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Life Sciences 77 (2005) 2770–2782

Life Sciences

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Effects of emodin treatment on mitochondrial ATP generation capacity and antioxidant components as well as susceptibility to ischemia–reperfusion injury in rat hearts: Single versus multiple doses and gender difference

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Received 28 December 2004; accepted 15 March 2005

Abstract

Effects of emodin (EMD) treatment on mitochondrial ATP generation capacity and antioxidant components as well as susceptibility to ischemia–reperfusion (I–R) injury were examined in male and female rat hearts. Isolated-perfused hearts prepared from female rats were less susceptible to I–R injury than those of male rats. I–R caused significant decreases in ATP generation capacity and reduced glutathione (GSH) and α-tocopherol (α-TOC) levels as well as glutathione reductase, Se-glutathione peroxidase and Mn-superoxide dismutase (SOD) activities. The lower susceptibility of female hearts to myocardial I–R injury was associated with higher levels of GSH and α-TOC as well as activity of SOD than those of male hearts. EMD treatment at 3 daily doses (0.6 or 1.2 mmol/kg) could enhance myocardial mitochondrial ATP generation capacity and antioxidant components in both male and female rat hearts, but it only significantly protected against I–R injury in female hearts. Treatment with a single dose of EMD invariably enhanced mitochondrial antioxidant components and protected against I–R injury in both male and female hearts. The gender-dependent effect of EMD treatment at multiple

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0024-3205/$ - see front matter
doi:10.1016/j.lfs.2005.03.027

doses may be related to the differential antioxidant response in the myocardium and/or induction of drug metabolizing enzymes in the liver.

Keywords: Emodin; Myocardial ischemia–reperfusion injury; Mitochondrion; Glutathione; α-Tocopherol; ATP

Introduction

Emodin (EMD, Fig. 1) is an active anthraquinone constituent of the root of *Polygonum multiflorum* Thunb. (Polygonaceae), a traditional Chinese herb that has long been used in tonic and anti-aging remedies (Chen and Li, 1993; Huang, 1993). Early pharmacological studies have demonstrated that EMD possesses anti-mutagenic, anti-cancer, anti-diuretic, vasorelaxant and immunosuppressive activities (Su et al., 1995; Koyama et al., 1988; Zhou and Chen, 1988; Huang et al., 1991). EMD is considered as a generator of reactive oxygen species owing to its ability to carry out one-electron transfer (Shi et al., 1994). Our laboratory has also shown that EMD pretreatment can protect against myocardial ischemia–reperfusion (I–R) injury in rats (Yim et al., 1998). The cardioprotection was associated with an enhancement in tissue glutathione status (Yim et al., 1998). While whether EMD possesses any in vitro and in vivo free radical scavenging activities is still unknown, it has been shown that the immunosuppressive and anti-cancer actions of EMD are mediated by its pro-oxidant activity (Huang et al., 1991; Yang et al., 2004; Yi et al., 2004). However, the biochemical mechanism involved in the cardioprotection afforded by EMD pretreatment remains unclear. In this connection, we have recently demonstrated the ability of EMD to enhance the hepatic mitochondrial glutathione status in mice (Chiu et al., 2002). Given the crucial role of mitochondria for cell survival (Green and Reed, 1998; Lemasters et al., 1999), we endeavored to investigate the effects of EMD treatment on mitochondrial ATP generation capacity and antioxidant components in rat hearts in relation to the changes in susceptibility to I–R injury. Epidemiological studies indicated that the risk of developing cardiovascular disease, particularly myocardial infarction, is considerably less in premenopausal females than in age-matched males in humans (Hayward et al., 2000). It is therefore possible that a sexual dimorphism in the extent of myocardial damage induced by ischemia also exists in rats. Experimental studies also showed that mitochondria from females exhibited higher antioxidant capacity and lower oxidative damage than males in rats (Borrás et al., 2003). Given the possible gender difference in myocardial susceptibility to oxidative challenge such as I–R, we also compared the effects of EMD treatment on the myocardium in both male and female rats. Since antioxidants are widely used for protecting against

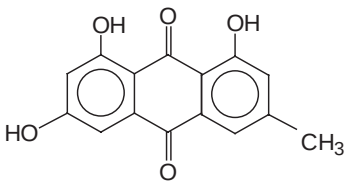

Fig. 1. Chemical structure of emodin (EMD).

myocardial I–R injury (Marczin et al., 2003), α-tocopherol (α-TOC), a lipid-soluble free radical scavenging antioxidant (Kagan et al., 1990), and α-lipoic acid (α-LA), a thiol-enhancing antioxidant (Parker et al., 1997), were also included in the study for comparison.

Materials and methods

Chemicals

EMD was purchased from ACROS Organics (New Jersey, USA). Reduced glutathione (GSH), oxidized glutathione, glutathione reductase (GRD), xanthine oxidase, xanthine, cytochrome *c*, α-TOC and α-LA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. Solvents used for high-performance liquid chromatography were of HPLC grade.

Animal pretreatment

Male and female adult Sprague–Dawley rats (8–10 weeks old; 250–300 g) were maintained under a 12-h dark/light cycle at about 22 °C and allowed food and water ad libitum. Experimental protocols were approved by the Research Practice Committee at the Hong Kong University of Science and Technology, Hong Kong. Animals were randomly divided into groups, with 5 animals in each. In the pretreatment groups, rats were treated intragastrically with EMD, α-TOC or α-LA (dissolved/suspended in olive oil) at a daily dose of 0.6 or 1.2 mmol/kg for 3 days. Preliminary dose–response studies indicated that pretreatment with EMD, α-TOC or α-LA at a daily dose of 1.2 mmol/day produced optimal protection against myocardial I–R injury as assessed by enzyme leakage in female rats. Control (i.e. untreated) animals received oil only (10 ml/kg). For the non-I–R groups, 24 h after the last dosing, control or drug-pretreated hearts were excised from pentobarbital-anesthetized rats and subjected to biochemical analysis. For the I–R groups, isolated hearts were subjected to Langendorff perfusion as described below.

To further investigate the gender-dependent effect, male and female rats were given a single dose of EMD at 1.2 mmol/kg. The hearts were isolated at increasing post-dosing time intervals (24–96 h) and then subjected to biochemical analysis and I–R experiment.

Isolated-perfused rat heart

The heart was excised quickly and immediately immersed in ice-cold saline containing heparin (50 unit/ml). The aorta was cannulated and then transferred to a warm and moistured chamber of the perfusion apparatus. The perfusion buffer (a modified Krebs–Henseleit bicarbonate solution (pH 7.4) containing 120 mM NaCl, 25.4 mM $NaHCO_3$, 4.8 mM NaCl, 1.2 mM KH_2PO_4, 0.86 mM $MgSO_4$, 1.25 mM $CaCl_2$ and 11 mM glucose) was maintained at 37 °C and gassed with 95% O_2–5% CO_2 gas mixture. The heart was retrogradely perfused at a constant pressure of 60 mm Hg (maintained by a peristaltic pump) to give a coronary flow rate of 6–11 ml/min and a heart rate of 160–200 beats/min during the equilibration period. The apex of the heart was attached via a metal hook to an unextendable cotton thread that was connected to a force displacement transducer (Grass FT03). The isometric contractions of the heart were recorded on a polygraph (Grass Model 7-8P), with a resting tension of 1.5 g.

Myocardial I–R

After an initial 30 min of perfusion for equilibration, the isolated heart was subjected to a 40-min period of 'no-flow' global ischemia. This was achieved by clamping the retrograde aortic perfusion. After ischemia, flow was restored and the heart was reperfused for a 20-min period. Coronary effluent was collected in 1-min fraction every 10 min or 1 min during the course of equilibration and reperfusion, respectively. The fractions were immediately put on ice until assay for lactate dehydrogenase (LDH) activity. The extent of LDH leakage during the reperfusion period, an indirect index of myocardial injury, was estimated by computing the area under the curve of the graph plotting the percent LDH activity released per min (with respect to the mean of pre-ischemic values measured during the equilibration period at 10, 20 and 30 min) against the reperfusion time (1–20 min), as described (Li et al., 1996). During the period of reperfusion, a gradual recovery of contractile force was observed. The contractile force recovery of isolated-perfused heart following the I–R challenge was expressed as a percent of the pre-ischemic value measured at the end of the 30-min equilibrium period.

Preparation of mitochondrial fractions

Myocardial ventricular tissue samples were rinsed with ice-cold isotonic buffer (50 mM Tris, 0.32 M sucrose, 1 mM Na_2EDTA, 0.2 mg/ml soybean trypsin inhibitor, 0.2 mg/ml bacitracine, 0.16 mg/ml benzamidine). Tissue homogenates were prepared by homogenizing 0.8 g of myocardial tissue in 8 ml ice-cold isotonic buffer and the homogenates were used for the preparation of mitochondrial fractions by differential centrifugation, with the purity being determined by measuring the relative specific activities of succinate dehydrogenase and LDH in the supernatant and mitochondrial pellet, as described previously (Evans, 1992). Yields of mitochondrial fractions, as reflected by protein content, were similar in various experimental groups. The mitochondrial pellets were resuspended in 1.5 ml of isotonic buffer containing 150 μl of 2 mg/ml soybean trypsin inhibitor and constituted the mitochondrial fractions.

Biochemical analysis

Myocardial homogenates were centrifuged at 13,000 rpm for 1 min using a bench-top centrifuge to obtain the nucleus-free homogenates. Myocardial mitochondrial ATP generation capacity, an indirect measure of mitochondrial oxidative phosphorylation (Konig et al., 1993), was measured by incubating 200 μl of nucleus-free tissue homogenate with 200 μl of substrate solution (containing 100 mM glutamate and 34 mM malate) and 20 μl ADP (2.3 mM) for 10 min at 37 °C, as described in Chiu and Ko (2003). The reaction was terminated by the addition of 35 μl perchloric acid (30%), and the reaction mixtures were then centrifuged at $600\times g$ for 10 min. An aliquot (120 μl) of the supernatant was mixed with 90 μl of 1.4 M $KHCO_3$ for neutralization. The mixtures were centrifuged again at $600\times g$ for 10 min and the ATP level of the supernatant was measured by HPLC analysis (Nova Pak column: 3.0×300 mm; gradient elution with a two-component mobile phase: (A) 1 mM EDTA, 6 mM tetrabutylammonium hydrogen sulfate in 35 mM KPO_4, pH 6.0; (B) acetonitrile/A (50:50, v/v); flow rate: 1.0 ml/min), as modified from the method of Ally and Park (1992). A preliminary study indicated that a linear increase in ATP production in the reaction mixture occurred with increasing periods of incubation up to 20 min (data not shown). LDH activity was spectrophotometrically measured as described (Li et al., 1996).

Aliquots (500 ml and 200 ml) of mitochondrial fractions were taken for measuring mitochondrial GSH and α-TOC levels by enzymatic and HPLC method, respectively, as described by Griffith (1980) and Sadrzadeh et al. (1994). Aliquots (400 μl) of mitochondrial fractions were mixed with 933 μl Triton X-100 solution (0.3%, v/v, in isotonic buffer) and sonicated for 2 min on ice. The mixtures were then subjected to measurements of mitochondrial GRD and Se-glutathione peroxidase (GPX) activities by spectrophotometric methods, as described (Chiu and Ko, 2003). Mn-superoxide dismutase (SOD) activity in the mitochondrial fraction was measured by monitoring the oxidation of cytochrome *c* caused by superoxide radicals generated from the xanthine–xanthine oxidase reaction, as modified from the method of McCord and Fridovich (1969). Protein concentrations of mitochondrial fractions were determined using a BioRad protein assay kit.

Statistical analysis

Data obtained from animal experiments were analyzed by one-way ANOVA followed by Duncan's multiple range test to detect the inter-group difference. Significant difference was determined when $p < 0.05$.

Results

I–R caused tissue damage in isolated-perfused rat hearts, as evidenced by the significant increase in LDH leakage (13- to 18-fold) (Fig. 2a) and a decrease in contractile force recovery (~86%) (Fig. 2b). Male hearts were more susceptible to I–R injury than female hearts, as indicated by a larger extent of LDH leakage (18-fold versus 13-fold). However, there was no difference between male and female hearts in the degree of contractile force recovery following the I–R challenge.

The mitochondrial ATP generation capacity was not different between male and female hearts, but female hearts exhibited higher levels of GSH (50%) and α-TOC (41%) levels as well as activity of SOD (100%) (Table 1). However, mitochondrial GRD and GPX activities were similar in male and female hearts. The myocardial I–R injury was associated with decreases in mitochondrial ATP generation capacity (17–25%), GSH (38–45%) and α-TOC (60–65%) levels as well as GRD (11–23%) and SOD (13–30%) activities in both male and female hearts. However, I–R increased the mitochondrial GPX activity by 21% in female hearts.

EMD treatment (0.6 or 1.2 mmol/kg/day) for 3 days increased mitochondrial ATP generation capacity, GSH and α-TOC levels as well as GRD and GPX activities, with the extent of stimulation being 16–18%, 15–19%, 52–55%, 11–15% and 15–40%, respectively, at 1.2 mmol/kg in male and female hearts (Fig. 3a). While male hearts had a larger increase in GPX activity, female hearts showed a dose-dependent and significant increase in SOD activity (10–29%).

α-TOC treatment (0.6 or 1.2 mmol/kg/day × 3) slightly affected the mitochondrial ATP generation capacity in male hearts. However, it significantly increased the ATP generation capacity by 22% at a dose of 1.2 mmol/kg in female hearts (Fig. 3b). α-TOC treatment increased mitochondrial GSH (22–30%) and α-TOC (104–149%) levels as well as GRD (10–18%) and GPX (12–15%) activities at a dose of 1.2 mmol/kg in male and female hearts. In contrast, the mitochondrial SOD activity was significantly decreased by 15–24% in male hearts. However, the mitochondrial SOD activity remained relatively unchanged in female hearts after α-TOC treatment.

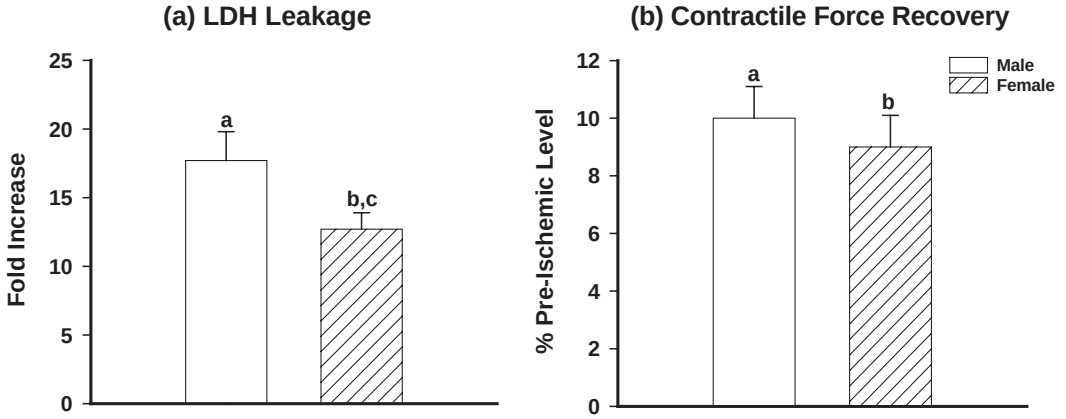

Fig. 2. Myocardial ischemia–reperfusion injury in isolated-perfused hearts from male and female rats. Isolated-perfused rat hearts were subjected to a 40-min period of ischemia followed by 20 min of reperfusion (I–R). The extent of lactate dehydrogenase (LDH) leakage and contractile force recovery were measured as described in Materials and methods. Each bar represents the mean ± S.E.M., with $n = 5$. [a]Significantly different from the female non-I–R group; [b]significantly different from the female non-I–R group; [c]significantly different from the male I–R group.

α-LA treatment (0.6 or 1.2 mmol/kg/day × 3) slightly increased the ATP generation capacity in both male and female hearts (Fig. 3c). However, it significantly increased mitochondrial GSH (20–58%) and α-TOC (24–121%) levels at a dose of 1.2 mmol/kg in male and female hearts. While GRD and GPX activities were only slightly affected by α-LA treatment in both male and female hearts, the SOD activity was significantly reduced by 21–33% in female hearts only.

Table 1
Effects of myocardial ischemia–reperfusion on mitochondrial functional status and antioxidant components in male and female rats

	ATP-GC (μmol/mg protein)	Mitochondrial antioxidant components				
		GSH (nmol/mg protein)	α-TOC (pmol/mg protein)	GRD (mU/mg protein)	GPX (mU/mg protein)	SOD (U/mg protein)
Non-I–R						
Male	914 ± 18	4.78 ± 0.11	756 ± 59.8	4.12 ± 0.42	51.2 ± 0.72	39.1 ± 4.29
Female	892 ± 58	7.15 ± 22[a]	1067 ± 25.1[a]	4.33 ± 0.22	48.0 ± 2.77	77.9 ± 2.21[a]
I–R						
Male	689 ± 41[a] (−25%)	2.65 ± 0.11[a] (−45%)	211 ± 16.2[a] (−65%)	3.66 ± 0.78	50.8 ± 0.38	34.0 ± 0.52[a] (−13%)
Female	740 ± 15[b] (−17%)	4.45 ± 0.26[b] (−38%)	428 ± 9.26[b] (−60%)	3.32 ± 0.15[b] (−23%)	58.1 ± 0.30[b] (21%)	54.5 ± 1.67[b] (−30%)

Isolated-perfused hearts were subjected to I–R challenge as described in Fig. 2. Myocardial mitochondrial ATP generation capacity (ATP-GC), reduced glutathione (GSH) and α-tocopherol (α-TOC) levels as well as glutathione reductase (GRD), Se-glutathione peroxidase (GPX) and Mn-superoxide dismutase (SOD) activities were measured as described in Materials and methods. Values given are mean ± S.E.M., with $n = 5$. The number in parentheses is the percent change when compared with the respective non-I–R control.

[a] Significantly different from the male non-I-R group.

[b] Significantly different from the female non-I-R group.

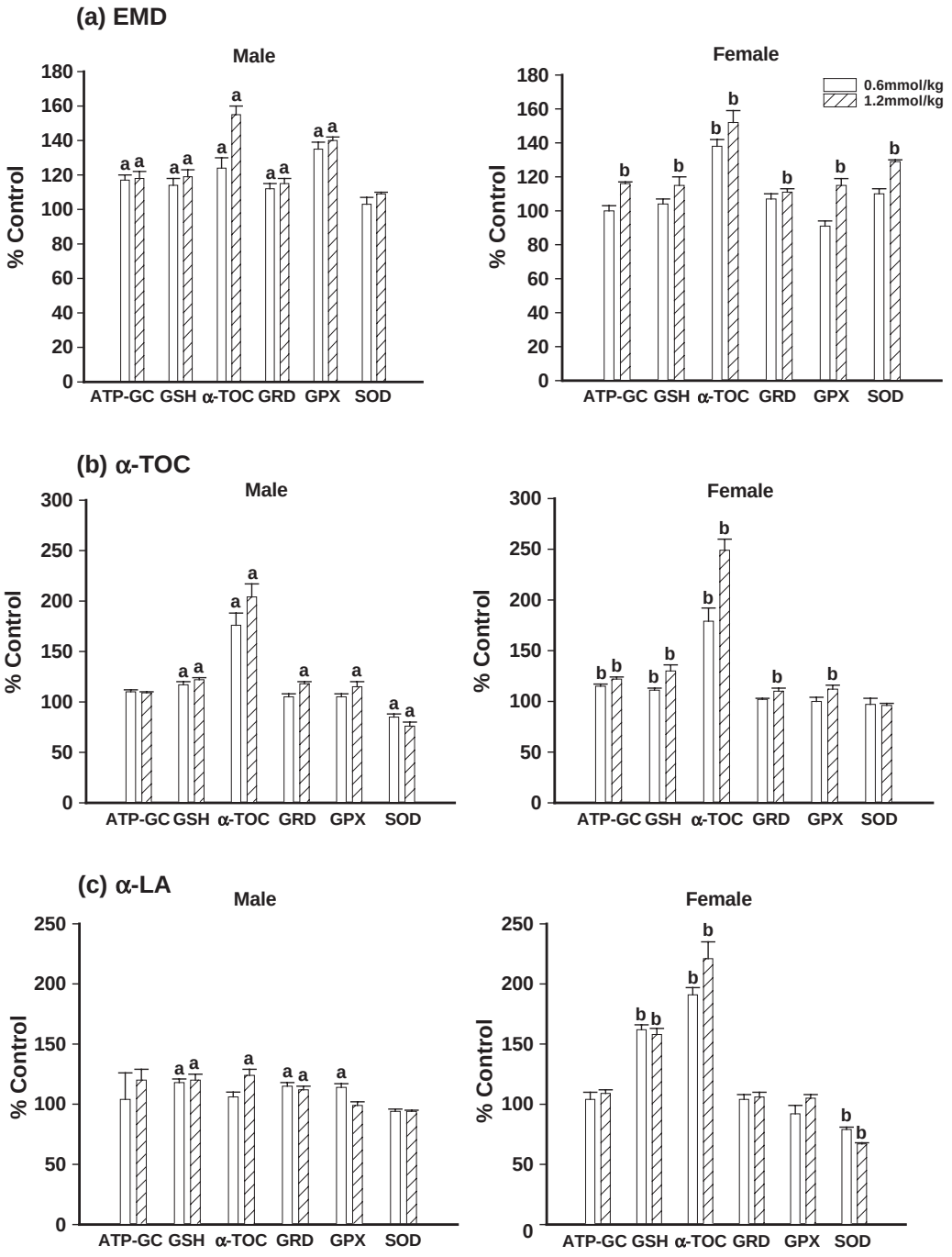

Fig. 3. Effects of EMD and antioxidant treatment on myocardial functional status and antioxidant components in male and female rats. Animals were orally treated with emodin (EMD), α-tocopherol (α-TOC) and α-lipoic acid (α-LA) at the indicated doses for 3 days as described in Materials and methods. Mitochondrial functional and antioxidant parameters were measured as described in Table 1. Values are expressed in percent control with reference to untreated and non-I–R values (i.e., basal levels) shown in Table 1, and each bar represents the mean ± S.E.M., with $n = 5$. [a]Significantly different from the male untreated group; [b]significantly different from the female untreated group.

EMD and α-LA pretreatment did not significantly protect against myocardial I–R injury in male hearts, as assessed by LDH leakage. However, α-TOC pretreatment significantly protected against I–R injury in male hearts, with the percent protection being 26% at a dose of 1.2 mmol/kg (Fig. 4a). All drug-pretreated male hearts showed increases in contractile force recovery after the I–R challenge relative to untreated controls, but the difference did not achieve statistical significance (Fig. 4b). As for female hearts, all drug pretreatments significantly protected against I–R injury, with the percent protection being 31%, 24% and 12% for EMD, α-TOC and α-LA, respectively, at a dose of 1.2 mmol/kg. The degree of contractile recovery after the I–R challenge in drug-pretreated female hearts also increased relative to untreated controls, but the difference did not achieve statistical significance (Fig. 4b).

A single dose of EMD (1.2 mmol/kg) increased mitochondrial GSH and α-TOC levels in a time-dependent manner in both male and female hearts, with the stimulation being maximum at 72 h post-dosing and returning to control levels thereafter (Table 2). While EMD treatment caused a

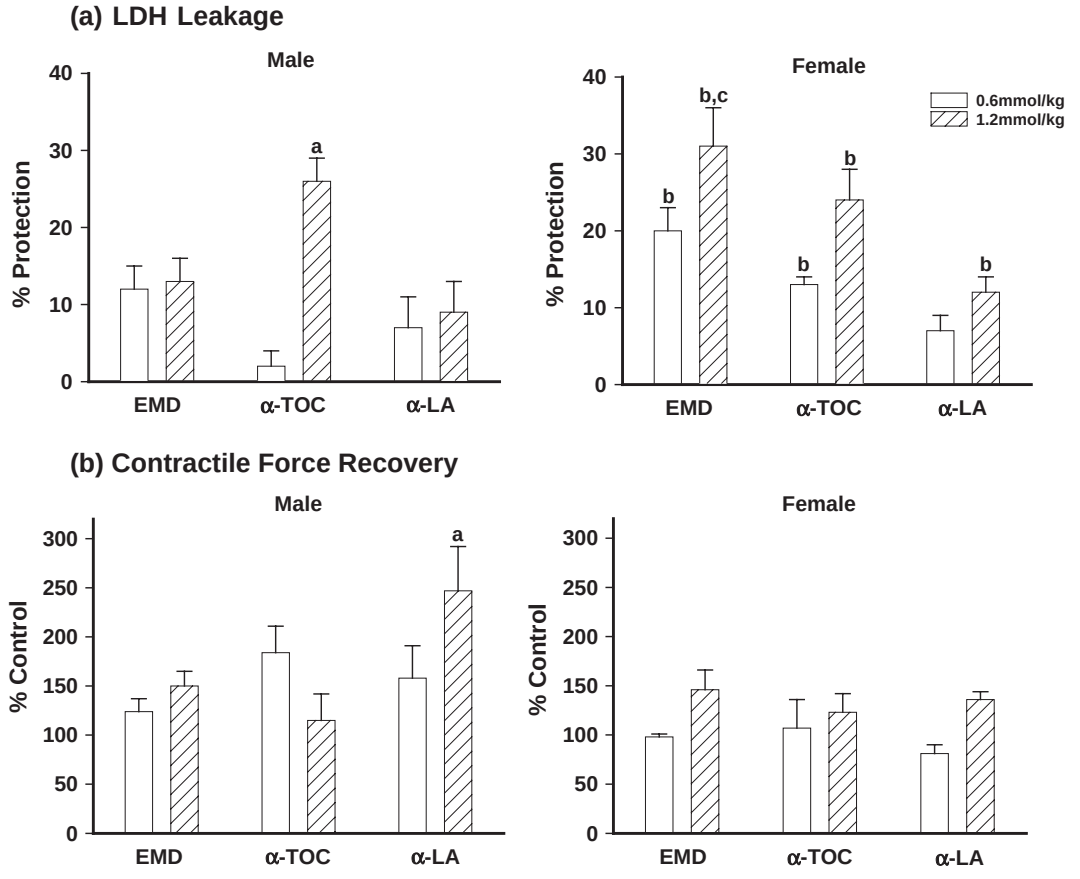

Fig. 4. Effects of EMD and antioxidant pretreatment on myocardial ischemia–reperfusion injury in male and female rats. Drug pretreatment was performed as described in Fig. 3. Isolated hearts were subjected to I–R challenge as described in Fig. 2. The extent of LDH leakage and contractile force recovery were expressed as percent protection and percent control, respectively, when compared with the respective unpretreated and I–R control group. Each bar represents the mean ± S.E.M., with $n = 5$. [a]Significantly different from the male unpretreated I–R group; [b]significantly different from the female unpretreated I–R group; [c]significantly different from the male EMD-pretreated and I–R group.

Table 2
Time-course of EMD-induced changes in mitochondrial antioxidant components in male and female rat hearts

	Post-dosing time (h)			
	24	48	72	96
Male				
GSH	89 ± 7	111 ± 22	123 ± 18[a]	107 ± 17
α-TOC	111 ± 1	110 ± 5	126 ± 9[a]	102 ± 5
SOD	104 ± 3	81 ± 3[a]	77 ± 4[a]	83 ± 3
Female				
GSH	111 ± 6	101 ± 6	125 ± 11[b]	119 ± 11
α-TOC	110 ± 7	123 ± 7[b]	145 ± 1[b]	84 ± 7
SOD	116±4[b]	120±4[b]	104±7	107±7

Animals were orally administered with a single dose of EMD at 1.2 mmol/kg. Myocardial mitochondrial antioxidant parameters (GSH, α-TOC and SOD) were measured, as described in Materials and methods, at increasing periods (24–96 h) of post-dosing time, and the values are expressed in percent control with reference to the untreated and non-I–R values. Each bar represents the mean ± S.E.M., with $n = 5$.

[a] Significantly different from the male untreated I-R group.

[b] Significantly different from the female untreated I-R group.

time-dependent decrease in mitochondrial SOD activity in male hearts, with the maximal inhibition occurring at 72 h post-dosing, it produced a biphasic change in the SOD activity in female hearts, with the value reaching the maximum at 48 h post-dosing and then returning to the control level (Table 2).

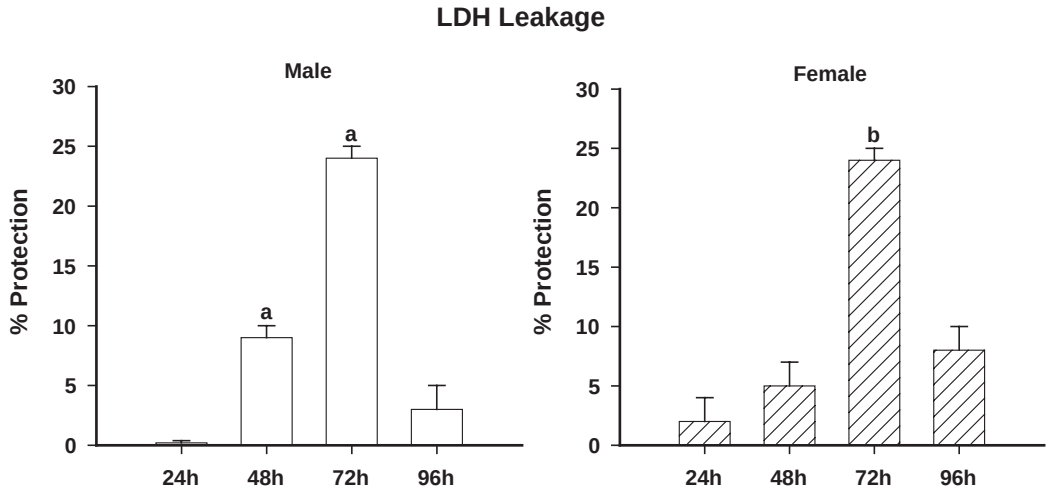

Fig. 5. Time-dependent effect on ischemia–reperfusion injury in EMD-pretreated male and female rat hearts. Animals were pretreated with EMD as described in and then subjected to I–R challenge at increasing periods of post-dosing time, as described in Fig. 2. The extent of LDH leakage was measured as described in Materials and methods, and the values are expressed in percent protection when compared with the unpretreated I–R group. Each bar represents the mean ± S.E.M., with $n = 5$. [a]Significantly different from the male unpretreated I–R group; [b]significantly different from the female unpretreated I–R group.

A single dose of EMD treatment also caused a time-dependent decrease in susceptibility of male and female hearts to I–R injury to a similar extent, with the percent protection reaching the maximum at 72 h post-dosing and declining thereafter (Fig. 5).

Discussion

Reperfusion of the previously ischemic myocardium can cause tissue injury (Takemura et al., 1993; Kaul et al., 1993). The I–R-induced increase in the extent of LDH leakage, as observed in the present study, was associated with an impairment in contractile force up to 20 min of reperfusion, both of which were indicative of tissue damage. Early experimental findings have demonstrated the increased formation of oxygen-derived free radicals (oxy-radicals) in the myocardium during post-ischemic reperfusion (Baker et al., 1988; Maupoil and Rochette, 1988), wherein mitochondria are the major source of oxy-radical production (Ambrosio et al., 1993; Venditti et al., 2001). Consistent with these, our findings showed that myocardial I–R injury was accompanied by decreases in mitochondrial ATP generation capacity and antioxidant levels/activities, which are indirect indices of mitochondrial functional and antioxidant status. While the decline in mitochondrial ATP generation capacity may be caused by oxidative damage on protein complexes involved in the electron transport process (Paradies et al., 1999; Jha et al., 2000; Lesnefsky et al., 2001), the depletion of non-enzymatic antioxidants, such as GSH and α-TOC, and inhibition of antioxidant enzymes, such as GRD and SOD, can aggravate the mitochondrial oxidative injury. These changes can culminate in the disruption of mitochondrial structural and functional integrity, leading to cellular necrosis and/or apoptosis (Broekemeier et al., 1998; Lemasters et al., 1999; Halestrap, 2002). Our finding of myocardial protection by antioxidant pretreatment associated with enhancement in mitochondrial antioxidant components supports the involvement of oxy-radicals in the pathogenesis of I–R injury. In this connection, the observation of higher susceptibility of male hearts to I–R injury may also be causally related to their smaller mitochondrial antioxidant capacity than that of female hearts, as reflected by lower antioxidant levels or activities.

Three daily doses of EMD treatment invariably enhanced mitochondrial ATP generation capacity and antioxidant components in male and female rat hearts. The mitochondrion possesses its own antioxidant defense system, which is comprised of both non-enzymatic components, such as GSH and α-TOC, and enzymatic components, such as GRD, GPX and SOD. SOD catalyzes the dismutation of superoxide into hydrogen peroxide and water molecules. After the decomposition of hydrogen peroxide by GPX at the expense of GSH, the GRD-catalyzed regeneration of GSH from its oxidized form can sustain the GSH-dependent oxy-radical scavenging activity (Meister, 1988). α-TOC, which scavenges lipid peroxyl radicals generated in lipid membranes (Kagan et al., 1990), can also be regenerated by GSH (Leedle and Aust, 1990). Thus, the dynamic interplay among various antioxidant components enables the maintenance of mitochondrial structural and functional integrity under normal and oxidative stress conditions (Sastre et al., 2003). As regards the mitochondrial function, the stimulation of ATP generation capacity by EMD treatment may be a consequence of enhancement in antioxidant capacity. This is supported by the observation that α-TOC treatment could increase both mitochondrial α-TOC level and ATP generation capacity, particularly in female hearts. On the other hand, the observation that the large rise in mitochondrial GSH level by α-LA treatment not paralleled by the increase in ATP generation capacity did not support a causative role of GSH in stimulating mitochondrial ATP generation.

EMD pretreatment produced a more prominent protection against myocardial I–R injury in female than in male hearts, despite its similar effect on mitochondrial ATP generation capacity and antioxidant components. The gender-dependent effect on myocardial I–R injury was also observed in animals receiving multiple doses of α-LA pretreatment. In contrast, α-TOC pretreatment at multiple doses protected both male and female hearts against I–R injury to a similar extent. While the biochemical mechanism involved in this gender-dependent protection against myocardial I–R injury remains to be determined, it is possible that multiple doses of EMD can cause differential cellular response(s) from male and female hearts, particularly under I–R conditions. This is supported by the finding that EMD treatment increased myocardial mitochondrial SOD activity in female but not male hearts. Similarly, the differential effect of α-LA pretreatment on I–R injury may be related to its ability to preferentially enhance mitochondrial α-TOC level in female hearts. While the differential susceptibility of males and females to tissue oxidative damage, as implicated in experimental and clinical observations, is likely attributed to biochemical event(s) secondary to the action of estrogen action (Borrás et al., 2003; Camper-Kirby et al., 2001), the gender-dependent myocardial protection afforded by EMD pretreatment at multiple doses may be related to the differential antioxidant response to I–R challenge under the influence of estrogen. In addition, the gender difference in liver metabolism of EMD may affect the bioavailability of orally administered EMD to the myocardium. In this regard, it has been demonstrated that EMD can induce cytochrome *P*-450 monooxygenase (P-450) 1A1 and 1B1 in human adenocarcinoma cells (Wang et al., 2001) and undergo P-450 1A1-dependent transformation in rat liver microsomes (Mueller et al., 1998). Moreover, estrogen was found to suppress the induction of P-450 1A1 expression in rainbow trout primary hepatocytes (Elskus, 2004). Taken together, the lack of a gender difference in the protection against I–R injury after a single oral dose of EMD supports the involvement of hepatic metabolism of EMD in causing the differential cardioprotection when administered at multiple doses. It is possible that EMD preferentially induces hepatic P-450 isoenzyme(s) in male rats so that the hepatic metabolism of the later administered drug in the multiple dosing regimen is enhanced, thereby decreasing the drug concentration attainable in the myocardium. According to Chinese medicine theory, *Polygonum* root belongs to a category of tonic herbs with "blood-enriching" activities (SATCMP, 1995). The "blood" is essential for maintaining normal physiological function, particularly in women who are more vulnerable to "blood-deficiency" than men. Our finding of a gender-dependent effect of EMD, an active ingredient of *Polygonum* root, suggests that "blood-enriching" Chinese tonifying herbs, which contain active ingredients similar to EMD, may produce a differential effect on men and women when given in multiple doses.

In conclusion, EMD treatment at 3 daily doses could enhance myocardial mitochondrial ATP generation capacity and antioxidant components in both male and female rat hearts, but it only significantly protected against I–R injury in female hearts. Treatment with a single dose of EMD invariably enhanced mitochondrial antioxidant system and protected against I–R injury in both male and female hearts. The gender-dependent effect of EMD treatment at multiple doses may be related to the differential antioxidant response in the myocardium and/or induction of drug metabolizing enzymes in the liver.

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