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Protective effects of EGCg or GCg, a green tea catechin epimer, against postischemic myocardial dysfunction in guinea-pig hearts

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Abstract

The protective effects of (−)-epigallocatechin-3-gallate (EGCg) or the C-2 epimer, (−)-gallocatechin-3-gallate (GCg), afforded by their antioxidative activity among green tea catechins were investigated in perfused guinea-pig Langendorff hearts subjected to ischemia and reperfusion. The recovery (%) of the left ventricular developed pressure from ischemia by reperfusion was 34.4% in the control, while in the presence of EGCg (3×10^{-5} M) or GCg (3×10^{-6} M, a more diluted concentration than that of EGCg), it led to a maximal increase of 78.4% or 76.2%, consistent with a significant preservative effect on the tissue level of ATP at the end of ischemia or reperfusion. In the perfused preparation of mitochondria, EGCg (10^{-5} M) inhibited mitochondrial Ca^{2+} elevation by changes in the Ca^{2+} content or the acidification of perfusate, similarly to findings with cyclosporin A, a well known inhibitor of the mitochondrial permeability transition pore. By in vitro electron paramagnetic resonance (EPR), EGCg or GCg was found to directly quench the activity of active oxygen radicals, with the strongest activity in tea catechins. EGCg or GCg decreased the caspase-3 activity induced apoptosis. Therefore, it is concluded that the beneficial effects of EGCg or GCg play an important role in ischemia–reperfusion hearts in close relation with nitric oxide (NO), active oxygen radicals and biological redox systems in mitochondria.

Keywords: Langendorff hearts; Green tea catechin; Electron paramagnetic resonance (EPR); Mitochondrial permeability transition pore (MPTP); Caspase 3

Introduction

(−)-Epigallocatechin-3-gallate (EGCg) is one of the major components of green tea leaves, and (−)-gallocatechin-3-gallate (GCg) is an artifact due to the epimerization of EGCg during the heating procedure. EGCg is the most abundant catechin and has received the most attention (Lim et al., 2003; Giakoustidis et al., 2006). Green tea has recently received much attention as a protective agent against cardiovascular disease (Imai and Nakachi, 1995; Kono et al., 1996; Tsubono and Tsugane, 1997; Aneja et al., 2004; Stephanou, 2004; Townsend et al., 2004), as important targets of preventive medicine. Early evidence showed the beneficial effects of green tea on the cardiovascular system; rabbits or rats given a tea supplement to an atherogenic diet had reduced serum lipoproteins and incidence of atherosclerosis (Young et al., 1967; Akinyanju and Yudkin, 1967). Green tea extract was also shown to lower plasma cholesterol and triglyceride levels in rats (Muramatsu et al., 1986; Ikeda et al., 2005). This effect may be due to the potent antioxidative activity of catechin derivatives in green tea (Zhang et al., 1997; Guo et al., 1999; Nakagawa and Yokozawa, 2002; Xu et al., 2004).

We have recently reported that in the isolated right atria, the contractile force of GCg produced a higher pD_2 value than EGCg, and neither catechin affected the heart rate (Hotta et al., 2006). The high dose 10^{-4} M, of EGCg, or the lower dose, 10^{-5} M, of GCg than that of EGCg, produced a maximum LVDP with decreases in the basal level of T_{Ca} in a manner

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similar to the administration of the Ca-sensitizer pimobendan (Fujino et al., 1988). Therefore, catechins have a new mechanism of a positive inotropic effect (PIE) similar to Ca-sensitizer drugs without the increases of intracellular Ca^{2+} concentration, while the level of the transient NO signal of GCg showed greater increases in the response than that of EGCg. Hence, GCg showed a more prolonged hypotensive effect on rabbits than EGCg. Each catechin (GCg or EGCg), like the NO donor, FK409 (Cao et al., 2001) and two serotonin derivatives from safflower seeds with potent antioxidative activity (Hotta et al., 2002), may have therapeutic use as an NO-mediated vasorelaxant, and may have an additional protective action in myocardial ischemia–reperfusion-induced injury.

Therefore, in the present study, we attempted to define the cardioprotective effects of EGCg or catechin epimer GCg, a more diluted concentration than that of EGCg, on sequential changes in contractility or stiffness of the left ventricle, and the cellular metabolism of high phosphorous energy in isolated guinea-pig Langendorff hearts subjected to normothermic global ischemia and subsequent reperfusion. Additionally, in relation to our investigation of Ca^{2+} systems, mitochondrial Ca^{2+}($[Ca^{2+}]_m$) uptake following the change of the acidity or Ca^{2+} content of the perfusate, similar to the end of ischemia or reperfusion after global ischemia in Langendorff hearts, was investigated prior to the administration of each optical isomer of the epimer (Hotta et al., 2004). The changes of $[Ca^{2+}]_m$ were measured as to their contribution to injury due to the mitochondrial permeability transition pore (MPTP) by treatment with openers or inhibitors of MPTP (Griffiths et al., 2000).

These results suggest that catechin likely inhibit the opening of the MPTP by preventing $[Ca^{2+}]_m$ overload-induced apoptosis in ischemia–reperfusion hearts (Hausenloy et al., 2004). Additionally, the ability of catechin to scavenge active oxygen radicals was measured. There is now evidence that apoptosis, or programmed cell death, is an important response of the myocardium to ischemia, which precedes cell necrosis and appears to contribute to the overall sequelae of cardiac injury. Therefore, we examined which protective action is associated with the beneficial effects of EGCg or GCg, with a new mechanism in PIE similar to Ca-sensitizers, with respect to the reduction in caspase-3 activity-induced apoptosis.

Materials and methods

Langendorff guinea-pig hearts

Heart preparation and examination procedures

Hartley strain guinea pigs of either sex, weighing 300–350 g, were anesthetized with diethyl ether and heparinized (250 IU, i.p.). The heart was rapidly excised and the aorta was cannulated. The Langendorff heart preparations were then perfused with Krebs–Henseleit solution (KH solution, pH 7.4, at 37 °C) containing in mM: NaCl 115, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 2.0, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 10. The KH solution was presaturated with a gas mixture containing 95% O_2 and 5% CO_2, and the heart was perfused at a constant pressure of 75 cm H_2O for NMR spectrometry or at a flow rate of 7 ml/min, using a peristaltic pump, for NO measurement. Subsequent to an equilibration period of 30 min required to stabilize the mechanical function, the perfused hearts were exposed to 40 min of global ischemia by clamping the perfusion flow line, and were then reperfused for 40 min with the control medium of perfusate. In the pretreatment groups, EGCg (3×10^{-5} M) or GCg (3×10^{-6} M) was introduced into the perfusate 4 min before ischemia to the perfusate during reperfusion. To examine the caspase-3 activity, the hearts were subjected to 1 h of ischemia and 5 h of reperfusion. EGCg or GCg was administered for 4 min before ischemia and for 5 h throughout reperfusion.

Measurement of myocardial function

Coronary flow rate (FR) in the NMR analysis was measured continuously with an in-line flow probe connected to an ultrasonic flow meter (Transonic T101, Advance, Ithaca, NY, USA). A latex balloon (Hirokawa, Niigata, Japan) was introduced into the left ventricle via the left atrium and connected to a strain-gauge transducer (MK12030US, Edwards Lifesciences, Tokyo, Japan) for measurement of the isovolumic left ventricular pressure. The left ventricular end diastolic pressure (LVEDP) as an index of stiffness of the left ventricle was adjusted to 10 mmHg during the equilibration period in each heart, and the volume of the balloon was unchanged during the experiments. The left ventricular developed pressure (LVDP) as an index of contractility of the left ventricle was calculated by subtracting the LVEDP from the left ventricular systolic pressure.

^{31}P-NMR measurement and data analysis

The myocardial temperature was maintained at 37±0.5 °C with a water-jacketed perfusion line and a continuous stream of air around the NMR sample tube. The heart connected to the Langendorff perfusion line was placed in a standard 20-mm NMR tube with the apex approximately 2.5 cm from the bottom of the tube, and the tube was inserted into the NMR coil. The effluent was removed from a level above the heart with a peristaltic pump, leaving the heart submerged in a fixed volume of the perfusate. In the ischemia–reperfusion experiments, ^{31}P-NMR spectra were monitored along with simultaneous recordings of ventricular pressure, as described previously (Koike et al., 1996; Hotta et al., 1998, 2001a,b), and paced at a rate of 3–4 Hz stimulation via a 3 M KCl agar electrode. ^{31}P-NMR spectra were obtained at 161.8 MHz on a GSX 400 spectrometer (JEOL Datum Co. Ltd., Tokyo, Japan) equipped with a 9.4-Tesla vertical-bore magnet. For each spectrum, 90 free-induction decays (4 min) were accumulated after 45° flip-angle pulses (18 μs) using 4096 data points and 15.015 kHz spectral widths with a repetition time of 2 s. Accumulated free-induction decays were exponentially filtered, resulting in a 30 Hz line broadening.

The phosphocreatine (PCr), inorganic phosphate (Pi) and β-ATP were quantified by comparison with a capillary tube of standard methylenediphosphonic acid (MDP, 0.25 M) fixed inside the NMR tube. Phosphate peaks, expressed as percentages of the control values, were determined by measuring the area under each resonance peak. The relative intensity of each peak was used for quantitative analysis. Datum Station ALICE

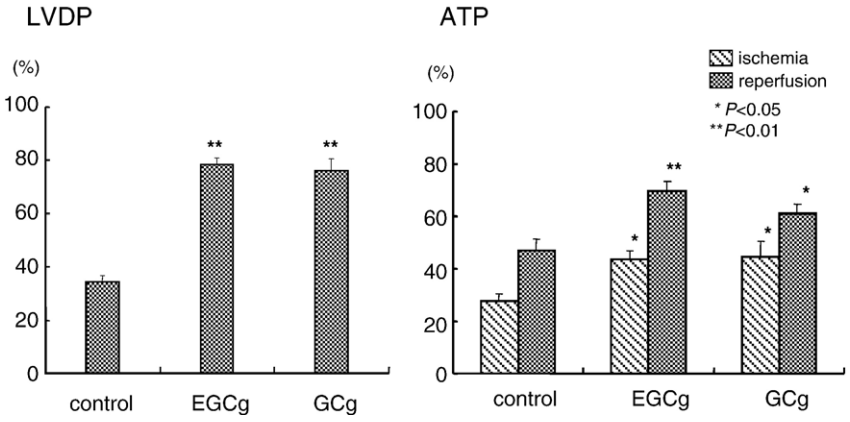

Fig. 1. Relation of the left ventricular developed pressure (LVDP) and high-energy phosphates (ATP) estimated by ^{31}P-NMR spectra in the presence and absence of (−)-epigallocatechin-3-gallate (EGCg) or (−)-gallocatechin-3-gallate (ECg) during ischemia–reperfusion. At the end stage of ischemia of the pre-treated hearts with GCg or EGCg, the level of ATP was partially restored and showed significantly greater recovery during reperfusion than the control (drug-free). The data of the LVDP, the left ventricular end diastolic pressure (LVEDP) and the coronary flow rate (FR) mentioned together in Table 1. *$P<0.05$, **$P<0.01$, significantly different from the control value. Data are expressed as the mean values of 5 preparations; vertical lines represent S.E.M.

software (JEOL Datum, Tokyo, Japan) was used to determine the area under each peak using a personal computer. The intracellular pH (pH*i*) was calculated from the chemical shift between PCr and Pi resonances using the following equation: $pH = 6.90 - \log[(\delta o - 5.85)/(3.29 - \delta o)]$, where δo is the chemical shift of Pi from PCr expressed as parts per million (ppm).

Measurement of the change in cytosolic Ca^{2+} and NO content in Langendorff hearts

Heart preparation and examination procedures. Hartley strain guinea pigs of either sex, weighing 270–320 g, were anesthetized with diethyl ether and heparinized (250 IU, i.p.). The heart was rapidly excised and the aorta was cannulated. The Langendorff heart preparations were then perfused with KH solution (pH 7.4, at 30 °C). The KH solution was presaturated with a gas mixture containing 95% O_2 and 5% CO_2, and the heart was perfused at a flow rate of 7 ml/min, using a peristaltic pump, for cytosolic Ca^{2+} and NO measurement.

Cytosolic Ca^{2+} measurement. Cytosolic Ca^{2+} fluorometry was performed with a fluorometer (CAF100; Japan Spectroscopic Co., Tokyo, Japan) as previously described (Hotta et al., 1995). The changes in contents of Ca^{2+} during ischemia and reperfusion in guinea-pig Langendorff hearts, pre-loaded with a specific fluorescent indicator, fura-2 AM for Ca^{2+}, were measured with simultaneous recording of the mechanical performance (LVDP). After the loading of the fura-2 Ca^{2+} indicator (added at 0.025% cremophore EL) for 30 min, the heart was perfused in a non-recirculating mode for 5 min to remove excess indicator fura-2 AM from the extracellular space. Subsequent to an equilibration period of 20 min required to stabilize the mechanical performance, the changes in ion contents and LVDP during ischemia and reperfusion were measured. The hearts that were incubated with fura-2 AM (2 μM, for Ca^{2+} measurements, 30 min as loading time) were measured at 500 nm as the ratio of fluorescence strength (R340/380) excited at 340 nm (F340) and 380 nm (F380). Due to the difficulty in determining the real dissociation constant of fura-2 for Ca^{2+} in myocardial cells, we used only the ratio of R340/380 as an indicator of cytosolic Ca^{2+}.

NO measurement. NO was measured with a commercially available NO meter (model NO-51 monitoring device, Inter Medical Co., Nagoya, Japan). The experimental set-up for NO and Ca^{2+} using the NO electrode and cytosolic Ca^{2+} fluorometry

Table 1
Left ventricular developed pressure (LVDP), ventricular end diastolic pressure (LVEDP), and coronary flow rate (FR) changes during ischemia–reperfusion of Langendorff hearts

	LVDP (mmHg)			LVEDP (mmHg)			FR(ml/min)		
	Pre	Isch	Rep	Pre	Isch	Rep	Pre	Isch	Rep
Control	57.2±5.4	0	19.7±3.9	10	37.4±2.8	43.4±2.8	12.2±1.3	0	5.6±0.7
EGCg	52.2±2.6	0	43.2±2.0[a]	10	24.4±2.1[b]	15.6±1.7[a]	12.8±1.0	0	9.4±0.5[b]
GCg	53.4±6.1	0	41.0±6.5[a]	10	24.8±2.6[b]	14.4±3.1[a]	13.8±1.3	0	9.8±1.2[b]

Changes during 40 min of ischemia and a subsequent 40 min of reperfusion.
Values are the mean±S.E.M, $n=5$–6.
[a]$P<0.01$; [b]$P<0.05$ significantly different from the control values.
Pre, preischemia; Isch, ischemia; Rep, reperfusion, Drug was introduced into the perfusate for reperfusion.

for Langendorff heart preparations was designed. The principles behind the measurements and the method to measure the NO concentrations using the device have been described previously (Ichimori et al., 1994; Muto et al., 2005; Huang et al., 2006). The concentration of NO was determined with an NO meter by measuring the redox current between the following two electrodes. The NO-selective electrode was made of Pt/Ir alloy (0.2 mm diameter; Pt, 90%; Ir, 10%) coated with a three-layered membrane consisting of KCl, NO-selective resin, and normal silicone membranes. A counter electrode was made of carbon fiber. An improved NO electrode was combined with a counter electrode to minimize the electrical noise. An NO-selective electrode combination was placed in the right atrium to measure the NO concentration of the effluent, which flowed from the heart.

Once the basic current had become stable, the NO response current was recorded continuously, and the level of the stable current, the baseline NO level in the experiments, was taken and recorded as the zero level. The electrode was calibrated using *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), a standard NO donor, following methods described previously. With this measuring instrument, a current of 1000 pA is approximately equal to an NO concentration of 1 μM. Then, fura-2 AM was loaded for 30 min as previously described. When the hemodynamic stability and the stability of the NO measurement system were achieved, LVDP, NO and Ca^{2+} signals were monitored simultaneously throughout the period of the Langendorff heart being treated with each drug.

Intramitochondrial fluorometric measurements according to the change in the Ca^{2+} concentration or pH in the perfusate

Mitochondria were isolated from guinea-pig hearts as previously described (Hotta et al., 1999, 2001a,b). The suspensions were incubated at 24 °C in a normal medium with a composition similar to the intracellular ionic composition (100 nM Ca^{2+}, 10 mM Na^+ and 110 mM K^+) containing respiratory substrates (composition in mM: sucrose 250, $MgCl_2$ 1, KH_2PO_4 1, succinate 10, malate 5, and MOPS (3-morpholinopropanesulfonic acid) 20 adjusted to pH 7.4 with KOH) and containing the Ca^{2+} fluoroprobe fura-2 AM (10 μM) and 0.025% cremophor EL. A 0.5-ml aliquot of mitochondria was allowed to settle on a glass coverslip on the stage of an inverted microscope (CAM230, Japan Spectroscopic Co., Tokyo, Japan) for 30 min, which was treated with poly-L-lysine to promote mitochondrial adhesion. After dye loading, the mitochondria on the coverslip were mounted on the stage of an inverted microscope and washed continuously in a dye-free solution for 10 min, followed by continuous circulation in 10 ml of medium solution. Fura-2 Ca^{2+} signals were measured at 500 nm as the ratio of fluorescence strengths (R340/380) excited at 340 nm (F340) and 380 nm (F380). After fura-2 was loaded for 30 min, the amplitude of F340 from the mitochondria increased 5–7-fold compared with that of the unloaded mitochondria. The intramitochondrial Ca^{2+} signal following preloading with high Ca^{2+} concentrations (10 μM) for over 5 min was markedly increased by lowering the Ca^{2+} concentration to a low physiological level (100 nM) or by acidifying the perfusate (pH 7.5→6.5). We then determined whether EGCg (10^{-5} M, 3×10^{-5} M) suppressed

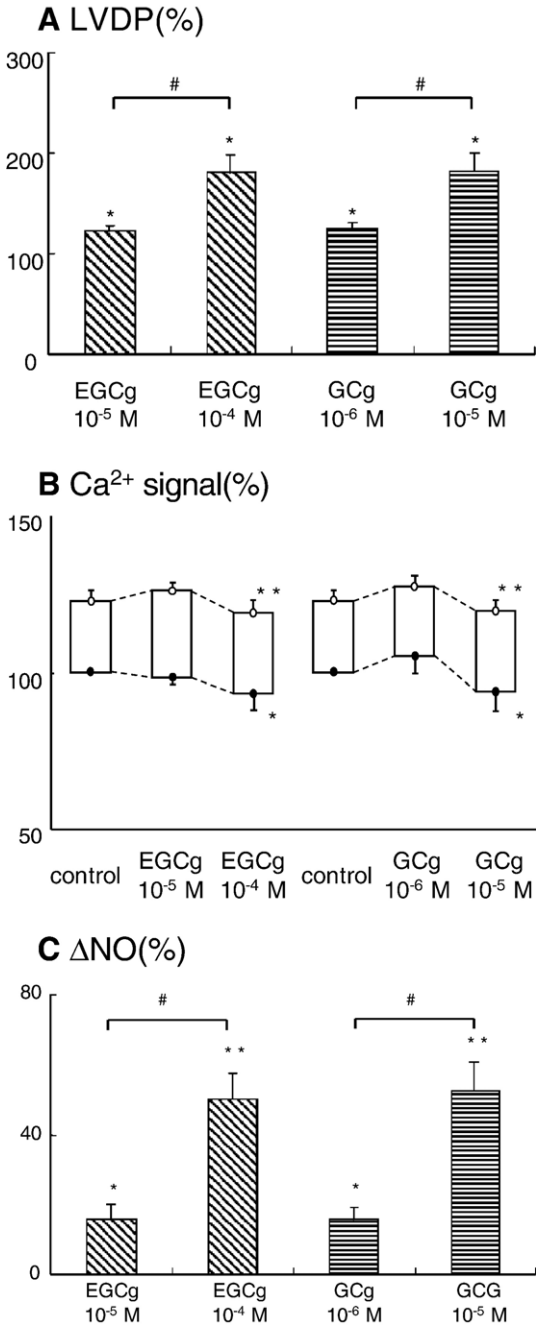

Fig. 2. Effects of EGCg at 10^{-5} M and 10^{-4} M or GCg at 10^{-6} M and 10^{-5} M on the left ventricular developed pressure (A) (as 100% drug-free value) and Ca^{2+} transient signals (B) (the systolic Ca^{2+} signal (○) and the diastolic Ca^{2+} signal (●) as 100% with the diastolic Ca^{2+} signal of drug-free value). (C) Effect of EGCg at 10^{-5} M and 10^{-4} M or GCg at 10^{-6} M and 10^{-5} M on the dose dependent response of NO signals in normal Langendorff hearts. The elevation of NO signals detected maximally with post-treatment of 5-HT 10^{-4} M, and was calculated as 100%. Data are the mean values of 5 preparations; vertical lines represent S.E.M. *$P<0.05$, **$P<0.01$, significantly different from the control (drug-free) value. #$P<0.05$ significant difference between EGCg 10^{-5} M and 10^{-4} M or GCg 10^{-6} M and 10^{-5} M treated groups.

the Ca^{2+} increase induced by these procedures. The final mitochondrial protein concentration was adjusted to 30–35 mg/ml by dilution.

Measurement of caspase-3 activity

The Langendorff heart preparations were perfused with normal Krebs–Henseleit solution (KH solution, pH 7.4, at 37 °C) presaturated with a gas mixture containing 95% O_2 and 5% CO_2. Subsequent to an equilibration period of 30 min required to stabilize the mechanical function, the preparations were perfused with a modified KH solution, in which glucose 10 mM was replaced by 2-deoxy-D-glucose 5 mM and glucose 5 mM 4 min before ischemia for 1 h. After reperfusion for 5 h, the left ventricle was cut into several pieces. EGCg (3×10^{-5} M) or GCg (3×10^{-6} M), a more diluted concentration than that of EGCg, was introduced into the modified perfusate for 4 min before the start of ischemia and was added to the drug-containing medium used for reperfusion. The changes in the cellular levels of high-energy phosphates in the heart, together with simultaneous recordings of LVDP, LVEDP and FR were monitored using ^{31}P-NMR. The proximal portions of the isolated Langendorff hearts were stored in liquid nitrogen for the measurement of the caspase-3 activity using a CPP32/Caspase-3 fluorometric protease assay kit (Casciola-Rosen et al., 1996). The assay is based on the detection of the cleavage of substrate DEVD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). DEVD-AFC emits blue light (λ_{max}=400 nm), but upon cleavage with CPP32 or related caspases, free AFC emits a yellow-green fluorescence (λ_{max}=505 nm), which can be quantified using a fluorometer. Comparison of the fluorescence of AFC from an apoptotic sample with an uninduced control allows determination of the fold-increase in CPP32 activity. The uninduced control was a heart preparation that had been perfused with normal solution (control-1).

Electron paramagnetic resonance (EPR) spectrometry

Measurement of the quenching effect on superoxide anion radicals and hydroxyl radicals

The activities of each catechin in systems producing superoxide anion radical (O_2^- radical) and hydroxyl radical (OH

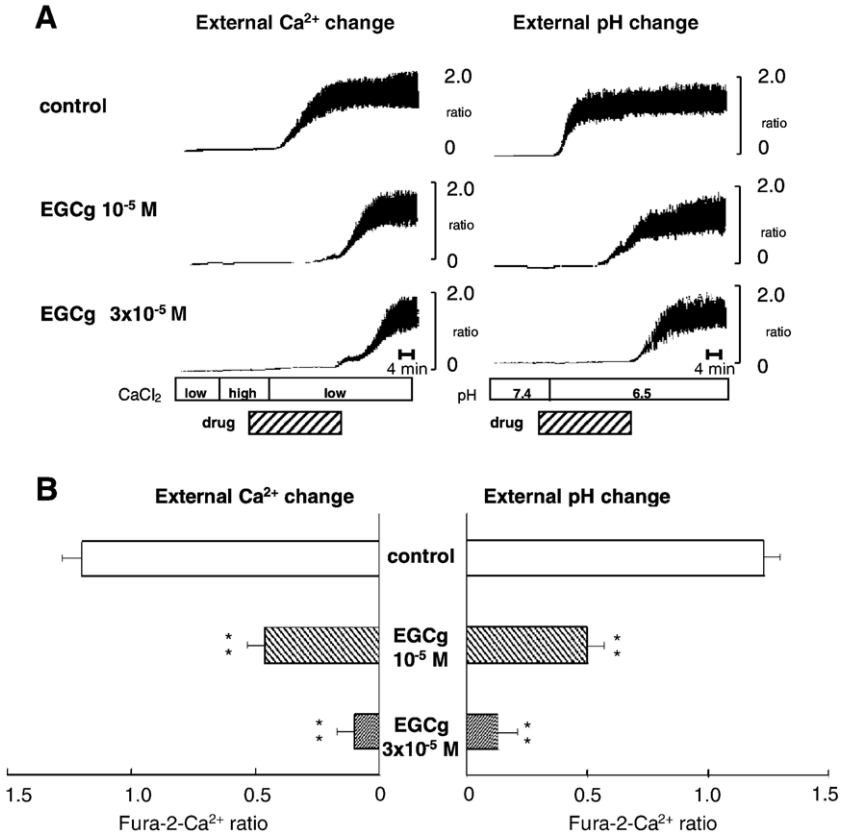

Fig. 3. (A) Recordings of intramitochondrial Ca^{2+} from the fura-2-loaded mitochondria preparations affected by changing the Ca^{2+} concentrations or acidification of the perfusate. The mitochondrial Ca^{2+} signal ($[Ca^{2+}]_m$) gradually increased with extremely high Ca^{2+} (10 μM), and perfusion with low Ca^{2+}-content solution (100 nM) rapidly increased the intramitochondrial Ca^{2+} level (left, top). Perfusate acidification (pH 6.5) in the low Ca^{2+}-content solution (100 nM) produced a more rapid Ca^{2+} signal elevation than that seen with changes in the Ca^{2+} content (right, top). (−)-Epigallocatechin-3-gallate (EGCg) (10^{-5} M and 3×10^{-5} M) reduced the increase in intramitochondrial Ca^{2+} signals induced by a change of perfusate. (B) Comparison of inhibition of the Ca^{2+}-uptake into mitochondria in the control and in the presence of EGCg. The mitochondrial Ca^{2+} level was elevated by an external Ca^{2+} concentration change (left) or external acidification (right). The $[Ca^{2+}]_m$ elevation (A and B) was reduced by EGCg (10^{-5} M and 3×10^{-5} M). **$P<0.01$, significantly different from the control (drug-free) values. The difference between EGCg at 10^{-5} M and at 3×10^{-5} M treated groups is not significant (n.s.). Data are the mean values of 3–5 preparations; vertical lines represent S.E.M.

radical) were determined using EPR spectrometry, as reported previously (Hotta et al., 2004), with some modifications. The final concentration to test the activity to quench the production of both radicals was 10^{-8}–10^{-2} M. The conditions for EPR spectrometry (JES-RE, JEOL Co., Ltd., Tokyo, Japan) to estimate the concentration of O_2^- and OH radicals were as follows: magnetic field: 335.7±5 mT; power: 4 mW 9.414 GHz; modulation: 100 kHz 1×0.079 mT; response: 0.1 s; temperature: 25 °C; amplitude: 160; sweep time: 2 min, respectively.

Superoxide anion radicals (O_2^- radicals)

For the analysis of O_2^- radicals, 50 μl of 2 mM hypoxanthine (Sigma), 35 μl of 10.98 mM diethylene triamine penta acetic acid (DETAPAC, Sigma), 50 μl of each catechin or vehicle (DMSO), 15 μl of 5, 5-dimethyl-1-pyrroline *N*-oxide (DMPO; Labotec, Tokyo, Japan) and 50 μl of xanthine oxidase (XOD, Sigma) were added to a test tube and mixed for 10 s. The mixture was transferred to a special flat cell for the analysis of the DMPO spin adducts of O_2^- radicals. The assay was performed 45 s after the addition of XOD. The signal intensities were evaluated from the peak height of the first signal of the DMPO-O_2^- spin adduct relative to the intensity of the Mn^{2+} signal, with an internal standard used to correct the measurement error.

Hydroxyl radicals (OH radicals)

For the analysis of OH radicals, 75 μl of a 0.1 mM $FeSO_4$ solution, 75 μl of 1 mM hydrogen peroxide (H_2O_2), 50 μl of each catechin or vehicle (methylcellulose), and 50 μl of 4.6 mM DMPO were mixed in a test tube. The amount of DMPO-OH spin adduct that formed was estimated exactly 45 s after the DMPO was added. The signal intensity was evaluated from the peak height of the second signal of the quartet of the DMPO-OH spin adduct relative to the intensity of the Mn^{2+} signal. Standard hydroxyl radical scavengers, such as ascorbic acid and glutathione, were also measured in this system to estimate the concentrations of agents required to inhibit the relative peak height of the DMPO-OH spin adduct.

Chemicals

The following reagents were obtained from the sources indicated: Each purified catechin was obtained from Nagara Science Co. (Gifu, Japan). Cyclosporin A was obtained from

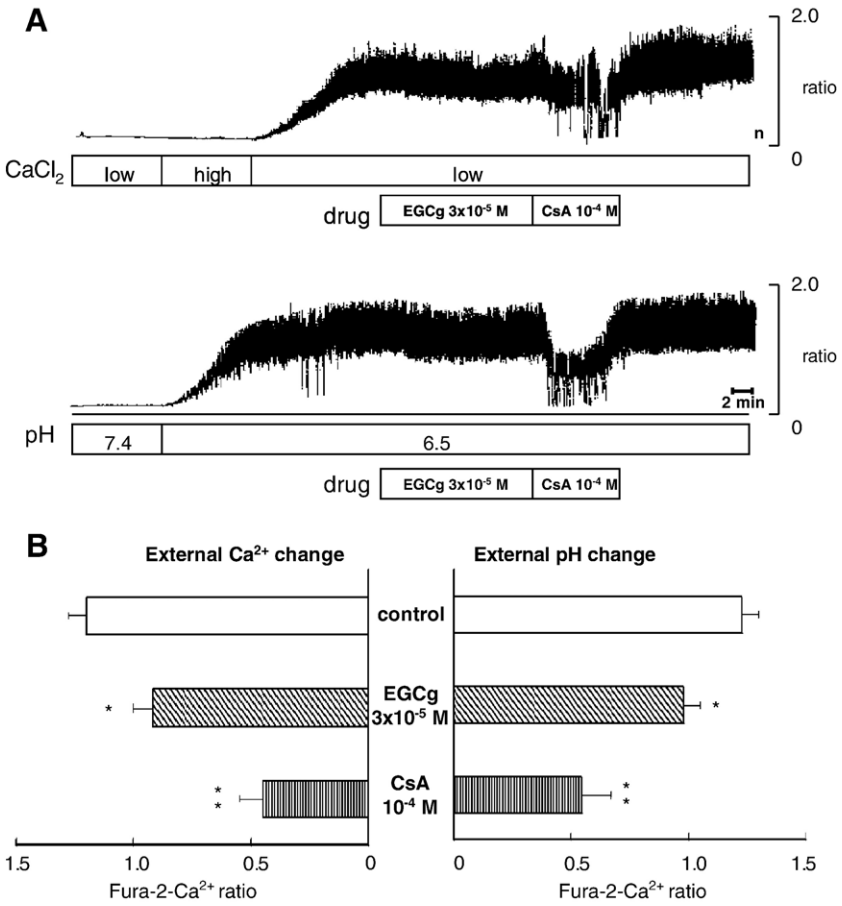

Fig. 4. (A) The mitochondrial Ca^{2+} signal ($[Ca^{2+}]_m$) elevation that brought an external Ca^{2+} concentration change or acidification was reduced by the infusion of (−)-epigallocatechin-3-gallate (EGCg) at 3×10^{-5} M. Further addition of the mitochondrial permeability transition pore inhibitor, cyclosporin A (CsA, 10^{-4} M) decreased the $[Ca^{2+}]_m$ elevation. (B) Comparison of the inhibition of Ca^{2+}-uptake into mitochondria in the controls and in the presence of EGCg 3×10^{-5} M or CsA 10^{-4} M. The $[Ca^{2+}]_m$ elevation was reduced by EGCg 3×10^{-5} M or CsA 10^{-4} M. *$P<0.05$, **$P<0.01$; significantly different from the control (drug-free) values. Data are the mean values of 3–5 preparations; vertical lines represent S.E.M.

Sigma (St. Louis, MO, USA). Fura-2 AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Methylenediphosphonic acid (MDP) and cremophore EL were obtained from Molecular Probes (Eugene, OR, USA). The CPP32/Caspase-3 fluorometric protease assay kit was purchased from NBL (Nagoya, Japan). All other chemicals used in this study were of analytical grade and obtained from commercial sources.

Animals

Throughout the experiments, all animals were handled in accordance with the guidelines for animal experimentation set by the Japanese Association for Laboratory Animal Science.

Statistical analysis

All values are presented as the mean±S.E.M. unless otherwise specified. The unpaired *t* test or analysis of variance (ANOVA), followed by Dunnett's method, was used for the comparison of means between the groups. Statistical significance was defined as $P<0.05$.

Results

Beneficial effects of EGCg or GCg during ischemia–reperfusion in Langendorff heart preparations

Postischemic recovery of contraction

Fig. 1 and Table 1 show the changes in contractility (LVDP) in the drug-free group (control) and the pretreated group during ischemia–reperfusion. In the control hearts, LVDP recovered to less than 34.4±2.8% of the preischemic level (as 100%) at 40 min of reperfusion. In the EGCg (3×10^{-5} M) or GCg (3×10^{-6} M) treated hearts, there was a greater recovery of LVDP, to 78.4±2.5% or 76.2±4.4% of the preischemic value ($P<0.01$), than in the control hearts. The postischemic recovery of EGCg-pretreated hearts was shown with ten times the concentration of GCg.

Postischemic end diastolic pressure

The values of LVEDP as an index of stiffness of the left ventricle obtained at the end of 40 min ischemia (ischemic contracture) in hearts pretreated with EGCg and GCg during ischemia and reperfusion were lower than those in the untreated hearts (Table 1). The main feature in the recovery of the postischemic function was the difference in end-diastolic pressure between the control and drug-treated hearts. In the control group, LVEDP tended to increase during the first 5 min of reperfusion, but thereafter it decreased again and remained low throughout the period of reperfusion. During reperfusion, in the hearts pretreated with EGCg or GCg, there was no further increase in the diastolic pressure, which gradually returned to the preischemic value. The LVEDP values obtained at the end of reperfusion were significantly lower than those in the control group and improved the recovery of LVDP during the first 30 min of reperfusion. The actual values (mmHg) of LVDP and LVEDP are shown in Table 1.

Postischemic coronary flow rate

At the end of reperfusion in the control hearts, the coronary flow rate (FR) was significantly lower than the preischemic level. However, compared to the controls, EGCg or GCg treated hearts showed significant differences at the end of reperfusion (see Table 1).

Effect of EGCg and GCg on high phosphorous energy during ischemia and reperfusion determined with ^{31}P-NMR spectroscopy

As mentioned before, these experiments were carried out in Krebs–Henseleit (KH)-perfusated hearts; ischemia and reperfusion were both measured for 40 min. The effects of EGCg and GCg were investigated, and the drug was introduced into the perfusate 4 min before global ischemia was induced by stopping the perfusate. High phosphorous energy, β-ATP, values are presented graphically as percentages relative to the preischemic baseline with the postischemia recovery rate of LVDP in Fig. 1. During 20 min of ischemia in the pretreated group, β-ATP and PCr did not change significantly. At the end of 40 min ischemia, significant differences in the preservation of ATP were obtained only in the hearts pretreated with EGCg (43.6±3.1% of preischemia as 100%), and GCg (44.4±5.9%) compared with the control (27.6±2.9%). The preservation of ATP on EGCg-pretreated hearts was similar to that of GCg-pretreated hearts. During reperfusion, there was a resynthesis of ATP in the pretreated hearts. Differences between the drug-treated and control hearts were significant at 20 min reperfusion and thereafter. The level of ATP at the end of 40 min reperfusion was significantly higher in the EGCg (69.5±3.5%), and GCg-groups (61.1±3.8%) than in the control group (46.9±4.6%). The recovery of ATP on EGCg-pretreated hearts was not significantly different to that of the GCg-pretreated hearts. A rapid decrease in PCr was observed during ischemia in all groups.

Changes in pH*i* during ischemia and reperfusion were calculated using the equation described in the Materials and methods. The initial (preischemia) level of pH*i* was almost

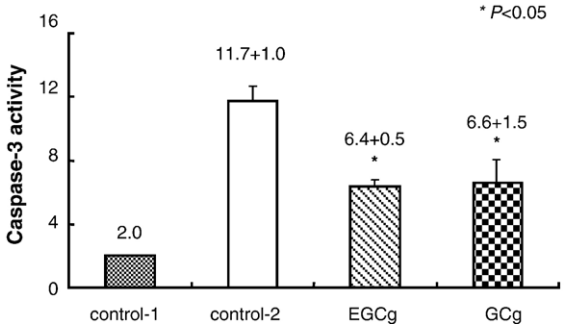

Fig. 5. Control-1: The uninduced control was a heart preparation perfused at 30 min with normal KH solution. Control-2: (−)-epigallocatechin-3-gallate (EGCg) or (−)-gallocatechin-3-gallate (ECg) and the same heart tissue were prepared during 5 h of reperfusion after 1 h of ischemia with KH solution containing 2-deoxy-D-glucose. Values are expressed as the means for 3–5 preparations±S.E.M and indicate the caspase-3 activity. *$P<0.05$; significantly different from the control (drug-free) values.

identical in each group (7.43±0.02). At the end of 36–40 min ischemia, the pH*i* did not differ significantly between the control, pre- or post-treated hearts (control: 6.00±0.04, EGCg: 6.13±0.06, GCg: 6.18±0.08). During reperfusion, the recovery from intracellular acidification observed in the pre- and post-treated hearts was faster than that of the control. However, the pH*i* values in these hearts at 40 min of reperfusion were not significantly different from the control values.

Effects of EGCg or GCg on Ca^{2+} transition signals, NO transient signals and left ventricular developed pressure of Langendorff guinea-pig hearts

EGCg at 10^{-5} M or GCg 10^{-6} M increased the amplitude of transient Ca^{2+} signals (T_{Ca}) concurrently with increases in the left ventricular developed pressure (Fig. 2A, B). On the other hand, high concentration at 10^{-4} M of EGCg or 10^{-5} M GCg decreased the Ca^{2+} level of T_{Ca} similar to a Ca^{2+}-sensitizing agent, pimobendan, with elevation of the left ventricular developed pressure (details of the mechanisms of inotropy will be reported in another paper, Hotta et al., 1995, 2006). Changes in the transient NO signals (T_{NO}) released from the heart tissue in one contraction (LVDP) were observed to be upside-down with respect to the T_{Ca} and transient O_2 signals detected with the pO_2 electrode (Hotta et al., 2002). The level of T_{NO} was increased dose-dependently by the addition of each catechin (EGCg or GCg) without a significant elevation of the heart rate at higher concentrations (Fig. 2C). GCg, having a more positive inotropic effect than EGCg in the experiment with guinea-pig atria, increased the systolic left ventricular developed pressure to the same degree without any change in the diastolic pressure at low concentrations of 10^{-6} M in Langendorff hearts. GCg at 10^{-5} M decreased the level of T_{Ca} with an elevation in the level of T_{NO}. The further addition of

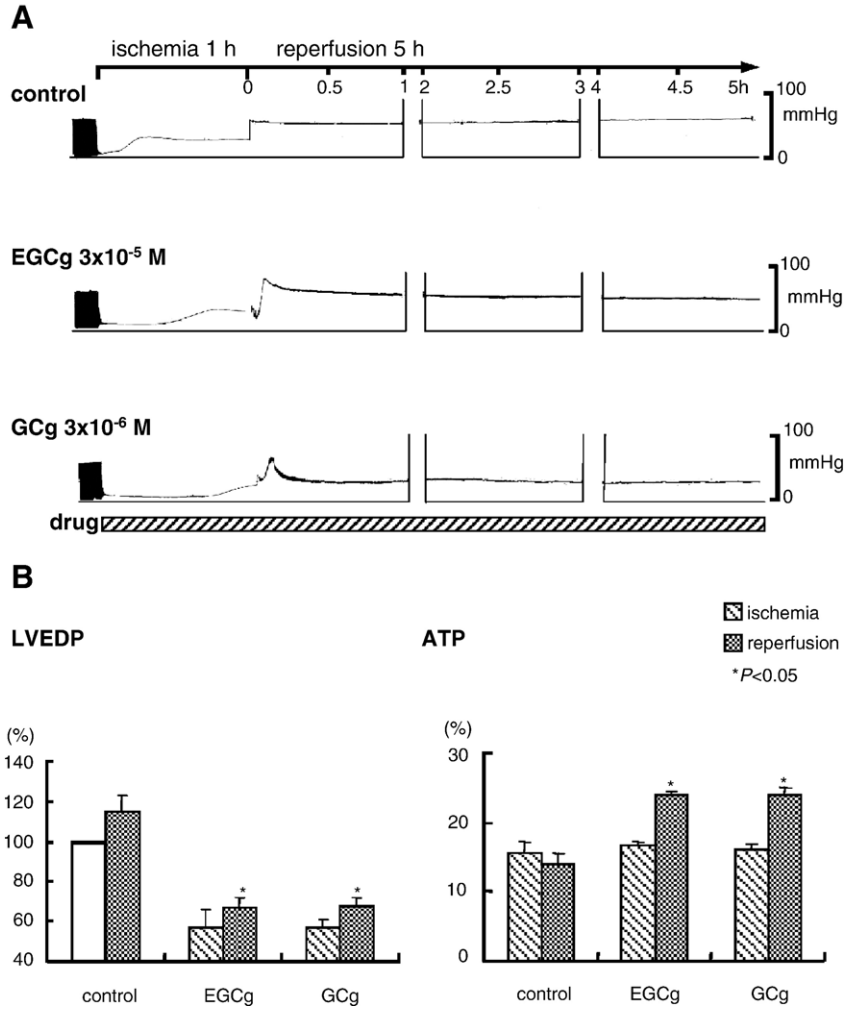

Fig. 6. (A) Beneficial effects of (−)-epigallocatechin-3-gallate (EGCg) or (−)-gallocatechin-3-gallate (ECg) during 1 h of ischemia and 5 h of reperfusion injuries in Langendorff heart preparation for the caspase-3-activity measurements. Control: no drug was treated during ischemia–reperfusion. EGCg 3×10^{-5} M or GCg 3×10^{-6} M was introduced into the perfusion on-line 4 min before the start of ischemia. (B) Relation of the left ventricular end diastolic pressure (LVEDP) and high-energy phosphates (ATP) estimated by ^{31}P-NMR spectra in the presence and absence of (−)-epigallocatechin-3-gallate (EGCg) or (−)-gallocatechin-3-gallate (ECg) during ischemia-reperfusion. The level of ATP showed greater recovery during reperfusion in the pre-treated hearts with GCg or EGCg than the control (drug-free). *$P<0.05$, significantly different from the control value. Data are expressed as the mean values of 5 preparations; vertical lines represent S.E.M. All data of (A) and (B) are described together in Table 2.

Table 2
Left ventricular developed pressure (LVDP), ventricular end diastolic pressure (LVEDP), coronary flow rate (FR) and high phosphorous energy (PCr, β-ATP) changes during ischemia–reperfusion of Langendorff hearts for the TUNEL method and caspase-3-activity measurements

	LVDP (mmHg)			LVEDP (mmHg)			FR(ml/min)		
	Pre	Isch	Rep	Pre	Isch	Rep	Pre	Isch	Rep
Control	51.2±7.4	0	2.0±0.4	10	37.6±3.7	43.2±3.1	15.2±0.4	0	0.4±0.7
EGCg	55.3±5.3	0	3.0±0.6	10	21.3±3.7	25.3±1.8[a]	13.3±0.9	0	2.7±0.3[a]
GCg	53.0±4.0	0	2.5±0.3	10	21.5±1.5	25.5±1.5[a]	13.5±0.5	0	2.5±0.5[a]

ATP (%)			PCr (%)		
Pre	Isch	Rep	Pre	Isch	Rep
100	15.6±1.7	14.0±1.6	100	0	12.3±7.9
100	16.6±0.6	23.9±0.6[a]	100	0	22.3±1.6[a]
100	16.1±0.7	24.1±1.0[a]	100	0	23.4±3.9[a]

Changes during 1 h of ischemia and a subsequent 5 h of reperfusion.
Values are the mean±S.E.M, n=4–6.
[a]$P<0.05$; significantly different from the control values.
Pre, preischemia; Isch, ischemia; Rep, reperfusion.

GCg at 10^{-4} M showed additional decreases in the level of T_{Ca} signals in response to the elevation of T_{NO}.

Intramitochondrial Ca^{2+} measurements with fura-2 AM

The intramitochondrial fura-2 Ca^{2+} signal ($[Ca^{2+}]_m$) increased steadily and linearly, but was only 10% higher at extremely high perfusate Ca^{2+} concentrations (1 μM–1 mM) in the perfusate (Fig. 3A). The displacement of the normal matrix Ca^{2+} concentration (100 nM) with perfusate produced a rapid and intense increase of up to about 8.6-fold (1.2±0.07), while the change in $[Ca^{2+}]_m$ caused by displacement of the perfusate to a high Ca^{2+} concentration was regarded as 100%. Pretreatment of mitochondria with EGCg 10^{-5} M and 3×10^{-5} M markedly suppressed the increase in $[Ca^{2+}]_m$, which occurred in the drug-free perfusate by 41.7% (0.5±0.07) and 8.3% (0.1±0.07) compared with the control (drug-free) values (Fig. 3B).

Changing the pH from 7.4 to 6.5 in the low-Ca^{2+} content perfusate (100 nM) elevated the $[Ca^{2+}]_m$ to the maximal extent, up to about ten times as much relative to the pH 7.4 perfusate, which was regarded as 100% (Fig. 3A). Furthermore, it was greater and more rapid than that caused by perfusion with a low-Ca^{2+} perfusate. The appreciable $[Ca^{2+}]_m$ elevation induced by acidification (1.25±0.05) was reduced by the pretreatment with EGCg 10^{-5} M by 40.0% (0.5±0.07) or EGCg 3×10^{-5} M by 10.4% (0.13±0.08) than the control (drug-free) values (Fig. 3B).

As shown in Fig. 4A, the addition of EGCg 3×10^{-5} M to the perfusate increased steadily and constantly the change in the concentration of perfusate Ca^{2+} or the acidity of the perfusate released from mitochondria. This effect was similar to that seen

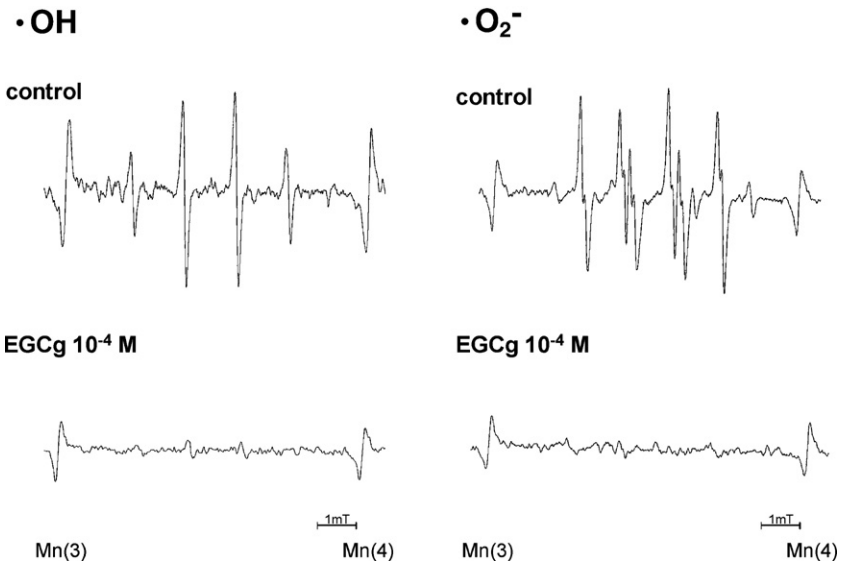

Fig. 7. Representative EPR spectra of a spin adduct showing hydroxyl radical (OH radical, left) or super oxide anion radical (O_2^- radical, right) by (−)-epigallocatechin-3-gallate (EGCg) at 10^{-4} M compared with the controls (drug-free). Each catechin at final concentrations from 10^{-8} M to 10^{-2} M quenched the generation of OH and O_2^- radicals and the IC_{50} value of each catechin is mentioned in the Results.

Table 3
In vitro free radical quenching activity of various catechins

Drug	OH–IC_{50}	O_2^-–IC_{50}
(−)-Epicatechin	1.3×10^{-3} M	6.1×10^{-5} M
(−)-Epigallocatechin	1.1×10^{-4} M	8.2×10^{-6} M
(−)-Epicatechin gallate	1.2×10^{-5} M	2.9×10^{-5} M
(−)-Gallocatechin gallate	1.3×10^{-5} M	9.0×10^{-6} M
(−)-Epigallocatechin gallate	6.6×10^{-6} M	7.6×10^{-6} M
Trolox (VE derivative)	2.3×10^{-4} M	1.5×10^{-3} M
Vitamin C	1.0×10^{-4} M	9.0×10^{-5} M

The IC_{50} (concentration causing a 50% scavenging effect) values for catechins, trolox (vitamin E derivative) and vitamin C.

with cyclosporin A (CsA, 10^{-4} M), which inhibits the mitochondrial permeability transition pore (PTP). The decrease in $[Ca^{2+}]_m$ caused by the administration of CsA was greater than that produced by EGCg [External pH change: EGCg 3×10^{-5} M by 80.0% ($0.14\rightarrow1.01\pm0.07$), CsA 10^{-4} M by 75.2% ($0.14\rightarrow0.80\pm0.04$, $P<0.05$), External Ca^{2+} change: EGCg 3×10^{-5} M by 74.0% ($0.14\rightarrow0.92\pm0.08$, $P<0.05$), CsA 10^{-4} M by 64.0% ($0.14\rightarrow0.80\pm0.06$, $P<0.01$)] (Fig. 4B).

The caspase-3 activity in the control and apoptotic samples

Examples of apoptotic tissues obtained after the various treatments are shown in our previous report (Hotta et al., 2004). Apoptotic cells were first observed in the ventricle after 5 h of reperfusion after 1 h of ischemia with KH solution containing 2-deoxy-D-glucose. The caspase-3 activity in the control and apoptotic samples was measured using the same frozen tissues with the TUNEL method. EGCg and GCg were not measured using the TUNEL method. Control-1 was a heart preparation perfused for 30 min with normal KH solution (considered to be 2.0), while control-2 was a heart subjected to 1 h of ischemia and 5 h of reperfusion with a drug-free solution (control). There was a decrease in caspase-3 activity in the heart pretreated with EGCg or GCg [control-1: 2.0, control-2: 11.7 ± 1.0, EGCg: 6.4 ± 0.5 ($P<0.05$ for control-2), GCg: 6.6 ± 1.5 ($P<0.05$ for control-2)] (Fig. 5).

As shown in Fig. 6A, B and Table 2, which lists the changes in mechanical function and high-energy phosphates, the LVEDP, β-ATP and PCr were significantly different in the hearts treated with EGCg at 3×10^{-5} M and GCg at 3×10^{-6} M, a more diluted concentration than that of EGCg, after 5 h of reperfusion compared with the control ($P<0.05$). From these results, the preventive effects of EGCg or GCg on the tissue levels of high-energy phosphate were greater than those of the control.

In vitro free radical quenching activity of each catechin

In the experiment on quenching activity, each purified catechin, trolox (vitamin E derivative) and vitamin C at final concentrations from 10^{-7} M to 10^{-2} M, dose-dependently quenched the generation of OH radicals and the generation of O_2^- radicals. Those respective EPR spectra of a spin adduct showing OH radicals and O_2^- radicals of EGCg at 10^{-4} M were compared with the controls (Fig. 7). The IC_{50} (concentration causing a 50% scavenging effect) values for catechin, trolox (vitamin E derivative) and vitamin C are shown in Table 3. The O_2^- and OH radical quenching activities of each purified catechin were attenuated more than those of vitamin C and E.

Discussion

In this present study we investigated the beneficial effects of purified original catechin EGCg (2*R*, 3*R* type) and the C-2 epimeric isomer GCg (2*S*, 3*R* type), which is produced during heat processing at high temperatures (Yoshino et al., 2004), on ischemia–reperfusion injury in isolated guinea-pig Langendorff hearts. EGCg at 3×10^{-5} M and GCg at 3×10^{-6} M, a more dilute concentration than that of EGCg, had a similar pronounced effect on the recovery of LVDP (EGCg: 78.4%, GCg: 76.2% vs. drug-free heart, control 34.4%) and a significant preserving effect (EGCg: 69.5%, GCg: 61.1% with the preischemia control as 100%) on the tissue levels of high-energy phosphate (ATP and Phosphocreatine) measured at the final stage of ischemia and after reperfusion compared with that (control: 46.9%) of drug-free hearts (Fig. 1). These findings strongly indicated that mitochondrial function, in close association with the synthesis of high phosphorous energy, ATP, constituted an important part of the regulatory mechanisms involved in myocardial ischemic injury.

On reperfusion in Langendorff guinea-pig hearts, the cytosolic Ca^{2+} concentration that had increased during global ischemia rapidly returned to the control level, as previously reported (Hotta et al., 1998, 2001a). The fura-2 Ca^{2+} signals in isolated myocardial mitochondria ($[Ca^{2+}]_m$), preloaded with abnormally high Ca^{2+} levels using a superfusion technique, increased rapidly, reaching a maximum level during perfusion of a solution containing a cytosolic level of Ca^{2+} (~100 nM) (Fig. 3). In addition, the elevation of $[Ca^{2+}]_m$ was quickly detected on acidification of the perfusate, similar to the final stage of global ischemia in Langendorff hearts. These increases in the perfused mitochondrial preparation were attenuated dose-dependently by pretreatment with EGCg 10^{-5} M or 3×10^{-5} M and drugs having a beneficial effect on Langendorff hearts (Hotta et al., 1998, 1999, 2001a). From these findings, it was suggested that for Ca^{2+} pumping at the time of reperfusion, mitochondria play an essential role in cellular Ca^{2+} homeostasis for the maintenance of cellular function of the heart, acting as a Ca^{2+} scavenger in the cytosol (Tani and Neely, 1989; Brooks et al., 1995). Mitochondrial Ca^{2+} uptake due to acidification or a Ca^{2+} concentration change of the perfusate was significantly suppressed compared with the control on treatment with EGCg 3×10^{-5} M (Fig. 4). These two $[Ca^{2+}]_m$ elevations with the change of the Ca^{2+} content and acidification of the perfusate in the mitochondrial preparation were similar to findings with a mitochondrial permeability transition pore (MPTP) opener, atractyroside (Hotta et al., 2006). Meanwhile, the depression of $[Ca^{2+}]_m$ elevation by treatment with EGCg gave the same results as with cyclosporin A (Fig. 4A), pH 8.5 or Mg^{2+}, a well-known inhibitor of MPTP. We also confirmed that K_{ATP} channel openers, nicorandil and diazepoxide, likely inhibit the opening

of MPTP by preventing $[Ca^{2+}]_m$ overload-induced apoptosis in ischemia–reperfusion guinea-pig hearts (Huang et al., 2005). Therefore, these observations of $[Ca^{2+}]_m$ by changing the perfusate in the mitochondrial preparation might make it possible to anticipate mitochondrial function associated with the regulation of MPTP.

Irrespective of its exact composition, the MPTP complex contains multiple targets for pharmacological investigations involved in different pathways of apoptosis induction as sensors for stress and damage, as well as for certain signals connected to the receptors (Kroemer et al., 1998; Green, 1998). There is now evidence that apoptosis, or programmed cell death, is an important response of the myocardium to ischemia and reperfusion, and appears to contribute to the overall sequelae of cardiac injury. Substances that prevent the opening of MPTP induced by cardiac injury may preserve mitochondrial function and may thus have potential as beneficial agents.

The proportion of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay (TUNEL)-positive myocytes in drug-free guinea-pig hearts subjected to 1 h of ischemia followed by 5 h of reperfusion with KH solution containing 5 mM 2-deoxy-D-glucose was approximately 30%, and thus a longer period of reperfusion may lead to accelerated apoptosis (Hotta et al., 2004). The administration of EGCg or GCg following 5 h of reperfusion significantly affected caspase-3 activity in the tissue used for the TUNEL assay compared with that in drug-free hearts. The effect of EGCg or GCg on caspase-3 activity after ischemia and reperfusion was less than that of the control (Fig. 5). However, EGCg or GCg significantly improved the recovery of left ventricular end diastolic pressure (LVEDP) and coronary flow rate (FR), with increases in the levels of high-energy phosphates (ATP, PCr), compared with those in drug-free hearts during a 5-h longer reperfusion (Fig. 6A, B and Table 2). The beneficial effect of EGCg or GCg provided greater protection of the ischemic myocardium than that of hearts subjected to reperfusion procedures. With in vitro EPR spectrometry (Fig. 7), the OH and O_2^- radical-quenching activities (IC_{50}) of each catechin were attenuated more than those of vitamin C or E. The opening of MPTP might have reset the mitochondrial Ca^{2+} overload, allowing mitochondrial Ca^{2+} efflux (Guarnieri et al., 1997). Petit et al. (1996) showed that MPTP opening causes release into the cytosol of an intermembrane protein able to trigger apoptosis. Recent studies seem to indicate cytochrome C as this factor (Kluck et al., 1997). Therefore, we speculate that under conditions of mitochondrial reactive oxygen species (ROS) accumulation, cells can be damaged either by ROS-mediated reactions or by starting a suicide program leading to self-elimination, through the activation of long-lasting MPTP opening. These mechanisms could represent the hypothetical role of mitochondrial ROS in prolonged myocardial stunning leading to cardiac failure. On the other hand, a lower level of mitochondrial H_2O_2 could induce a series of reversible ROS-mediated reactions, including MPTP opening, followed by its closure, which could favor the recovery of mitochondrial and cardiac function.

In addition to these observations, EGCg or the C-2 epimer GCg, showed dose-dependent NO release from a Langendorff heart with a positive inotropic effect (Hotta et al., 2006). In another study of the right atria, the contractile force of GCg produced a higher pD_2 value than EGCg, and neither catechin affected the heart rate. The high dose of EGCg (10^{-4} M) or GCg (10^{-5} M) produced maximum LVDP with decreases in the basal level of T_{Ca} in a manner similar to the administration of the Ca-sensitizer pimobendan. The change in NO released from the heart by treatment with GCg was shown to increase more than with EGCg (Fig. 2). In another of our studies of guinea-pig hearts, the NO supplied by serotonin derivatives with antioxidative activity from safflower oil (Hotta et al., 2002), or the specific NO donor FK409, had a cardioprotective action, presumably by scavenging cytosolic superoxide anion (Cao et al., 2001). In addition to the most potent antioxidant effect, EGCg or GCg, like the NO donor, may have therapeutic use as an NO-mediated vasorelaxant (Huang et al., 1999; Sanae et al., 2002) and have additional protective action in myocardial ischemia–reperfusion-induced injury. Additionally, other extracts of red wine, grapes and French maritime pine were shown to exhibit endothelium-dependent relaxing (EDR) effects to varying degrees and beneficial effects on heart injury (Fitzpatrick et al., 1998). In the EDR response, the constituent endothelial nitric oxide (eNOS) enzyme is stimulated to produce NO. The effects on NO enhancement of catechins may help to obtain information regarding the identity of these compounds for the prevention of myocardial injury.

In summary, in ischemic–reperfusion injury hearts, we found that the C-2 epimer GCg, which is produced due to the heating process, showed equal cardioprotective effects by administration of the lower concentration than that of EGCg associated with the inhibition of cardiomyocyte apoptosis (caspase-3 activity) (Fig. 5). These findings strongly indicated that mitochondrial function, in close association with the synthesis of high-energy phosphates, is an important part of the regulatory mechanisms involved in myocardial ischemic injury. Identification of the factors regulating cardiomyocyte cell death is important to understand the pathogenesis of congenital heart diseases. The link between the signaling circuity of external stimuli and mitochondrial apoptotic machinery is of wide interest in cardiac diseases (Aneja et al., 2004; Stephanou, 2004). Further investigation of the different cardiac effects of another purified catechin, which was synthesized as the lead compound GCg should provide valuable information on the normal physiology of cell death with reference to cardiomyocyte apoptosis, and help in the design of cardioprotective drugs. Additional studies are also needed to better understand the dose-response relationships in the plasma levels of catechin or in urinary excretion of EGCg or GCg after the ingestion of tea to understand the bioavailability of tea catechins in humans.

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References

Akinyanju, P., Yudkin, J., 1967. Effect of coffee and tea on serum lipids in the rat. Nature 214, 426–427.

Aneja, R., Hake, P.W., Burroughs, T.J., Denenberg, A.G., Wong, H.R., Zingarelli, B., 2004. Epigallocatechin, a green tea polyphenol, attenuates myocardial ischemia reperfusion injury in rats. Molecular Medicine 10, 55–62.

Brooks, W.W., Conrad, C.H., Morgan, J.P., 1995. Reperfusion induced arrhythmias following ischaemia in intact rat heart: role of intracellular calcium. Cardiovascular Research 29, 536–542.

Cao, Y., Hotta, Y., Shioi, K., Nagata, Y., Kawai, N., Ishikawa, N., 2001. Protective effects of FK409, a novel NO-donor, against post-ischemic myocardial dysfunction in guinea-pig hearts. Journal of Cardiovascular Pharmacology 38, 593–605.

Casciola-Rosen, L., Nicholson, D.W., Chong, T., Rowan, K.R., Thornberry, N.A., Miller, D.K., Rosen, A., 1996. Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. Journal of Experimental Medicine 183, 1957–1964.

Fitzpatrick, D.F., Bing, B., Rohdewald, P., 1998. Endothelium-dependent vascular effects of Pycnogenol. Journal of Cardiovascular Pharmacology 32, 509–515.

Fujino, K., Sperelakis, N., Solaro, R.J., 1988. Sensitization of dog and guinea pig heart myofilaments to Ca^{2+} activation and the inotropic effect of pimobendan: comparison with milrinone. Circulation Research 63, 911–922.

Giakoustidis, A.E., Giakoustidis, D.E., Iliadis, S., Papageorgiou, G., Koliakou, K., Kontos, N., Taitzoglou, I., Botsoglou, E., Papanikolaou, V., Atmatzidis, K., Takoudas, D., Antoniadis, A., 2006. Attenuation of intestinal ischemia/reperfusion induced liver and lung injury by intraperitoneal administration of (and)-epigallocatechin-3-gallate. Free Radical Research 40, 103–110.

Green, D.R., 1998. Apoptotic pathways: the roads to ruin. Cell 94, 695–698.

Griffiths, E.J., Ocampo, C.J., Savage, J.S., Stern, M.D., Silverman, H.S., 2000. Protective effects of low and high doses of cyclosporin A against reoxygenation injury in isolated rat cardiomyocytes are associated with differential effects on mitochondrial calcium levels. Cell Calcium 27, 87–95.

Guarnieri, C., Finelli, C., Zini, M., Muscari, C., 1997. Effects of trimetazidine on the calcium transport and oxidative phosphorylation of isolated rat heart mitochondria. Basic Research in Cardiology 92, 90–95.

Guo, Q., Zhao, B., Shen, S., Hou, J., Hu, J., Xin, W., 1999. ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers. Biochimica et Biophysica Acta 1427, 13–23.

Hausenloy, D., Wynne, A., Duchen, M., Yellon, D., 2004. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. Circulation 109, 1714–1717.

Hotta, Y., Ando, H., Fujita, M., Nakagawa, J., Takeya, K., Sakakibara, J., 1995. Different effects of isoproterenol and dihydroouabain on cardiac Ca^{2+} transients. European Journal of Pharmacology 282, 121–130.

Hotta, Y., Fujita, M., Nakagawa, J., Ando, H., Takeya, K., Ishikawa, N., Sakakibara, J., 1998. Contribution of cytosolic ionic and energetic milieu change to ischemia- and reperfusion-induced injury in guinea pig heart: fluorometry and nuclear magnetic resonance studies. Journal of Cardiovascular Pharmacology 31, 146–156.

Hotta, Y., Otsuka-Murakami, H., Fujita, M., Nakagawa, J., Yajima, M., Liu, W., Ishikawa, N., Kawai, N., Masumizu, T., Kohno, M., 1999. Protective role of nitric oxide synthase against ischemia–reperfusion injury in guinea pig myocardial mitochondria. European Journal of Pharmacology 380, 37–48.

Hotta, Y., Nakagawa, J., Ishikawa, N., Wakida, Y., Ando, H., Takeya, K., Ohashi, N., Matsui, K., 2001a. Protective effect of SM-20550, a selective Na^{+}–H^{+} exchange inhibitor, on ischemia–reperfusion-injured hearts. Journal of Cardiovascular Pharmacology 37, 143–154.

Hotta, Y., Ishikawa, N., Ohashi, N., Matsui, K., 2001b. Effects of SM-20550, a selective Na^{+}–H^{+} exchange inhibitor, on the ion transport of myocardial mitochondria. Molecular and Cellular Biochemistry 219, 83–90.

Hotta, Y., Nagatsu, A., Wei, L., Muto, T., Narumiya, C., Xiuli, L., Yajima, M., Ishikawa, N., Ohashi, N., Miyazeki, K., Kawai, N., Mizukami, H., Sakakibara, J., 2002. Protective effects of antioxidative serotonin derivatives isolated from safflower against postischemic myocardial dysfunction. Molecular and Cellular Biochemistry 238, 151–162.

Hotta, Y., Nishimaki, H., Takeo, T., Itoh, G., Yajima, M., OtsukaMurakami, H., Ishikawa, N., Kawai, N., Huang, L., Yamada, K., Yamamoto, S., Matsui, K., Ohashi, N., 2004. Differences in the effects of Na^{+}–H^{+} exchange inhibitors on cardiac function and apoptosis in guinea-pig ischemia-reperfused hearts. European Journal of Pharmacology 503, 109–122.

Hotta, Y., Huang, L., Muto, T., Yajima, M., Miyazeki, K., Ishikawa, N., Fukuzawa, Y., Wakida, Y., Thusima, H., Ando, H., Nonogaki, T., 2006. Positive inotropic effect of purified green tea catechin derivative in guinea pig hearts: the measurements of cellular Ca^{2+} and nitric oxide release. European Journal of Pharmacology 552, 123–130.

Huang, Y., Chan, N.W., Lau, C.W., Yao, X.Q., Chan, F.L., Chen, Z.Y., 1999. Involvement of endothelium/nitric oxide in vasorelaxation induced by purified green tea (−)epicatechin. Biochimica et Biophysica Acta 1427, 322–328.

Huang, L., Hotta, Y., Wakida, Y., Feng, G.G., Ando, H., Ishikawa, N., 2005. Protective effects of nicorandil against cardiac function and apoptosis in ischemia–reperfusion guinea-pig myocardial injury. Journal of Pharmacological Sciences 97 (Suppl. I) (191 pp).

Huang, L., Hotta, Y., Miyazeki, K., Ishikawa, N., Miki, Y., Sugimoto, Y., Yamada, J., Nakano, A., Nishiwaki, K., Shimada, Y., 2006. Different effects of optical isomers of the 5-HT_{1A} antagonist pyrapyridolol against postischemic guinea-pig myocardial dysfunction and apoptosis through the mitochondrial permeability transition pore. European Journal of Pharmacology 534, 165–177.

Ichimori, K., Ishida, H., Fukahori, M., Nakazawa, H., Murakami, E., 1994. Practical nitric oxide measurement employing a nitric oxide-sensitive electrode. Review of Scientific Instruments 65, 2714–2718.

Ikeda, I., Tsuda, K., Suzuki, Y., Kobayashi, M., Unno, T., Tomoyori, H., Goto, H., Kawata, Y., Imaizumi, K., Nozawa, A., Kakuda, T., 2005. Tea catechins with a galloyl moiety suppress postprandial hypertriacylglycerolemia by delaying lymphatic transport of dietary fat in rats. Journal of Nutrition 135, 155–159.

Imai, K., Nakachi, K., 1995. Cross sectional study of effects of drinking green tea on cardiovascular and liver diseases. British Medical Journal 310, 693–696.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., Newmeyer, D.D., 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275, 1132–1136.

Koike, A., Akita, T., Hotta, Y., Takeya, K., Kodama, I., Murase, M., Abe, T., Toyama, J., 1996. Protective effects of dimethyl amiloride against postischemic myocardial dysfunction in rabbit hearts: phosphorus 31-nuclear magnetic resonance measurements of intracellular pH and cellular energy. The Journal of Thoracic and Cardiovascular Surgery 112, 765–775.

Kono, S., Shinchi, K., Wakabayashi, K., Honjyo, S., Todoroki, I., Sakurai, Y., Imanishi, K., Nishikawa, H., Ogawa, S., Katsurada, M., 1996. Relation of green tea consumption to serum lipids and lipoproteins in Japanese men. Journal of Epidemiology 6, 128–133.

Kroemer, G., Dallaporta, B., Resche-Rigon, M., 1998. The mitochondrial death/life regulator in apoptosis and necrosis. Annual Review of Physiology 60, 619–642.

Lim, D.Y., Lee, E.S., Park, H.G., Kim, B.C., Hong, S.P., Lee, E.B., 2003. Comparison of green tea extract and epigallocatechin-3-gallate on blood pressure and contractile responses of vascular smooth muscle of rats. Archives of Pharmacal Research 26, 214–223.

Muramatsu, K., Fukuyo, M., Hara, Y., 1986. Effect of green tea catekins on plasma cholesterol level in cholesterol-fed rats. Journal of Nutritional Science and Vitaminology 32, 613–622.

Muto, T., Hotta, Y., Miyazeki, K., Ando, H., Ishikawa, N., Hasegawa, T., Sugimoto, Y., Yamada, J., Miki, Y., 2005. Protective effects of sarpogrelate, a 5-HT_{2A} antagonist, against postischemic myocardial dysfunction in guinea-pig heart. Molecular and Cellular Biochemistry 272, 119–132.

Nakagawa, T., Yokozawa, T., 2002. Direct scavenging of nitric oxide and superoxide by green tea. Food and Chemical Toxicology 40, 1745–1750.

Petit, P.X., Susin, S.A., Zamzami, N., Mignotte, B., Kroemer, G., 1996. Mitochondria and programmed cell death: back to the future. FEBS Letters 396, 7–13.

Sanae, F., Miyaichi, Y., Kizu, H., Hayashi, H., 2002. Effects of catechins on vascular tone in rat thoracic aorta with endothelium. Life Sciences 71, 2553–2562.

Stephanou, A., 2004. Role of STAT-1 and STAT-3 in ischaemia/reperfusion injury. Journal of Cellular and Molecular Medicine 8, 519–525.

Tani, M., Neely, J.R., 1989. Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H^+–Na^+ and Na^+–Ca^{2+} exchange. Circulation Research 65, 1045–1056.

Townsend, P.A., Scarabelli, T.M., Pasini, E., Gitti, G., Menegazzi, M., Suzuki, H., Knight, R.A., Latchman, D.S., Stephanou, A., 2004. Epigallocatechin-3-gallate inhibits STAT-1 activation and protects cardiac myocytes from ischemia/reperfusion-induced apoptosis. FASEB Journal 18, 1621–1623.

Tsubono, Y., Tsugane, S., 1997. Green tea intake in relation to serum lipid levels in Middle-aged Japanese men and women. Annals of Epidemiology 7, 280–284.

Xu, J.Z., Yeung, S.Y., Chang, Q., Huang, Y., Chen, Z.Y., 2004. Comparison of antioxidant activity and bioavailability of tea epicatechins with their epimers. British Journal of Nutrition 91, 873–881.

Yoshino, K., Ogawa, K., Miyase, T., Sano, M., 2004. Inhibitory effects of the C-2 epimeric isomers of tea catechins on mouse type IV allergy. Journal of Agricultural and Food Chemistry 52, 4660–4663.

Young, W., Hotovec, R.L., Romero, A.G., 1967. Tea and atherosclerosis. Nature 216, 1015–1016.

Zhang, A., Zhu, Q.Y., Luk, Y.S., Ho, K.Y., Fung, K.P., Chen, Z.Y., 1997. Inhibitory effects of jasmine green tea epicatechin isomers on free radical-induced lysis of red blood cells. Life Sciences 61, 383–394.