. To test this possibility, a small reduction in peak tension (approximately 10%) was first produced by 30 nM sodium nitroprusside before a subsequent concentration–response relaxation by sodium nitroprusside was performed. There were no significant difference between the data with a lower peak tension and those obtained with a normal peak tension (data not shown). Genistein at 3 μM or below with minimal direct effect on peak tension (≈ 6.5%, Fig. 1) is therefore insufficient to account for the shift in sodium nitroprusside concentration–relaxation curve. Higher concentrations of genistein (10 μM) would affect relaxation through both direct and indirect mechanisms. Indeed, higher concentrations of genistein also enhanced sodium nitroprusside induced relaxation (at 10 μM, the EC_{50} shifted from 1.39 to 0.14 μM; data not shown).

3.3. Role of nitric oxide synthase (NOS), endothelium and tyrosine kinase in the enhancement of the relaxation to sodium nitroprusside

Inhibition of NOS by L-NAME (300 μM) did not alter the effect of 3 μM genistein to enhance sodium nitroprusside relaxation when compared to vehicle (DMSO, 0.03%, Fig. 3A and B). Disruption of endothelium by 0.5% Triton X-100 perfusion also did not affect the enhancement of relaxation by genistein (Fig.

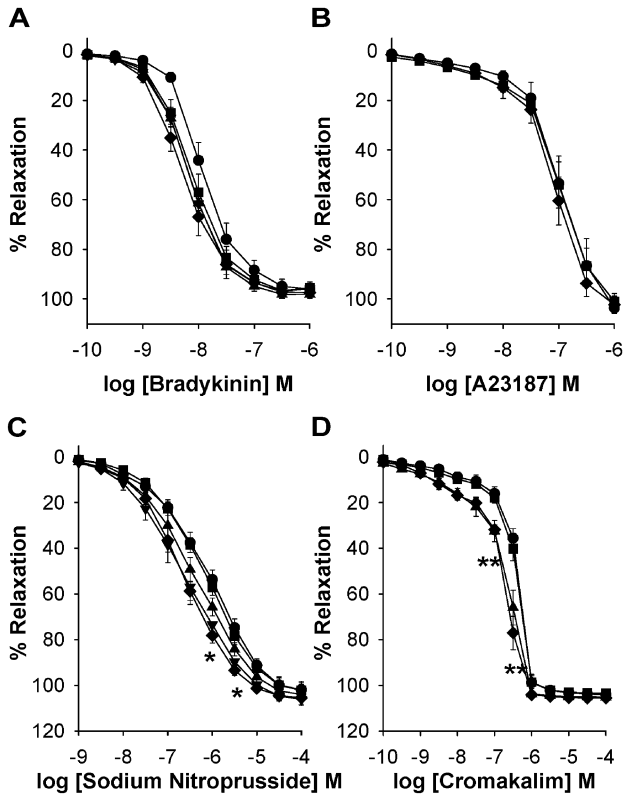

Fig. 2. Effects of genistein (▲, 0.3; ▼, 1; ♦, 3 μM) on concentration–relaxation curves in porcine coronary arterial rings contracted with U46619 by various relaxing agents. Panel (A) shows bradykinin-induced relaxation, panel (B) shows A23187-induced relaxation, panel (C) shows sodium nitroprusside-induced relaxation and panel (D) shows cromakalim-induced relaxation. Values are expressed as mean ± S.E.M. ($n=8–9$). Arterial rings without any treatment were used as control (●). No significant effect of genistein on relaxation to bradykinin and A23187 was observed in panels (A) and (B) when compared to data with DMSO (■). Significant effects of genistein on relaxation to sodium nitroprusside and cromakalim were observed in panels (C) and (D). *$P<0.05$ and **$P<0.01$ versus data with DMSO (■).

Table 1
Effects of acute treatment with genistein on the EC_{50} values of concentration–relaxation curves to sodium nitroprusside or cromakalim in porcine coronary arteries contracted by 30 nM U46619

	EC_{50} values (μM)				
	Control	DMSO (0.03%)	Genistein (0.3 μM)	Genistein (1 μM)	Genistein (3 μM)
Sodium nitroprusside	0.95	1.39	0.76	0.28[a]	0.25[b]
Cromakalim	0.42	0.40	ND	0.22[b]	0.19[b]

ND, not determined. $n=8–9$ in each group.
[a] $P<0.05$ versus data with DMSO.
[b] $P<0.01$ versus data with DMSO.

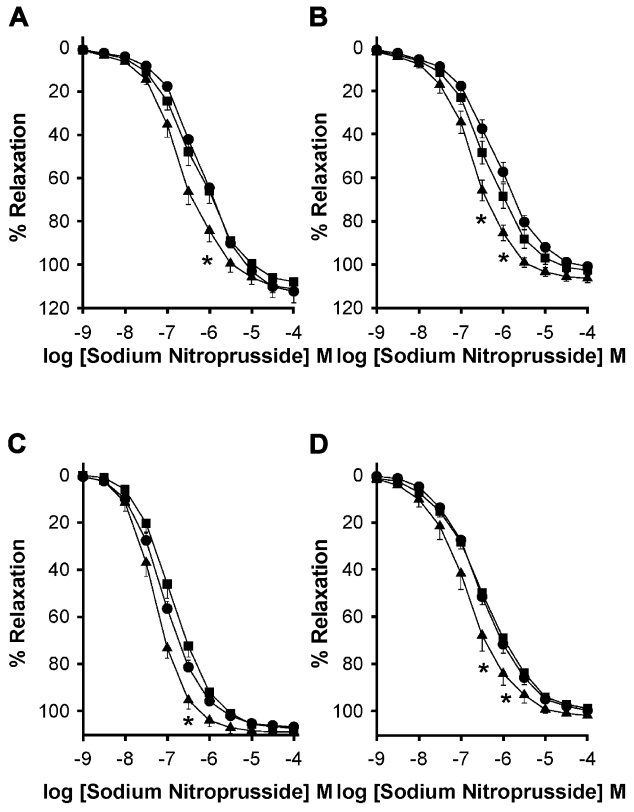

Fig. 3. Influence of NOS and endothelium on the effects of genistein (▲, 3 μM) on sodium nitroprusside-induced relaxation in U46619 contracted porcine coronary arterial rings. Panel (A) shows the effect of genistein in the absence and panel (B) presence of L-NAME (300 μM). Panel (C) shows the effect of genistein in the presence of intact endothelium (perfused with KHS) and panel (D) absence of intact endothelium (perfused with 0.5% Triton X-100). Values are expressed as mean ± S.E.M. ($n = 6–9$). Rings without any treatment were used as control (●). *$P < 0.05$ versus data with DMSO (■).

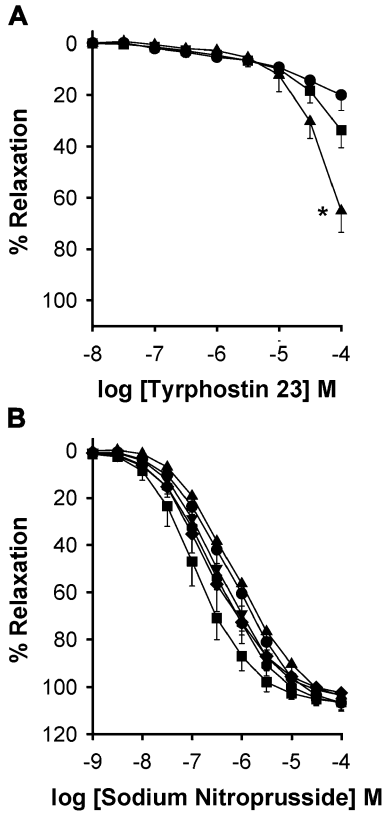

Fig. 4. Role of tyrosine kinase inhibition on sodium nitroprusside-induced relaxation. In panel (A), relaxation was determined by the direct addition of tyrphostin 23 (▲) or the equivalent volume of the vehicle, DMSO (■). Rings without any addition were used as control (●). *$P < 0.05$ versus data with DMSO. In panel (B), rings were contracted with U46619 and sodium nitroprusside-induced relaxation was determined after incubation with low concentrations of tyrphostin 23 (◆, ▼, ▲ for 10, 20 and 30 μM, respectively). Arterial rings without any addition and with DMSO (0.3%) were used for comparisons (●, ■, respectively). Values are mean ± S.E.M. ($n = 4$).

3C and D; Table 2). High concentrations of the tyrosine kinase inhibitor, tyrphostin 23, relaxed U46619-contracted rings (Fig. 4A). Incubation with low concentrations of tyrphostin 23 (≤ 30 μM), that had comparable direct effect on relaxation as 3 μM genistein, did not affect the sodium nitroprusside-induced relaxation (Fig. 4B).

Table 2
Effects of acute treatment with genistein on the EC_{50} values of concentration–relaxation curves to sodium nitroprusside in porcine coronary arteries contracted by 30 nM U46619

Treatment/condition	EC_{50} values (μM)	
	DMSO (0.03%)	Genistein (3 μM)
Without L-NAME	0.43	0.21[a]
With L-NAME	0.51	0.23[a]
With intact endothelium	0.31	0.13[a]
With disrupted endothelium	0.14	0.06[a]

Arterial rings with endothelium were treated with and without 300 μM L-NAME. Arterial rings without (perfused with 0.5% Triton X-100) or with intact endothelium (perfused with KHS) were also used.
$n = 9$ and 6 in L-NAME treatment and Triton X-100 treatment groups, respectively.
[a] $P < 0.05$ versus data with DMSO.

4. Discussion

Epidemiological studies suggested a beneficial effect of soy-rich diet in reducing cardiovascular diseases among Asian (Adlercreutz et al., 1995; Anderson et al., 1995; Messina et al., 1994; Setchell, 1998). For example, consumption of soybean products was associated with a reduction of cholesterol level in Japanese men and women (Nagata et al., 1998). Moreover, a hypocholesterolemic effect of soy protein concentrates was observed in normal premenopausal Japanese subjects (Takatsuka et al., 2000). The serum concentration of total cholesterol was reduced by 400 ml of soymilk (109.2 mg of total isoflavones) daily for two menstrual cycles.

Genistein (4,5,7-trihydroxyisoflavone) is one of the major isoflavones found in dietary soy proteins that may

contribute to the cardiovascular benefits. Foods like soymilk, miso (a paste made from soybean common in Japanese diet), soy protein isolate, soy flour, baked soybean powder and tofu are good sources of genistein (USDA-Iowa, 1999). The maximal attainable plasma concentration of genistein is about 1–4 μM for people taking a single soy meal, which contains about 53.5 mg of genistein (King and Bursill, 1997).

One of the beneficial effects of soy products might be due to a reduction of blood pressure (Honore et al., 1997). Genistein ($\geq$ 10 μM) relaxed phenylephrine-contracted rat aortic rings (Nevala et al., 1998). In the present studies, a full range of genistein (0.3–100 μM) was used. The present findings confirm the vascular relaxant effect of genistein (Herrera et al., 1996; Nevala et al., 1998). There are several proposed mechanisms for the actions of genistein including Ca^{2+} channel antagonism (Figtree et al., 2000; Wijetunge et al., 1992), inhibition of cyclic nucleotide phosphodiesterase activities (Satake et al., 1999) and enhanced release of nitric oxide with improved endothelial function (Squadrito et al., 2000). However, the direct relaxing effect of genistein in the rat mesenteric artery was independent of the endothelium activity (Nevala et al., 1998).

One of the emphases of the present study was to investigate physiologically relevant concentrations of genistein that can be achieved through dietary supplementation. A soy-rich diet or with soy product supplement results in low μM concentrations of genistein in the plasma (King and Bursill, 1997). Hence, 3 μM or lower concentrations of genistein were selected for further studies to investigate the modulation of vascular function by genistein. No significant difference was found in endothelium-dependent relaxation in the presence of these concentrations of genistein. In contrast, endothelium-independent relaxations to both sodium nitroprusside and cromakalim were significantly enhanced by genistein.

The present experimental findings showed that the enhancement of sodium nitroprusside-induced relaxation by genistein in porcine coronary arteries with intact endothelium was independent of NOS activity. Furthermore, enhancement of relaxation by genistein was not affected by the disruption of the endothelium by Triton X-100 perfusion.

Genistein is a nonselective tyrosine kinase inhibitor as well as an inhibitor of epidermal growth factor receptor kinase (10 μM or above, Akiyama et al., 1987). It also inhibits serotonin-induced vascular contraction via the protein tyrosyl phosphorylation pathway (Watts et al., 1996). A relationship exists between inhibition of tyrosine kinase and reduction of muscle contraction (Herrera et al., 1996; Nevala et al., 1998). The role of tyrosine kinase inhibition was therefore further tested in the present study. Tyrphostin 23 (30 μM), another tyrosine kinase inhibitor with similar relaxing effect as 3 μM genistein in porcine coronary artery, did not affect the sodium nitroprusside-induced relaxation. This suggests that low concentrations of genistein have little or no effect on tyrosine kinase activity while higher concentrations inhibit tyrosine kinase and directly relax the coronary smooth muscle. Furthermore, only low concentration of genistein but not tyrphostin 23 enhanced sodium nitroprusside relaxation. This is best explained by the proposals of Akiyama et al. (1987) and Mishra et al. (2000) that genistein acts as tyrosine kinase inhibitor at a relatively higher doses (>10 μM) while it exerts estrogenic or non-estrogenic activity at lower doses ($\leq$ 3 μM). This is in agreement with the previous work done by Nichols and Morimoto, 1999 that genistein as high as 100 μM showed minimal tyrosine kinase activity in tyrosine kinase assays of HT4 cell extract.

In conclusion, genistein produced relaxations of coronary arterial smooth muscles at high concentrations. Furthermore, we demonstrated for the first time that lower concentrations enhanced relaxation induced by endothelium-independent relaxants. This indirect action of genistein on the modulation of vascular function did not depend on the inhibition of tyrosine kinase. The enhancement of relaxation by genistein was not mediated via changes in NOS activity and was not affected by the disruption of endothelial function. Clearly, further investigations are required to elucidate the mechanism(s) for the indirect action of genistein on vascular relaxation.

Acknowledgements

The authors thank the support from the Institute of Molecular Technology for Drug Discovery and Synthesis, an Areas of Excellence scheme established under the University Grant Council of the Hong Kong Special Administrative Region, China. We also thank Professor Paul M. Vanhoutte for his comments on the manuscript. We would like to thank Godfrey S.K. Man, Dr. Hwee Teoh, Dr. Susan W.S. Leung and Adrian Quan for their support and discussions. Thanks are also due to the Kennedy Town Abattoir for providing us with the pig hearts for this study. Cromakalim was a gift from Beecham Pharmaceuticals.

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