

Acceleratory effect of ellagic acid on sarcoplasmic reticulum Ca^{2+} uptake and myocardial relaxation

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Abstract

The effects of ellagic acid, a phenolic phytochemical contained in fruits and vegetables, on sarcoplasmic reticulum Ca^{2+} uptake and myocardial contraction were examined in mouse ventricular myocardia. In cardiomyocytes loaded with Fura-2 (a fluorescent Ca^{2+} indicator), isoprenaline (a β -adrenoceptor agonist) produced a decrease in the basal fluorescence ratio. This decrease was inhibited by propranolol (a β -adrenoceptor antagonist) and cyclopiazonic acid (a sarcoplasmic reticulum Ca^{2+} -ATPase inhibitor). Ellagic acid produced a decrease in basal fluorescence ratio, which was completely inhibited by cyclopiazonic acid. In isolated myocardial tissue preparations, isoprenaline increased the contractile force and shortened the time required for relaxation. Ouabain, a cardiac glycoside, increased the contractile force but did not affect the time required for relaxation. Ellagic acid shortened the time required for relaxation but did not affect the contractile force. The beating rate of isolated right atria was increased by isoprenaline, but ellagic acid had no effect. In conclusion, ellagic acid accelerates sarcoplasmic reticulum Ca^{2+} uptake and myocardial relaxation without affecting the contractile force or the beating rate. Ellagic acid, which has such non-conventional mode of action, may be of value in the long-term maintenance of cardiac function.

1. Introduction

Phenolic phytochemicals are contained in fruits and vegetables and have been reported to have beneficial effects against oxidation-related chronic diseases including cancer and cardiovascular diseases^[1,2]. They have been reported to ameliorate cardiovascular disorders such as atherosclerosis and ischemic injury. The beneficial effects of polyphenols are considered to be the result of their scavenging action on active oxygen species and enhancement of antioxidant enzyme activity. On the other hand, mechanisms such as alteration of the activity and expression of enzymes and transporters have also been postulated.

Ellagic acid (Fig. 1) is a phynolytic lactone

compound contained in berries, grapes, walnuts and distilled spirits^[3]. It is contained in plants in the form of ellagitannins, esters of glucose and ellagic acid, which are hydrolyzed to form ellagic acid. Recent studies have shown the antimutagenic, antioxidant and anti-inflammatory activities of ellagic acid. In the cardiovascular system, ellagic acid was reported to have hypotensive and bradycardiac effects, which appear to be related to its vasodilative effects^[4,5].

In the present study, we examined the effect of ellagic acid on isolated mouse myocardial preparations. We first constructed a fluorescence microscopy-based method for measuring the uptake of cytoplasmic Ca^{2+} into the sarcoplasmic reticulum in cardiomyocytes. As

ellagic acid showed an acceleratory effect, we examined its effects on the contraction, relaxation and beating rate of isolated tissue preparations in comparison with two conventional cardiotonic agents, isoproterenol and ouabain.

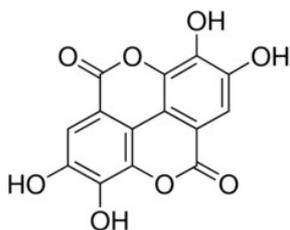


Fig. 1 Chemical structure of ellagic acid

2. Materials and Methods

2.1. General

All experiments were approved by the Toho University Animal Care and User Committee, and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. Male *ddy* strain mice were used at the age of 4 weeks after birth. The experimental methods were basically the same as those in our previous studies [6-8].

2.2. Measurement of basal Ca^{2+} concentration

Isolated ventricular cardiomyocytes were obtained by Langendorff perfusion and collagenase digestion. The cells were superfused with a HEPES-Tyrode solution of the following composition (mM): NaCl 143, KCl 5.4, $MgCl_2$ 1.8, $CaCl_2$ 2, NaH_2PO_4 0.33, glucose 5.5 and HEPES 20. The solution was gassed with 100% O_2 and warmed to $36^\circ C$. The cells were preincubated with the acetoxymethyl derivative of Fura-2 (Fura-2-AM 10 μM , 30min). An experimental chamber containing the cells was placed on a fluorescence microscope (IX70; Olympus Corp., Tokyo, Japan), and perfused with the HEPES-Tyrode solution. The basal Ca^{2+} fluorescence of single myocytes was measured and the time course on application of agents was analyzed. The cells were excited at 340 and 380 nm from a Xenon lamp and a high-speed excitation wavelength switcher (C7733, Hamamatsu Photonics) and emission (>500 nm) was separated with a dichroic mirror, detected by a

high-speed cooled CCD camera (C6790, Hamamatsu Photonics) at a time resolution of 5 s, and ratioed after correction of background fluorescence (Aquacosmos software, Hamamatsu Photonics).

2.3. Measurement of contractile force

The right ventricular free wall strips were placed horizontally in a 20 ml organ bath containing a modified Ringer solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, Mg_2SO_4 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 11.1 (pH7.4 at $36^\circ C \pm 0.5^\circ C$). The preparations were electrically driven by field stimulation delivered by a pair of platinum plate electrodes with voltage pulses (1Hz, 3ms, 1.5 x threshold voltage) generated from an electronic stimulator (SEN-3301; Nihon Kohden). Contractile force was recorded isometrically with a force-displacement transducer (TB-611T; Nihon Kohden) connected to a carrier amplifier (AP-621G; Nihon Kohden). This analog signal was digitized by an A/D converting interface (Power Lab/4SP, AD Instruments), and analyzed with a computer software (Chart 7; AD instruments). The resting tension on each preparation was applied so that the muscle was stretched to the peak of its length-tension curve. The contractile force, time from the onset of twitch to the peak tension (time to peak tension; TPT), and time from the peak tension to 90% relaxation (time to 90% relaxation; RT_{90}) were measured.

2.4. Measurement of beating rate

The action potentials of spontaneously beating right atria were detected by bipolar platinum electrodes, amplified by a bioelectric amplifier (AB-621G, Nihon Kohden) and counted by a pulse rate tachometer (AT-601G, Nihon Kohden).

2.5. Statistics

All the data were expressed as mean \pm S.E.M. The effect of agents on basal Ca^{2+} was analyzed with one way analysis of variance followed by Tukey's test for multiple comparisons. The effects of agents on contractile parameters and beating rate were analyzed with the paired t-test. A *P* value less than 0.05 was considered as statistically significant.

2.6. Drugs and chemicals

Isoprenaline (Wako Junyaku), propranolol (Wako Junyaku) and ouabain (Wako Junyaku) were dissolved in saline. Fura-2/AM (Dojindo Laboratories), ellagic acid (Sigma), cyclopiazonic acid (Sigma) were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the extracellular solution did not affect the parameters measured.

3. Results

The cardiomyocytes loaded with the Ca^{2+} indicator, Fura-2, showed an even fluorescence throughout the cytoplasm (Fig. 2Ab). The nuclei could be clearly distinguished from the cytoplasm by their higher fluorescence intensity. The cellular fluorescence completely disappeared after permeabilization of the sarcolemma with digitonin (Fig. 2Ad)

Isoprenaline (1 μM), a β -adrenergic agonist, decreased the basal fluorescence ratio (Fig. 2Ac). The maximum decrease was about 25% of the initial value which was reached at about 4 min after application (Fig. 2B). The isoprenaline-induced decrease in fluorescence was significantly inhibited by 1 μM propranolol, a blocker of the β -adrenergic receptor, or by 10 μM cyclopiazonic acid^[9], an inhibitor of the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) (Fig. 2C).

Ellagic acid (10 μM) decreased the basal fluorescence ratio (Fig. 3Ac). The maximum decrease was about 12% of the initial value which was reached at about 4 min after application (Fig. 3B). The ellagic acid-induced decrease in fluorescence ratio was inhibited by 1 μM cyclopiazonic acid (Fig. 3B).

In isolated ventricular tissue preparations, 100 nM isoprenaline produced an increase in contractile force and shortened the time required for relaxation (Fig. 4A; Table 1). Ouabain (1 μM), a cardiac glycoside, increased the contractile force but did not affect the time required for relaxation (Fig. 4B; Table 1). Ellagic acid (10 μM) shortened the time required for relaxation but did not affect the contractile force (Fig. 4C; Table 1). Cyclopiazonic acid decreased the contractile force and prolonged the time required for relaxation (Fig. 4D; Table 1). The accelerating effect of ellagic acid on relaxation was diminished in the presence of

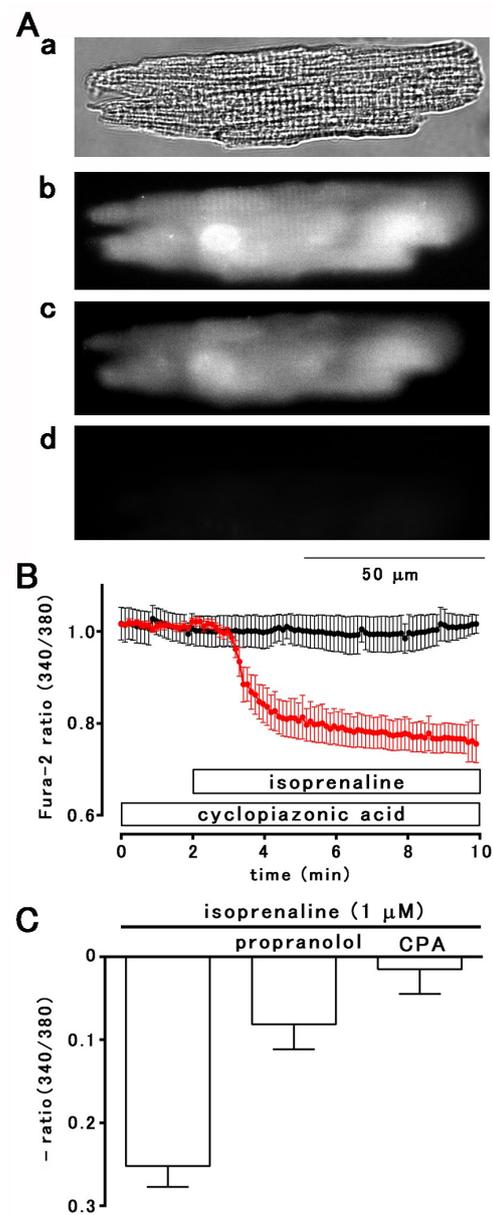


Fig.2 Effect of isoprenaline on Ca^{2+} fluorescence. A: Typical DIC images (a) and fluorescence images (340 nm excitation) before (b) and after (c) the application of 1 μM isoprenaline, and after membrane permeabilization by 20 $\mu\text{g}/\text{ml}$ digitonin (d). B: Typical time course of the effect of 1 μM isoprenaline on the fluorescence ratio in the absence (red line) and presence of 10 μM cyclopiazonic acid (black line). C: Summarized results for the effect of isoprenaline alone, in the presence of 1 μM propranolol, 10 μM cyclopiazonic acid (CPA).

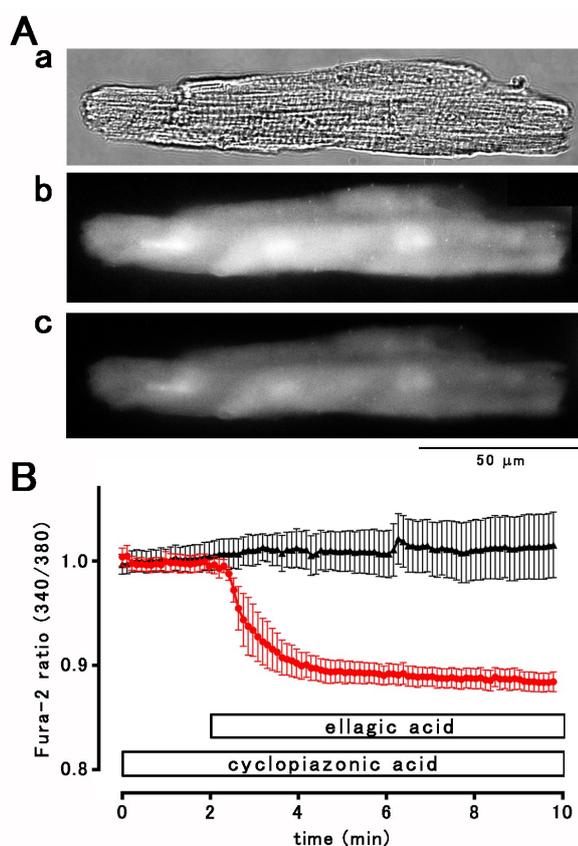


Fig.3 Effect of ellagic acid on Ca^{2+} fluorescence.

A: Typical DIC images (a) and fluorescence images (340 nm excitation) before (b) and after (c) the application of 10 μM ellagic acid. B: Typical time course of the effect of 10 μM ellagic acid in the absence (red line) and presence of 10 μM cyclopiazonic acid (black line).

cyclopiazonic acid (Fig. 4E; Table 1).

In the spontaneously beating isolated right atria, isoprenaline (10 nM) significantly increased the beating rate, in contrast, ellagic acid had no effect (Table 2).

4. Discussion

The present study was performed to clarify the effect of ellagic acid on sarcoplasmic reticulum Ca^{2+} uptake and myocardial relaxation. The Ca^{2+} sensitive fluorophore, Fura-2, was introduced into cardiomyocytes using its acetoxymethyl derivative,

Fura 2-AM. The fluorescence in the nuclear region was higher than in the cytoplasmic region indicating that the fluorophore was not taken up by intracellular organelle such as the mitochondria and sarcoplasmic reticulum. Thus, these cardiomyocytes were suitable for the measurement of Ca^{2+} uptake from the cytoplasm into the sarcoplasmic reticulum.

Under basal conditions, a small amount of Ca^{2+} leakage from the sarcoplasmic reticulum occurs which is observed as spontaneous Ca^{2+} sparks^[10]. This Ca^{2+} is sequestered into the sarcoplasmic reticulum by SERCA and thus a basal cytoplasmic Ca^{2+} concentration of about 100 nM is maintained. Theoretically, interventions which enhance SERCA would change the balance between Ca^{2+} release and uptake and result in a decrease in basal Ca^{2+} concentration. Indeed application of isoprenaline, a β -adrenoceptor agonist, induced a concentration-dependent decrease in the basal Ca^{2+} concentration. β -adrenoceptor stimulation is known to increase SERCA activity through phosphorylation of phospholamban, the regulatory protein of SERCA^[11]. The observed inhibition of the decrease by cyclopiazonic acid indicates that the decrease in basal Ca^{2+} fluorescence reflects SERCA activity. The result that isoprenaline shortened the time required for relaxation in tissue preparation could be explained by the SERCA activation.

We next evaluated the effect of ellagic acid on SERCA using the decrease in basal Ca^{2+} fluorescence as an index of SERCA activity. Ellagic acid (10 μM) induced a decrease in Ca^{2+} fluorescence similar in time course as 1 μM isoprenaline. In isolated tissue preparations, ellagic acid shortened the time required for relaxation. These effects were completely inhibited by cyclopiazonic acid, which indicates that the effect of ellagic acid was mediated by activation of SERCA. Ellagic acid was reported to enhance Ca^{2+} uptake into sarcoplasmic reticulum vesicles^[12], and was postulated to remove the inhibitory effect of phospholamban on SERCA^[13]. The present results provide evidence for the acceleratory effect of ellagic acid on SERCA in intact cardiomyocytes. In contrast to isoprenaline, which increases the contractile force by enhancement of transsarcolemmal Ca^{2+} influx

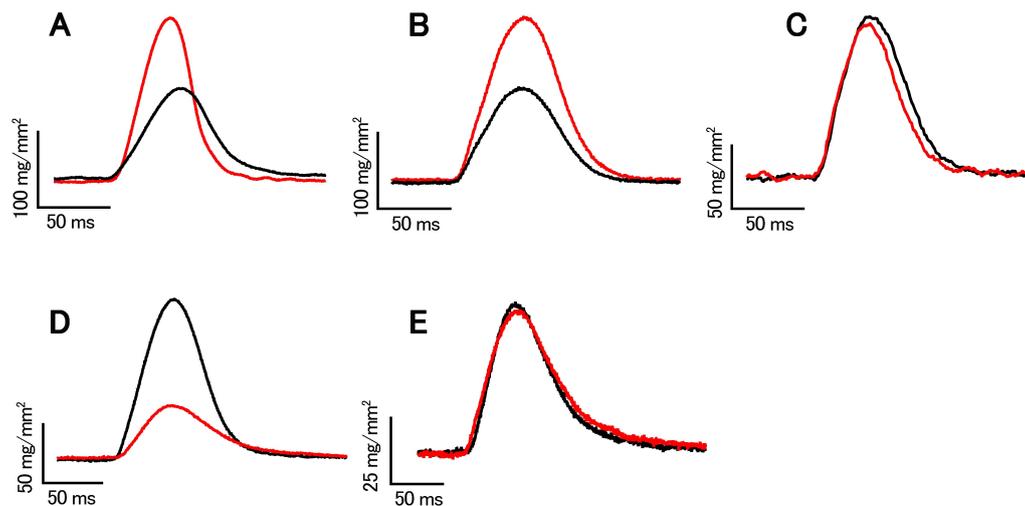


Fig.4 Effect of agents on contraction. Typical traces before (red lines) and after (black lines) application of 100 nM isoprenaline (A), 1 µM ouabain (B), 10 µM ellagic acid (C), 10 µM cyclopiazonic acid (D) and 10 µM ellagic acid in the presence of 10 µM cyclopiazonic acid (E).

Table 1 Effect of agents on the contractile parameters

| agent | | contractile force (mg/mm ²) | time to peak tension (ms) | time for 90% relaxation (ms) |
|--|--------|---|---------------------------|------------------------------|
| isoprenaline | before | 142.8 ± 20.8 | 41.4 ± 3.4 | 54.0 ± 1.0 |
| | after | 182.9 ± 27.2* | 40.3 ± 1.0 | 48.3 ± 1.3* |
| ouabain | before | 140.0 ± 21.1 | 43.8 ± 1.3 | 57.9 ± 2.5 |
| | after | 266.1 ± 43.9* | 44.6 ± 1.1 | 58.2 ± 3.6 |
| ellagic acid (alone) | | | | |
| | before | 139.4 ± 20.1 | 46.2 ± 4.5 | 58.5 ± 1.8 |
| | after | 126.0 ± 16.9 | 45.6 ± 4.3 | 52.2 ± 3.0* |
| ellagic acid (in the presence of cyclopiazonic acid) | | | | |
| | before | 60.7 ± 17.3 | 47.5 ± 5.3 | 96.1 ± 4.9 |
| | after | 54.7 ± 14.2 | 50.5 ± 4.2 | 101.5 ± 5.8 |

Values are the mean ± S.E.M. of 5 preparations. *P<0.05 compared with before agents.

through the Ca²⁺ channel^[11], ellagic acid had no effect on contractile force. Effects of ellagic acid on sarcolemmal ion channels have not been reported.

Concerning heart failure, systolic dysfunction has conventionally been considered to be the major contributing factor and the standard treatment of heart failure was improvement of systolic dysfunction through cardiotoxic agents^[14]. Using the mouse

ventricular myocardium, the enhancement of myocardial contractile force by standard cardiotoxic agents, isoprenaline and ouabain, was confirmed in the present study. These conventional agents, however, are now recognized to have adverse effects on cardiac function in the long run probably through their cardio-stimulating effect itself. At present, novel cardioactive agents with different mechanisms of

Table 2 Effect of isoprenaline and ellagic acid on the beating rate

| | before | after |
|--------------|--------------|---------------|
| isoprenaline | 435.0 ± 0.8 | 572.5 ± 10.6* |
| ellagic acid | 435.2 ± 10.1 | 435.3 ± 10.5 |

Values are the mean ± S.E.M. of 6 preparations.

*P<0.05 compared with before agents.

action are in need.

Recently, diastolic dysfunction has been recognized as a major deficit underlying heart failure^[15]. More than half of heart failure patients have a preserved left ventricular ejection fraction, but an increased left ventricular end-diastolic pressure, which indicates impaired diastolic function. Diastolic dysfunction accompanies common diseases such as hypertension, coronary artery disease and diabetes mellitus. Although treatment of diastolic dysfunction is now considered to be important for the preservation of cardiac function, the pathophysiology of diastolic dysfunction and the pharmacological agents for its treatment has not yet been sufficiently clarified. The present observation that ellagic acid accelerates myocardial relaxation through activation of SERCA implies that ellagic acid may be of value in the treatment of myocardial diastolic dysfunction^[6].

The beating rate of isolated right atria was increased markedly by isoprenaline. In contrast, ellagic acid did not affect the beating rate. The lack of effect on the beating rate is a desirable feature from the aspect of cardioprotection because the beating rate is known to correlate with myocardial oxygen consumption and a higher heart rate is often accompanied by increased cardiovascular events and mortality.

In conclusion, ellagic acid accelerates sarcoplasmic reticulum Ca²⁺ uptake and myocardial relaxation without affecting the contractile force and beating rate. Such profile of ellagic acid is distinct from conventional cardiotonic agents and may be of value in the treatment of cardiac diastolic dysfunction.

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摘出マウス心筋の筋小胞体へのCa²⁺取込と筋弛緩に対する エラグ酸の促進作用

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抄録

野菜や果物に含まれる植物化学物質である ellagic acid (エラグ酸) がマウス摘出心室筋の筋小胞体への Ca²⁺取込と筋収縮に与える影響を検討した。Ca²⁺感受性蛍光プローブを取込ませた単離心室筋細胞において、アドレナリン β 受容体作動薬の isoprenaline は非興奮状態での蛍光強度比を低下させた。この低下はアドレナリン β 受容体遮断薬の propranolol および筋小胞体 Ca²⁺-ATPase 阻害薬の cyclopiazonic acid により著明に抑制された。Ellagic acid は非興奮状態での蛍光強度比を低下させた。この低下は cyclopiazonic acid により完全に抑制されたが、propranolol によっては影響されなかった。摘出心室筋組織標本において、isoprenaline は収縮力を増大させ、弛緩時間を短縮した。強心配糖体の ouabain は、収縮力を増大させたが、弛緩時間に影響を与えなかった。Ellagic acid は、弛緩時間を短縮したが、収縮力は変化させなかった。Cyclopiazonic acid 存在下では、ellagic acid の作用は消失した。摘出右心房標本において、isoprenaline は拍動数を上昇させたが、ellagic acid は拍動数に影響しなかった。Ellagic acid は、心筋の筋小胞体への Ca²⁺取込や筋弛緩を促進する一方、心筋収縮力や拍動数には影響しないことが判明した。心筋に対して従来の強心薬と異なる作用様式を有する Ellagic acid は、心筋の長期的機能維持に有用である可能性がある。

キーワード：エラグ酸、筋小胞体、心筋弛緩

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東邦大学大学院薬学研究科博士後期課程修了。

専門は循環薬理学。細胞内事象の蛍光イメージング法と薬理的・電気生理学的手法を組み合わせた総合的検討により、心臓機能を包括的に理解することを目指して研究を行っている。