

Cardioprotective Effects of 9-Hydroxyellipticine on Ischemia and Reperfusion in Isolated Rat Heart

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ABSTRACT—We determined the effect of 9-hydroxyellipticine (9HE) on ryanodine receptor (RyR) and cardiac function after global ischemia in isolated rat hearts. The binding of [³H]-ryanodine in rabbit cardiac sarcoplasmic reticulum was displaced by 9HE in a biphasic manner corresponding to the two sites model with IC₅₀ values of 6.1 μ M and 55 mM. The increase of the intracellular Ca²⁺ concentration induced by caffeine in CHO cells expressing cardiac-type RyR was suppressed by 9HE in a concentration-dependent manner. Pretreatment of the heart with 9HE decreased the total duration of reperfusion-induced ventricular fibrillation (VF) and delayed the onset of VF. There was also a significant recovery of contractile force of ischemic hearts following 9HE. Unlike nifedipine, an L-type Ca²⁺-channel blocker, 9HE did not suppress the contraction of rat papillary muscles. Thus, 9HE exerts the cardioprotective effects against ischemia/reperfusion injury without changing hemodynamic indices.

Keywords: Ryanodine receptor, 9-Hydroxyellipticine, Arrhythmia, Ischemia, Reperfusion

Ischemic heart disease is one of the clinical problems causing myocardial damage, arrhythmia and stunning (1–3). Many researchers (4–6) have implicated that reperfusion injury after ischemia is associated with the elevation of intracellular Ca²⁺ concentration through sarcoplasmic reticulum (SR) membrane, and that the Ca²⁺ release channel, especially ryanodine receptor (RyR), on SR plays an important role in the development of intracellular Ca²⁺ overload. Therefore, the modulation of RyR should be a new cardioprotective strategy for ischemia/reperfusion injury.

Caffeine activates Ca²⁺ release via RyR from SR and increases the [³H]-ryanodine binding activity to cardiac SR (7). A previous study has shown that bromoeudistomin D (BED) has a powerful Ca²⁺-releasing effect on skeletal SR, exhibiting caffeine-like properties (8). On the other hand, its derivatives inhibit both Ca²⁺- and caffeine-induced Ca²⁺-release from skeletal SR (9). Assuming that an inhibitor of Ca²⁺-release from SR can be a novel cardio-

protectant, we conducted a series of investigations on compounds with a structure analogous to BED and found 9-hydroxyellipticine (9HE) (Fig. 1) as a RyR inhibitor. 9HE displays highly antitumor (10), antioxidant (11), and catecholamine-releasing activities as well (12). In the present study, we determined the interaction of 9HE with cardiac-type RyR (RyR2) and the protective action against ischemia/reperfusion-induced arrhythmias in the isolated rat heart.

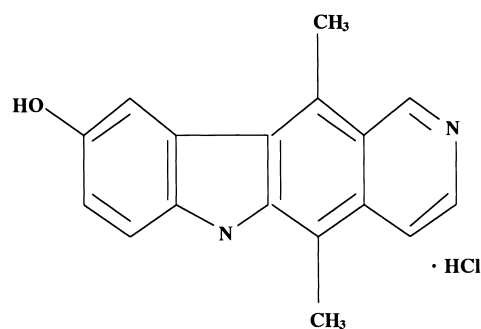


Fig. 1. Chemical structure of 9-hydroxyellipticine.

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MATERIALS AND METHODS

Measurement of intracellular Ca^{2+} concentration in RyR expressing CHO cells (CRR4 cells)

CRR4 cells, a stable cell line expressing rabbit RyR2 (13), were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum under 5% CO_2 – 95% air at 37°C and loaded with 11 μM Fluo-3 acetoxymethyl ester (Fluo-3AM) in a buffer (146 mM NaCl, 4 mM KCl, 0.2 mM MgCl_2 , 0.5 mM CaCl_2 , 10 mM glucose and 10 mM Hepes-Tris, pH 7.4) for 1 h at room temperature. After washing, the cells were harvested with 0.05% EGTA-PBS and collected by centrifugation. The cell suspension was prepared with the buffer at concentration of 5×10^5 cells/ml. The fluorescence signal was monitored with the excitation wavelength at 480 nm and emission wavelength at 540 nm using an intracellular ion analyzer (CAF-110; Japan Spectroscopic Co., Tokyo). The difference in the fluorescence intensity between before and after caffeine treatment was used as the increased intracellular Ca^{2+} signal (maximum signal with buffer treatment: F_{control} , maximum signal with 9HE treatment: $F_{9\text{HE}}$, baseline signal with buffer treatment: B_{control} , baseline signal with 9HE treatment: $B_{9\text{HE}}$). The antagonistic activity was calculated as follows: Antagonistic activity = $100 - (F_{9\text{HE}} - B_{9\text{HE}}) / (F_{\text{control}} - B_{\text{control}}) \times 100$

[^3H]-Ryanodine binding study

Cardiac SR (200 $\mu\text{g}/\text{ml}$) was incubated with 0.156 nM to 10 nM [^3H]-ryanodine for 1 h at 37°C (pCa 4.0). Non-specific binding was determined in the presence of 5 μM unlabeled ryanodine. For displacement studies, cardiac SR was incubated with 5 nM [^3H]-ryanodine for 1 h at 37°C in the presence of various concentrations of 9HE. Data analyses were performed using Graph Pad Prism version 2.00 for Windows.

Animals

This project was approved by the Ethical Committee at Tanabe Seiyaku and all efforts were made to minimize animal suffering and to reduce the number of animals used. Male Sprague-Dawley (SD) rats (Charles River Co., Kanagawa) were used. The animals were fed standard rat food (CE-2; Clea, Tokyo) and supplied with tap water ad libitum.

Isolated heart preparation

Male SD rats (10–20 weeks) were anesthetized with diethyl ether and heparin (100 IU/body) was injected intravenously. Hearts were quickly removed and placed in ice-cold Krebs-Henseleit solution (K-H solution: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.5 mM CaCl_2 , 25 mM NaHCO_3 and 11 mM glucose, pH 7.4) to cease spontaneous beating and then perfused

according to the Langendorff's method at a constant pressure of 90 cmH_2O with K-H solution continuously gassed with O_2/CO_2 (95:5) at 37°C. Following the start of perfusion, the hearts started spontaneous beating. The contractile force of hearts was monitored through a hook attached to the apex of the heart by means of a force transducer (TB-611T; Nihon Kohden, Tokyo) under an initial preload of 2 g (resting tension). The rates of force development ($+\text{d}f/\text{d}t$) and relaxation ($-\text{d}f/\text{d}t$) were calculated using a differentiator (ED-601G, Nihon Kohden). The perfusion flow rate was determined by an electromagnetic flow probe (Nihon Kohden) that was connected to the aortic cannula. The heart rate was calculated using a bioelectric amplifier (Nihon Kohden) from the electrocardiogram that was recorded by two electrodes placed on the apex of the heart and the right ventricle. Hemodynamic parameters were continuously recorded on a thermal pen recorder and were taken in a Macintosh computer using a MacLab system (AD Instruments, Castlehill, Australia). Data were analyzed by Chart Version 3.4 (AD Instruments).

Experimental protocols

The hearts were allowed to equilibrate for 15 min prior to each study. For non-ischemia studies (Fig. 2), the hearts were perfused with K-H solution for another 15 min, then the solution was changed to the K-H buffer with or without 9HE for 25 min, and finally perfused without 9HE up to 130 min. For ischemia/reperfusion studies (Fig. 2), after the equilibration for 15 min with K-H solution and the hearts were perfused for 5 min with or without 9HE, global ischemia was conducted by interrupting the aortic flow for 20 min followed by reperfusion with K-H solution up to 130 min. 9HE (10 mM) was prepared in distilled water and diluted with K-H solution to the desired concentration employed in each experiment.

Estimation of arrhythmia

Ventricular arrhythmia was defined in accordance with the Lambeth Conventions (14). Briefly, ventricular tachycardia (VT) was defined as four or more consecutive ventricular premature beats. Ventricular fibrillation (VF) was defined as no recognizable heart rates because of deformed QRS complex. After reperfusion, all of the hearts showed VT within a few seconds and VT immediately converted to the sustained VF. The contractile force was regarded to be zero when the heart was in sustained VF.

Inotropic effects of 9HE and nifedipine

Male SD rats (7–11 weeks) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the heart was removed and rapidly placed in ice-cold K-H solution saturated with 95% O_2 and 5% CO_2 . Left ventricular papillary muscles were dissected, placed vertically in a tempera-

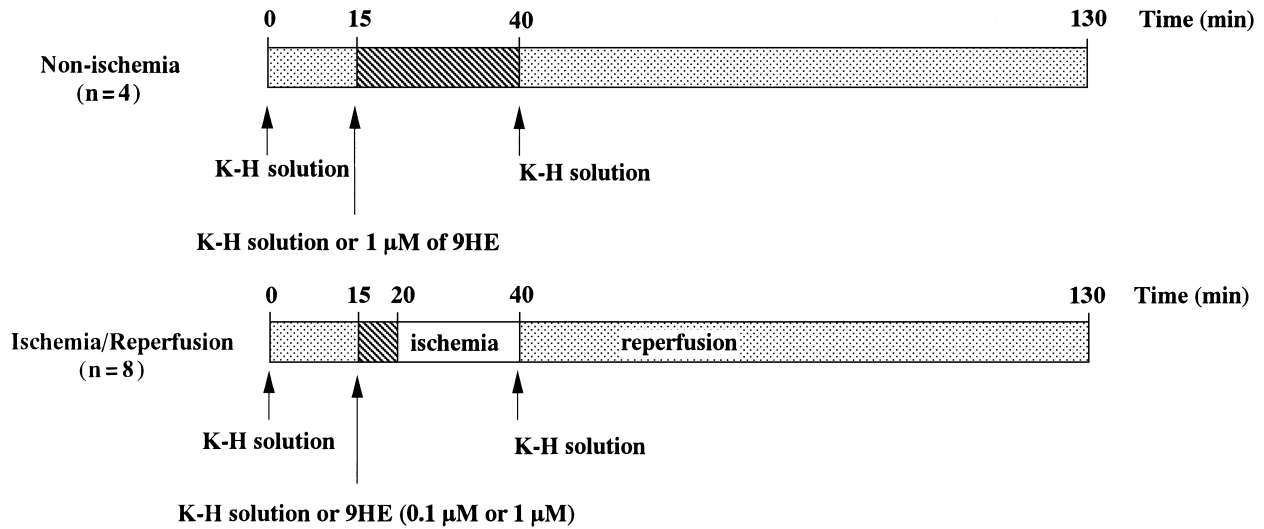


Fig. 2. Schematic protocol of ischemia/reperfusion.

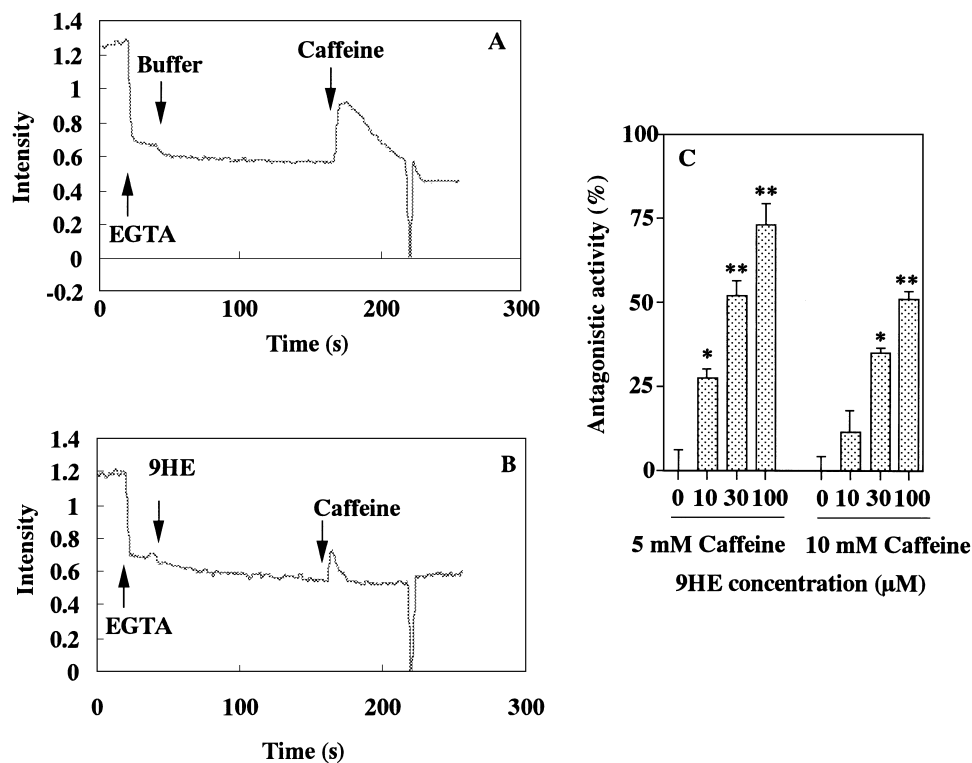


Fig. 3. Typical tracing of the intracellular Ca^{2+} signal in CRR4 cells. After treatment with 150 mM EGTA/10 mM Tris-HCl (pH 7.4), the buffer with or without 9HE was added to fluo-3-loaded cells and then 5 or 10 mM caffeine was added (A: control, B: 9HE). The inhibitory effects of 9HE on the caffeine-induced Ca^{2+} increase are summarized in the panel C. Results are shown as means \pm S.E.M. (n = 4). * $P < 0.05$, ** $P < 0.01$ vs control group.

ture-regulated chamber (37°C) containing the K-H solution and the resting tension of 0.5 g was applied. The papillary muscle was paced at 0.5 Hz via platinum contact electrodes with square wave pulses. The developed tension was monitored by a force transducer (TB-611T) and continuously

recorded on a thermal pen recorder and in a Macintosh computer using a MacLab system. Data analysis was performed by a Chart Version 3.4. Nifedipine (1 mM) was prepared in dimethylsulfoxide (DMSO) and diluted with K-H solution before the experiment.

Drugs

9HE was synthesized in the Organic Chemistry Laboratory of Tanabe Seiyaku Co., Ltd. Other drugs used in the present study were caffeine, DMSO, nifedipine (Wako, Osaka); Fluo-3AM (Dohjin Chemicals, Kumamoto); Ham's F-12 medium (Nissui Pharmaceutical Co., Ltd., Tokyo); and Heparin (Mochida Pharmaceutical Co., Ltd., Tokyo).

Statistical evaluation

Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison. A *P* value smaller than 0.05 was judged to indicate a significant difference.

RESULTS

Effects of 9HE on intracellular Ca^{2+} concentration of CRR4 cells

In the presence of excess EGTA to avoid the effect of Ca^{2+} influx from the extracellular space, we tested the effect of 9HE on caffeine-induced Ca^{2+} release in CRR4 cells. Figure 3 shows typical responses of Fluo-3-AM-loaded CRR4 cells to caffeine in the absence (A) or the presence (B) of 9HE. Although the fluorescent intensity was slightly decreased by 9HE, a similar response to 9HE was observed also in non-transfected CHO cells (data not shown). 9HE concentration-dependently inhibited the mobilization of Ca^{2+} elicited by 5 or 10 mM caffeine with IC_{50} values of 30 and 100 μ M, respectively (Fig. 3C).

Effects of 9HE on [3H]-ryanodine binding to rabbit cardiac SR

Scatchard analysis of [3H]-ryanodine binding to cardiac SR revealed a binding site with K_d and B_{max} values of 1.98 nM and 949 fmol/mg protein, respectively (Fig. 4A). The 9HE competition curve was flat with a pseudo-Hill coefficient of 0.38 against [3H]-ryanodine (Fig. 4B). Analysis of the competition curve indicated that a two-site model was significantly better ($P < 0.05$, F test) than a one-site model. IC_{50} values for the high- and the low-affinity binding sites of 9HE were 6.1 μ M and 55 mM, respectively.

Effects of 9HE on cardiac functions of isolated rat heart

9HE did not significantly affect hemodynamic indices in either the control group or 9HE group (Table 1).

Effects of 9HE on cardiac function after ischemia/reperfusion

There were no significant differences in hemodynamic indices between the control group and 9HE groups (data not shown). When global ischemia was induced by clamping the perfusion line, there were remarkable decreases in

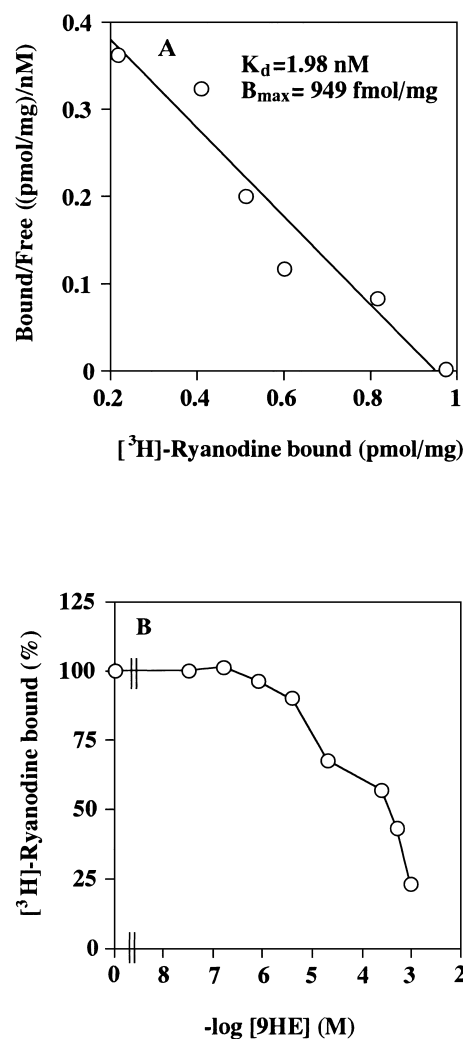


Fig. 4. Scatchard analysis of the [3H]-ryanodine binding to cardiac SR (A) and inhibitory effect of 9HE on [3H]-ryanodine binding to cardiac SR (B). A) Cardiac SR (200 μ g/ml) was incubated with 0.156 nM to 10 nM [3H]-ryanodine for 1 h at 37°C (pCa 4.0). Nonspecific binding was determined in the presence of 5 μ M unlabeled ryanodine. B) Cardiac SR was incubated with 5 nM [3H]-ryanodine for 1 h at 37°C in the presence of various concentrations of 9HE. Nonspecific binding was determined in the presence of 5 μ M unlabeled ryanodine. Values are means of duplicate determinations.

both contractile force and heart rate in all groups. After starting the reperfusion, all the hearts developed VT followed by sustained VF. Although there was no significant difference in the recovery rate from the VF among the control group and 9HE groups, it seemed to be increased by 9HE after reperfusion (Table 2). 9HE (1 μ M) significantly prolonged the average time to onset of the VF from 29 to 60 s ($P < 0.01$) (Table 2). In the control group, only 1 out of 8 hearts started spontaneous beating at 117 min following ischemia/reperfusion. In the 0.1 μ M of 9HE group, 3 out of 8 hearts started beating at 63, 68 and 98 min, respectively. In the 1 μ M of 9HE group, 5 out of 8 hearts started

Table 1. The time-dependent effects of 9HE on hemodynamic indices in isolated perfused heart

	Group	Contractile force (g)	+dF/dt (g/s)	-dF/dt (g/s)	Flow rate (ml/min)	Heart rate (beats/min)
Before 9HE treatment	Control	9.3 ± 0.7	200.0 ± 6.1	-205.0 ± 21.2	15.7 ± 2.4	262.7 ± 22.6
	9HE	8.2 ± 0.5	186.7 ± 22.7	-178.3 ± 14.3	15.8 ± 0.6	271.3 ± 19.2
45 min	Control	8.0 ± 0.1	178.3 ± 1.7	-177.0 ± 8.8	16.2 ± 2.3	265.3 ± 26.2
	9HE	7.8 ± 0.6	186.7 ± 20.3	-170.0 ± 15.3	16.7 ± 0.4	291.1 ± 12.4
70 min	Control	7.7 ± 0.3	165.0 ± 5.8	-153.3 ± 9.3	16.7 ± 2.8	263.3 ± 24.9
	9HE	7.5 ± 0.6	171.7 ± 19.2	-133.3 ± 11.7	16.6 ± 0.5	276.3 ± 4.9
130 min	Control	7.1 ± 0.1	150.0 ± 5.8	-120.0 ± 7.6	14.5 ± 2.1	268.7 ± 19.6
	9HE	6.8 ± 0.7	158.3 ± 19.2	-106.7 ± 14.8	15.3 ± 0.9	282.0 ± 9.0

Values are means ± S.E.M. (n = 4). Hemodynamic indices before 9HE treatment were taken at the point of 0 min. There were no significant differences in hemodynamic indices between the control and the 1 μ M of 9HE group.

Table 2. Recovery from the myocardial ischemia injury

Group	A	B (s)	C (min)
Control	1/8	29 ± 5	88 ± 1.6
9HE 10 ⁻⁷ M	3/8	34.8 ± 3.9	69.9 ± 10.4
9HE 10 ⁻⁶ M	5/8	60 ± 13**	51.9 ± 13.4*

Column A: Number of beating-restored hearts in 8 experiments by 130 min. Column B: Time to onset first VF after starting reperfusion (s). Values are means ± S.E.M. Column C: Duration of VF after reperfusion (min). Values are mean ± S.E.M. * P < 0.05, ** P < 0.01 vs control group.

beating at 52, 53, 55.5, 67 and 118 min, respectively. Thus, it seems likely that 9HE shortened the duration of VF (Table 2).

Effects of 9HE on cardiac functions during reperfusion after ischemia

There was a significant recovery of contractile force, +df/dt and -df/dt by 9HE (1 μ M) at 70 and 90 min (Fig. 5). Similarly, cardiac functions except heart rate were restored following 1 μ M 9HE at 100 min (Fig. 5).

Inotropic effects of 9HE and nifedipine

Nifedipine (10 μ M) reduced the developed tension of papillary muscles at 10 to 30 min (Fig. 6). After washing, there was no recovery of the developed tension. 9HE (100 μ M) did not change the developed tension of papillary muscles up to 60 min (Fig. 6).

DISCUSSION

Caffeine induces the release of Ca²⁺ via RyR in CHO cells expressing RyR2 (13). 9HE suppressed the Ca²⁺-mobilization induced by caffeine in a concentration-dependent manner. The specific binding of [³H]-ryanodine to

cardiac SR was concentration-dependently displaced by 9-HE. These data indicate that 9HE binds RyR2 and inhibits the Ca²⁺ release through RyR2. Thus, 9HE is a novel RyR2 antagonist.

Release of Ca²⁺ through RyR from cardiac SR plays an important role in ventricular arrhythmia during acute myocardial damage due to ischemia and reperfusion (15–17). Ryanodine and caffeine deplete Ca²⁺ from cardiac SR and pretreatment with these agents abolishes the ischemia-induced ventricular arrhythmias and reperfusion-induced VF (18). Dantrolene blocks Ca²⁺ release from SR and exerts cardioprotective action (19). Likewise, 9HE suppressed the VF after ischemia/reperfusion of the isolated, perfused rat hearts. These suggest that suppression of Ca²⁺ release from SR exerts cardioprotective effects during ischemia/reperfusion.

The displacement curve of [³H]-ryanodine binding to cardiac SR by 9HE showed that the pseudo Hill coefficient was less than unity (0.38) and 9HE bound to two different sites. IC₅₀ values for the high- and the low-affinity binding sites of 9HE were 6.1 μ M and 55 mM, respectively. On the other hand, 9HE protected against cardiac injury after ischemia/reperfusion at 1 μ M. Therefore, the high-affinity binding site of 9HE may contribute to the protection against ischemia/reperfusion injury.

Because Ca²⁺ release from SR plays a crucial role in cardiac contraction, depletion of Ca²⁺ in SR by caffeine and ryanodine is associated with the reduction of +LVdp/dt_{max} and -LVdp/dt_{max} in normal hearts (18). High concentrations of dantrolene (>16 μ M) shows a negative inotropic effect (19). In contrast, 9HE did not affect the flow rate, spontaneous beating rate, and contractile force in the present study. In isolated papillary muscles, the contractile force was not affected by 9HE. Therefore, it is likely that 9HE exerted the cardioprotective effects by combination of these diverse pharmacological properties. The probability that this is true is supported by the present observation that

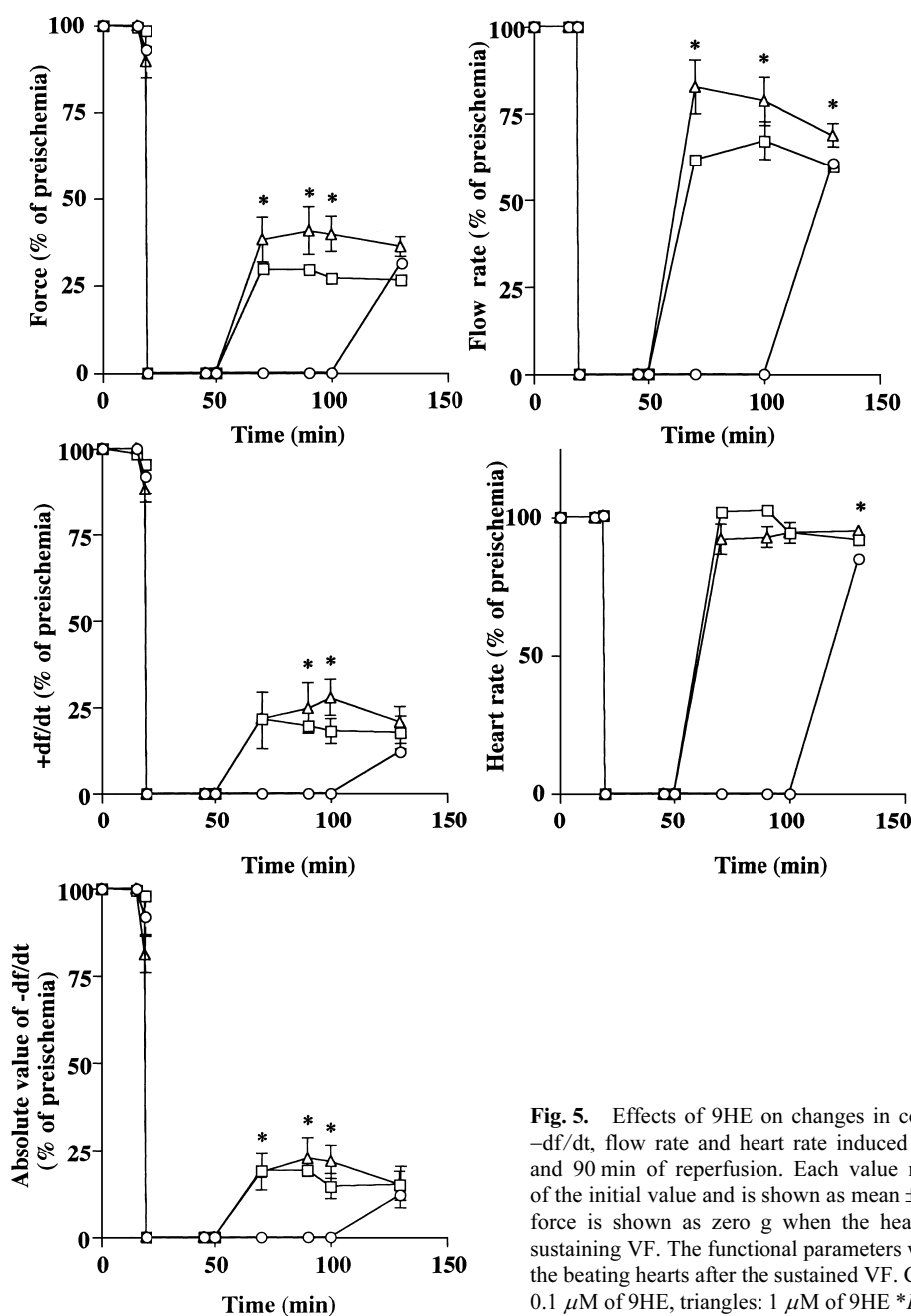


Fig. 5. Effects of 9HE on changes in contractile force, $+df/dt$, $-df/dt$, flow rate and heart rate induced by 20 min of ischemia and 90 min of reperfusion. Each value represents a percentage of the initial value and is shown as mean \pm S.E.M. The contractile force is shown as zero g when the heart was in the phase of sustaining VF. The functional parameters were obtained only from the beating hearts after the sustained VF. Circles: control, squares: 0.1 μ M of 9HE, triangles: 1 μ M of 9HE * P <0.05 vs control group.

9HE exerted the cardioprotective effects on the perfused heart at concentrations even lower than those required for suppression of caffeine-induced Ca^{2+} release in CHO cells expressing RyR2.

Although ryanodine receptors have been identified in smooth muscle cells and ryanodine-sensitive Ca^{2+} release might be involved in maintaining the vascular tone (20, 21), 9HE did not induce any coronary artery-dilating action before ischemia. Treatment with 9HE caused a marked increase in flow rate after reperfusion. While the underlying mechanism is not clear, the increase in flow rate

induced by pretreatment with 9HE during reperfusion may contribute significantly to the cardioprotective effect of the compound.

Early studies have shown 9HE has an antioxidant action of 9HE similar to vitamin E (11). Free radicals are formed during ischemia/reperfusion of hearts and lead to membrane injury through oxidation/peroxidation of phospholipids and proteins. Radical scavengers including SOD (22), vitamin E analogues (23) and CV-3611 (24) effectively reduce the ischemia/reperfusion injury. Among free radicals formed during ischemia/reperfusion, hydroxyl

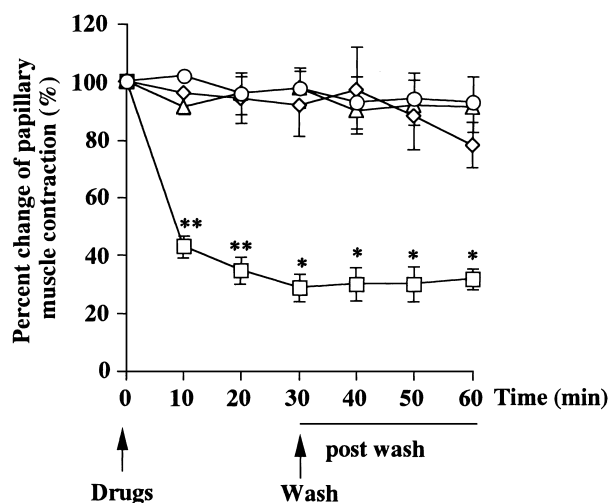


Fig. 6. Inotropic effects of 9HE and nifedipine, control and 0.1% DMSO in papillary muscle. The developed tension is expressed as a percentage of the initial value and is shown as means \pm S.E.M. ($n=7$). The 0.1% DMSO was used as the control for 10 μ M of nifedipine. Circles: control, diamonds: 0.1% DMSO, triangles: 100 μ M of 9HE, squares: 10 μ M of nifedipine. The initial values were as follows: control, 301 ± 88 mg; 0.1% DMSO, 252 ± 45 mg; 100 μ M of 9HE, 334 ± 97 mg; 10 μ M of nifedipine, 271 ± 105 mg. Nifedipine significantly reduced the developed tension. * $P<0.05$, ** $P<0.01$ vs 0.1% DMSO group.

radicals react with sulfhydryl groups on RyR2 to increase the open probability (25). These suggest that free radicals not only cause membrane injury but also modulate RyR2 function. Therefore, it is possible that the antioxidant activity of 9HE also participates in the cardioprotective effects.

In conclusion, 9HE is a novel cardioprotective agent, which attenuates myocardial cell damages during reperfusion after global ischemia. Combination of various effects, such as suppression of Ca^{2+} release via RyR2, the activity as a radical scavenger, and the increase in flow rate during reperfusion may be responsible for the cardioprotective effects of 9HE.

Acknowledgments

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REFERENCES

- Patel B, Kloner RA, Przyklenk K and Braunwald E: Postischemic myocardial "stunning": a clinically relevant phenomenon. *Ann Intern Med* **108**, 626–628 (1988)
- Kloner RA, Przyklenk K and Patel B: Altered myocardial states. The stunned and hibernating myocardium. *Am J Med* **86**, 14–22 (1989)
- Kloner RA and Przyklenk K: Hibernation and stunning of the myocardium. *N Engl J Med* **325**, 1877–1879 (1991)
- Kleber AG and Oetliker H: Cellular aspects of early contractile failure in ischemia. In *The Heart and Cardiovascular System*, Edited by Fozzard HA, Haber E, Jennings RB, Katz AM and Morgen HE, pp 1975–1996, Raven Press, New York (1992)
- Zucchi R, Ronca-Testoni S, Yu G, Galbani P, Ronca G and Mariani M: Postischemic changes in cardiac sarcoplasmic reticulum Ca^{2+} channels. A possible mechanism of ischemic preconditioning. *Circ Res* **76**, 1049–1056 (1994)
- Chen W, London R, Murphy E and Steenbergen C: Regulation of the Ca^{2+} gradient across the sarcoplasmic reticulum in perfused rabbit heart. A ^{19}F nuclear magnetic resonance study. *Circ Res* **83**, 898–907 (1998)
- Coronado R, Morrisette J, Sukhareva M and Vaughan DM: Structure and function of ryanodine receptors. *Am J Physiol* **266**, C1485–C1504 (1994)
- Nakamura Y, Kobayashi J, Gilmore J, Mascal M, Rinehart KL Jr, Nakamura H and Ohizumi Y: Bromo-eudistomin D, a novel inducer of calcium release from fragmented sarcoplasmic reticulum that causes contractions of skinned muscle fibers. *J Biol Chem* **261**, 4139–4142 (1986)
- Takahashi Y, Furukawa K, Ishibashi M, Kozutsumi D, Ishiyama H, Kobayashi J and Ohizumi Y: Structure-activity relationship of bromoeudistomin D, a powerful Ca^{2+} releaser in skeletal muscle sarcoplasmic reticulum. *Eur J Pharmacol* **288**, 285–293 (1995)
- Auclair C: Multimodal action of antitumor agents on DNA: the ellipticine series. *Arch Biochem Biophys* **259**, 1–14 (1987)
- Rousseau-Richard C, Auclair C, Richard C and Martin R: Free radical scavenging and cytotoxic properties in the ellipticine series. *Free Radic Biol Med* **8**, 223–230 (1990)
- Chanh PH, Xuong ND, Le Pecq JB and Paoletti C: Cardiovascular activity of 9-hydroxy-ellipticine. *Pharmacology* **14**, 490–498 (1976)
- Imagawa T, Nakai J, Takeshima H, Nakasaki Y and Shigekawa M: Expression of Ca^{2+} -induced Ca^{2+} release channel activity from cardiac ryanodine receptor cDNA in Chinese hamster ovary cells. *J Biochem (Tokyo)* **112**, 508–513 (1992)
- Walker MJA, Curtis MJ, Hearse DJ, Campbell RWF, Janse MJ, Yellon DJ, Cobbe SM, Coker SJ, Harness JB, Harron DWG, Higgins AJ, Julian DG, Lab MJ, Manning AS, Northover BJ, Parratt JR, Riemersma RA, Riva E, Russell DC, Sheridan DJ, Winslow E and Woodward B: The Lambeth Conventions: guidelines for the study of arrhythmia in myocardial ischemia, infarction and reperfusion. *Cardiovasc Res* **22**, 447–455 (1988)
- Ishide N: Intracellular calcium modulators for cardiac muscle in pathological conditions. *Jpn Heart J* **37**, 1–17 (1996)
- Zucchi R and Ronca-Testoni S: The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. *Pharmacol Rev* **49**, 1–51 (1997)
- Zucchi R, Ronca-Testoni S, Yu G, Galbani P, Ronca G and Mariani M: Effect of ischemia and reperfusion on cardiac ryanodine receptors-sarcoplasmic reticulum Ca^{2+} channels. *Circ Res* **74**, 271–280 (1994)
- Thandroyen FT, McCarthy J, Burton KP and Opie LH: Ryanodine and caffeine prevent ventricular arrhythmias during acute myocardial ischemia and reperfusion in rat heart. *Circ Res* **62**, 306–314 (1988)

- 19 Yu G, Zucchi R, Ronca-Testoni S and Ronca G: Protection of ischemic rat heart by dantrolene, an antagonist of the sarcoplasmic reticulum calcium release channel. *Basic Res Cardiol* **95**, 137 – 143 (2000)
- 20 Lesh RE, Nixon GF, Fleischer S, Airey JA, Somlyo AP and Somlyo AV: Localization of ryanodine receptors in smooth muscle. *Circ Res* **82**, 175 – 185 (1998)
- 21 Neylon CB, Richards SM, Larsen MA, Agrotis A and Bobik A: Multiple types of ryanodine receptor/ Ca^{2+} release channels are expressed in vascular smooth muscle. *Biochem Biophys Res Commun* **215**, 814 – 821 (1995)
- 22 Aoki N, Bitterman H, Brezinski ME and Lefer AM: Cardio-protective actions of human superoxide dismutase in two reperfusion models of myocardial ischaemia in the rat. *Br J Pharmacol* **95**, 735 – 740 (1988)
- 23 Walker MK, Vergely C, Lecour S, Abadie C, Maupoil V and Rochette L: Vitamin E analogues reduce the incidence of ventricular fibrillations and scavenge free radicals. *Fundam Clin Pharmacol* **12**, 164 – 172 (1998)
- 24 Tada H, Kutsumi Y, Misawa T, Shimamoto N, Nakai T and Miyabo S: Effects of pretreatment with 2-*O*-octadecylascorbic acid, a novel free radical scavenger, on reperfusion-induced arrhythmias in isolated perfused rat hearts. *J Cardiovasc Pharmacol* **16**, 984 – 991 (1990)
- 25 Anzai K, Ogawa K, Kuniyasu A, Ozawa T, Yamamoto H and Nakayama H: Effects of hydroxyl radical and sulfhydryl reagents on the open probability of the purified cardiac ryanodine receptor channel incorporated into planar lipid bilayers. *Biochem Biophys Res Commun* **249**, 938 – 942 (1998)