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Effects of pharmacological preconditioning by emodin/oleanolic acid treatment and/or ischemic preconditioning on mitochondrial antioxidant components as well as the susceptibility to ischemia-reperfusion injury in rat hearts

Ying Du and Kam Ming Ko

Department of Biochemistry, Hong Kong University of Science & Technology, Clear Water Bay, Hong Kong, China

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Abstract

Using an *ex vivo* rat heart model of ischemia-reperfusion (I-R) injury, we examined the effect of pharmacological preconditioning by chronic treatment with emodin (EMD)/oleanolic acid (OA) at low dose (25 μmol/kg/day $\times$ 15) and/or ischemic preconditioning (IPC) (4 cycles of 5 min ischemia followed by 5 min of reperfusion) on myocardial I-R injury. The results indicated that EMD/OA pretreatment, IPC, or their combinations (EMD+IPC and OA+IPC) protected against myocardial I-R injury, as assessed by lactate dehydrogenase leakage and contractile force recovery. The cardioprotection was associated with a differential enhancement in mitochondrial antioxidant components. The combined EMD/OA and IPC pretreatment produced cardioprotective action in a semi-additive manner. This suggested that EMD/OA pretreatment and IPC protected against myocardial I-R injury via a similar but not identical biochemical mechanism. (Mol Cell Biochem **288:** 135–142, 2006)

Key words: emodin, oleanolic acid, ischemic preconditioning, myocardial ischemia-reperfusion injury, mitochondria, glutathione, α-tocopherol, superoxide dismutase

Abbreviation: EMD, emodin; GSH, reduced glutathione; IPC, ischemia preconditioning; I-R, ischemia-reperfusion; LDH, lactate dehydrogenase; OA, oleanolic acid; Mn-SOD, Mn-superoxide dismutase; α-TOC, α-tocopherol

Introduction

Despite the recent advances in medical science, coronary heart disease remains a major health problem in industrialized countries. Over the past few decades, exploring possible therapeutic interventions aimed at ameliorating myocardial consequences arising from ischemia-reperfusion (I-R) has been an area of intensive research. In this regard, ischemic preconditioning (IPC) is able to elicit a protective endogenous adaptive response of the heart against a prolonged ischemic insult [1]. Apparently, oxy-radicals arising from episodes of ischemia are involved in triggering cellular processes leading to IPC [1]. However, the application of IPC, which requires a physical cut of the blood supply, can be difficult or impractical in many clinical situations. Over the past two decades, a large volume of research has focused on investigating the biochemical mechanism(s) of IPC in an effort to develop clinically useful agents that can mimic the effect of IPC [2, 3].

Address for offprints: K. Ming Ko, Department of Biochemistry, Hong Kong University of Science & Technology, Clear Water Bay, Hong Kong, China (E-mail: bcrko@ust.hk (K.M. Ko))

This endeavor has so far been not very successful because mimetic candidates often produce clinically intolerable side effects [4]. Since Chinese tonifying herbs have long been used for safeguarding health and delaying the onset of senility, a search for agents that can precondition the myocardium and protected against myocardial I-R injury from Chinese tonifying herbs represents a rational approach. In this regard, emodin (EMD, Fig. 1a) and oleanolic acid (OA, Fig. 1b) are active ingredients from the root of *Polygonum multiflorum* Thunb. and the fruit of *Ligustri Lucidum* Ait., respectively, both of which are commonly used Chinese tonifying herbs [5, 6]. EMD, an anthraquinone derivative, is considered as a generator of oxy-radicals owing to its ability to carry out one-electron transfer [7]. It has also been shown that the immunosuppressive and anti-cancer actions of EMD are mediated by its pro-oxidant activity [8]. While whether OA, a triterpenoid derivative, possesses any *in vitro* and *in vivo* free radical scavenging activities is still unknown, OA has been shown to produce anti-inflammatory and anti-hepatotoxic actions *in vivo* [9, 10]. Recently, our laboratory has shown that both EMD and OA pretreatments protected against I-R injury in isolated rat hearts [11–13]. The cardioprotection was associated with enhancements in mitochondrial antioxidant components, particularly under I-R condition [12, 13]. As previous studies on the cardioprotective effect of EMD and OA adopted a short-term and high dose treatment regimen, it is still unclear whether chronic treatment with a low dose of EMD or OA, which is therapeutically more feasible, can protect against myocardial I-R injury. In the present study, we endeavored to examine the effect of chronic treatment with EMD or OA at a low daily dose on mitochondrial antioxidant components and the susceptibility to I-R injury in isolated rat hearts. We also compared the pharmacological preconditioning afforded by EMD and OA treatment with IPC produced by repeated episodes of ischemia and reperfusion under non-I-R and I-R conditions. To further investigate the mechanistic difference in cardioprotection between pharmacological preconditioning and IPC, we examined the effect of combined EMD/OA and IPC pretreatment on myocardial I-R injury.

Materials and methods

Chemicals

EMD and OA were purchased from ACROS Organics (New Jersey, USA), with the purity being ≥97%. Reduced glutathione (GSH), oxidized glutathione, glutathione reductase, xanthine oxidase, xanthine, cytochrome c, and α-tocopherol (α-TOC) 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.

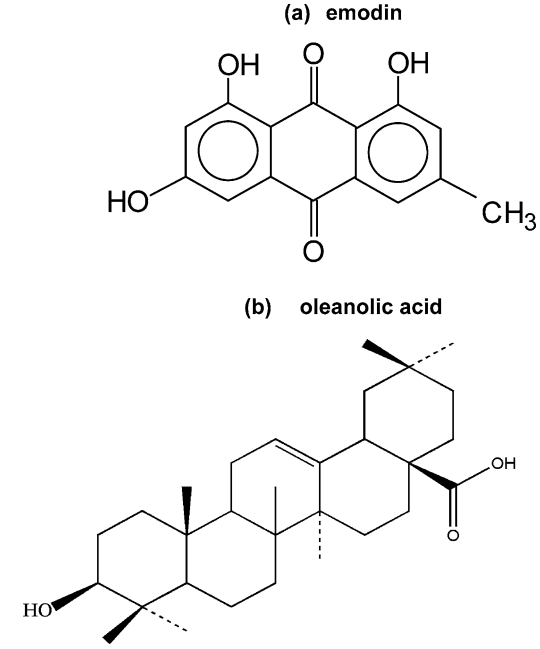

Fig. 1. Chemical structure of emodin (EMD) and oleanolic acid (OA).

Animal pretreatment

Adult female Sprague-Dawley rats (8–10 week-old; 220–250 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 & Technology, Hong Kong. Animals were randomly divided into groups, with 5 animals in each. For pharmacological preconditioning, animals were treated with EMD or OA (dissolved and/or suspended in olive oil) at an oral daily dose of 25 μmol/kg for 15 days. Untreated (i.e. control) animals received the olive oil only (10 ml/kg).

Isolated-perfused rat heart

Twenty-four hours after the last dosing, the heart was excised quickly from control or drug-treated animals and immediately immersed in ice-cold saline containing heparin (50 unit/ml). The aorta was cannulated and then transferred to a warm and moisturized 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

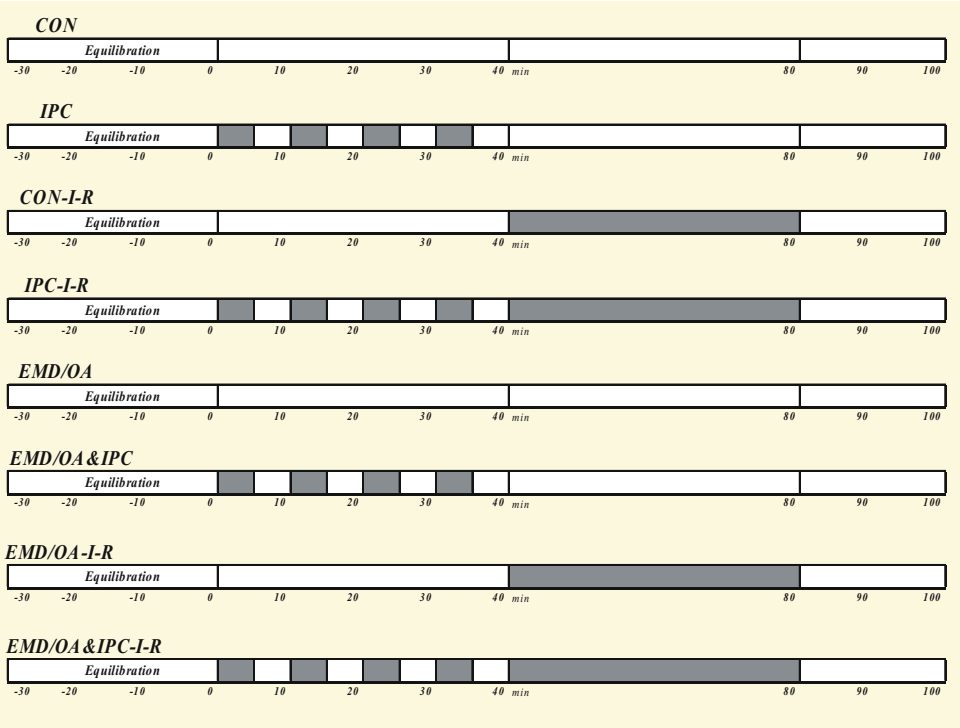

Fig. 2. Protocol of isolated heart experiments with or without ischemic preconditioning (IPC) /ischemia-reperfusion (I-R). Animals were treated with EMD/OA (25 μmol/kg/day) for 15 days. For non-I-R experiments, after 30-min of aerobic perfusion for equilibration, the isolated heart prepared from control or drug-pretreated animals was subjected to either 4 cycles of 5-min global no-flow ischemia followed by 5-min of reperfusion or 40-min perfusion (i.e. without IPC). The heart was then subjected to a prolonged 60-min of reperfusion. For I-R experiments, following the IPC or 40-min perfusion, the heart was subjected to a prolonged 40 min of ischemia followed by 20 min of reperfusion.

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

As shown in Fig. 2, for non-I-R experiments, after 30-min of aerobic perfusion for equilibration, the isolated heart prepared from control or drug-treated animals was subjected to 4 cycles of 5-min global no-flow ischemia followed by 5-min of reperfusion (i.e. IPC) or 40-min perfusion. The heart was then subjected to a prolonged 60-min of reperfusion. For I-R experiments, the 4 cycles IPC or 40 min perfusion was followed by a prolonged 40 min of ischemia followed by 20 min of reperfusion. 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 [14]. 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 [15]. 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

LDH activity was spectrophotometrically measured as described [14]. Aliquots (500 μl and 200 μl) of mitochondrial fractions were taken for measuring mitochondrial GSH and α-TOC levels by an enzymatic method and HPLC analysis, respectively, as described [16, 17]. Mn-superoxide dismutase (Mn-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 [18]. Protein concentrations of mitochondrial fractions were determined using a BioRad protein assay kit.

Statistical analysis

Comparisons between non-I-R or I-R control group and the respective preconditioned group were done using Student's t-test. The difference was regarded as statistically significant when p-value was smaller than 0.05.

Results

As shown in Table 1, a 40-min period of ischemia followed by 20 min of reperfusion caused a drastic increase in the extent of LDH leakage and a significant impairment (80%) in contractile recovery in isolated-perfused rat hearts. The myocardial I-R injury was associated with significant decreases in mitochondrial α-TOC (42%) and GSH (26%) levels as well as Mn-SOD activity (16%).

EMD/OA pretreatment (25 μmol/kg/day $\times$ 15), IPC (4 cycles of 5 min ischemia followed by 5 min of reperfusion), or their combinations (EMD+IPC and OA+IPC) did not affect the LDH leakage and contractile force recovery (data not shown). Fig. 3a shows that both EMD and OA pretreatments protected against I-R injury in rat hearts, as evidenced by the significant decrease in LDH leakage, with the percent protection being 33 and 22%, respectively, when compared with the unpretreated I-R control. IPC also decreased the extent of LDH leakage (by 36%). The combined EMD+IPC and OA+IPC pretreatments resulted in the protection against I-R injury, with the percent protection being 45 and 42%, respectively. While EMD pretreatment did not produce any detectable change in contractile force recovery after I-R challenge, OA pretreatment increased the degree of contractile force recovery (by 57%) in I-R hearts, when compared with the unpretreated I-R control (Fig. 3b). IPC caused a 37% increase in the degree of contractile force recovery after I-R challenge. The combined EMD+IPC and OA+IPC pretreatments enhanced the post-I-R contractile force recovery by 44 and 62%, respectively.

As shown in Fig. 4a, while EMD treatment caused significant increases in mitochondrial α-TOC (17%) and GSH (17%) levels as well as Mn-SOD activity (7%) in non-I-R hearts, OA treatment only produced a significant increase in mitochondrial α-TOC level (20%), when compared with the untreated non-I-R control. However, the mitochondrial GSH level was significantly decreased (12%) by OA treatment. IPC slightly decreased mitochondrial α-TOC and GSH levels in non-I-R hearts, but it significantly increased the Mn-SOD activity (by 9%). While the combined EMD+IPC treatment did not change mitochondrial α-TOC and GSH levels, it produced a significant increase in the Mn-SOD activity (15%). The combined OA+IPC

Table 1. Ischemia-reperfusion injury and the associated changes in mitochondrial antioxidant components in rat hearts

			Mitochondrial Antioxidant Components		
	LDH leakage (*AUC*)	Contractile force recovery(%)	α-TOC (pmol/mg protein)	GSH (nmol/mg protein)	Mn-SOD (mU/mg protein
Non-I-R	340 ± 9	68.9 ± 1.2	933.6 ± 3.7	5.37 ± 0.15	70.1 ± 1.2
I-R	$6686 \pm 213^*$ (19-fold)	$13.5 \pm 0.5^*$ (-80%)	$542.5 \pm 3.9^*$ (-42%)	$3.96 \pm 0.21^*$ (-26%)	$58.6 \pm 1.3^*$ (-16%)

Isolated-perfused hearts were subjected to ischemia-reperfusion (I-R) challenge as described in Materials and methods. Myocardial mitochondrial reduced glutathione (GSH) and α-tocopherol (α-TOC) levels as well as Mn-superoxide dismutase (Mn-SOD) activity were measured. Values given are mean $\pm$ S.E.M., with $n = 5$. The number in parentheses is the percent change when compared with the respective non-I-R (i.e. perfused) control.
*significantly different from the non-I-R group, with $p < 0.05$.

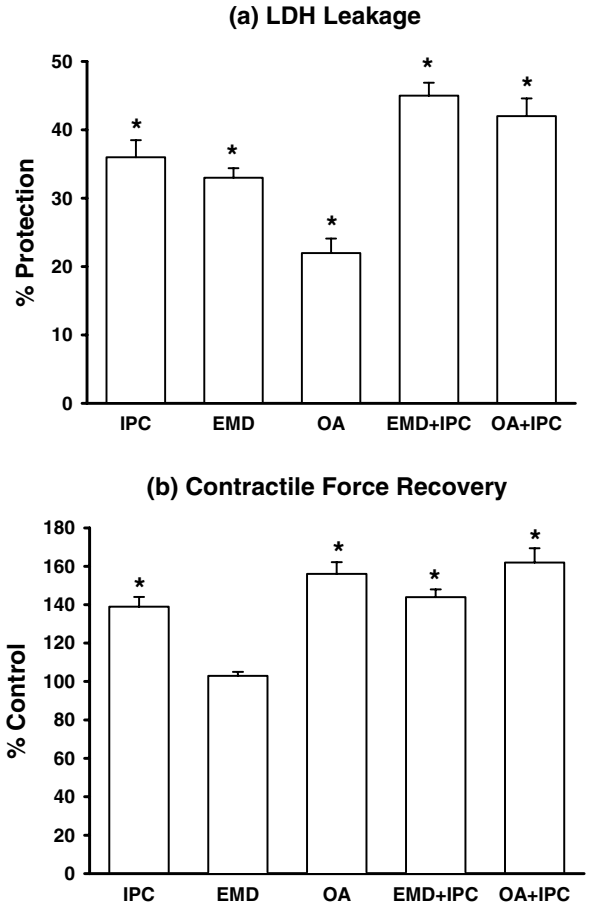

Fig. 3. Effect of EMD/OA pretreatment and/or IPC on I-R injury in rat hearts. Animals were treated with EMD/OA as described in Fig. 2. Twenty-four hours after the last dosing, isolated hearts were subjected to IPC (4 cycles) and then followed by I-R challenge as described in Fig. 2. The extent of I-R injury was assessed by measurements of (a) LDH leakage and (b) contractile force recovery. The percent protection with respect to LDH leakage was estimated by the equation: (unpretreated I-R value – pretreated I-R value/unpretreated I-R value – Non-I-R value) × 100%. The degree of contractile force recovery was expressed as percent control with reference to the unpretreated I-R value shown in Table 1. Values given are mean ± S.E.M, with $n = 5$. *significantly different from the I-R control group, with $p < 0.05$.

treatment increased mitochondrial α-TOC level (27%) and Mn-SOD activity (12%) in non-I-R hearts, but no detectable change in mitochondrial GSH level was observed. The cardioprotection afforded by EMD/OA pretreatment or IPC against I-R injury was associated with a significant increase in mitochondrial α-TOC level (40/56 or 19%, respectively), when compared with the unpretreated I-R control (Fig. 4b). The GSH level was only significantly increased (22%) in EMD-pretreated I-R hearts. EMD/OA pretreatment or IPC significantly increased the Mn-SOD activity (9–22%) in I-R hearts. The combined EMD+IPC and OA+IPC pretreatments caused a significant increase (61 and 39%, respectively) in α-TOC level in I-R hearts, but only the combined EMD+IPC pretreatment showed an increase in the mitochondrial GSH level (22%). However, both combined EMD+IPC and OA+IPC pretreatments increased the Mn-SOD activity (15 and 19%, respectively) in I-R hearts.

Discussion

Reperfusion of the previously ischemic myocardium can cause tissue injury [19, 20]. The I-R-induced increase in the extent of LDH leakage, which has been found to positively correlate with the degree of post-ischemic myocardial infarction in isolated-perfused rat hearts [21], was associated with the 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 [22, 23], wherein mitochondria are the major source of oxy-radical production [24, 25]. Consistent with these, the present finding showed that myocardial I-R injury was accompanied by decreases in level/activity of mitochondrial antioxidant components, which are indirect indices of mitochondrial antioxidant status. The membrane damage caused by oxy-radicals can result in the dysfunction of sarcoplasmic reticulum, leading to the excitation-contraction uncoupling of cardiac muscle and the consequent contractile force impairment [26].

Pharmacological preconditioning of the myocardium by chronic treatment with EMD/OA at low dose could differentially enhance mitochondrial antioxidant components. While chronic EMD treatment produced a generalized enhancement in mitochondrial antioxidant components, chronic OA treatment increased the mitochondrial α-TOC level only. Under the present experimental conditions, EMD/OA pretreatment protected against I-R injury in rat hearts, as assessed by LDH leakage and contractile force recovery. The cardioprotection was associated with enhancements in mitochondrial antioxidant components to varying degrees. Results obtained from the present study are consistent with findings of our previous studies which have demonstrated the cardioprotective effect of EMD and OA pretreatment with a short-term and high dose regimen (1.2 mmol/kg/day for 3 days) [12, 13]. Interestingly, the reciprocal change between mitochondrial GSH and α-TOC level in OA-pretreated heart, as observed in the present and previous studies [13], may be related to the GSH-mediated regeneration of α-TOC [27]. It has long been known that brief periods of ischemia decrease the myocardial damage resulting from a subsequent prolonged period of ischemia [28]. In the present study, IPC

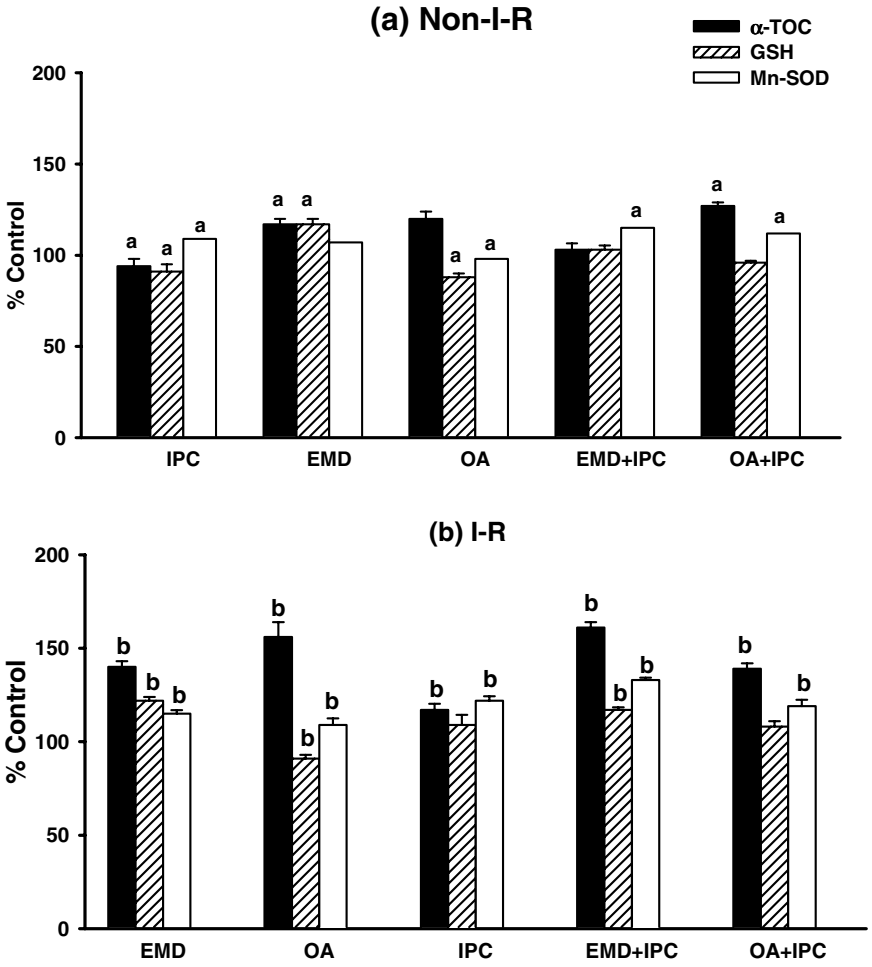

Fig. 4. Effect of EMD/OA pretreatment and/or IPC on mitochondrial antioxidant components in non-I-R and I-R hearts. Animals were treated with EMD/OA and subjected to non-I-R and I-R experiments as described in Fig. 2. Mitochondrial antioxidant parameters were measured as described in Table 1. All data were expressed as percent control with respect to non-I-R and I-R control values. Values given are mean ± S.E.M., with $n = 5$. [a]significantly different from the non-I-R control group; [b]significantly different for I-R control group, with $p < 0.05$.

(4 cycles of 5 min ischemia followed by 5 min of reperfusion) was found to protect against myocardial I-R injury, with the degree of protection comparable to those produced by pharmacological preconditioning (i.e. EMD/OA pretreatment). While the biochemical mechanism involved in the enhancement of mitochondrial antioxidant components by EMD/OA treatment remains to be determined, it is likely that the IPC-induced increase in Mn-SOD activity is due to oxy-radicals arising from the brief episodes of I-R. Presumably, oxy-radicals can trigger signal transduction pathways that prime the entry of mitochondria into a stress-resistant state [1], which is characterized by an enhanced mitochondrial antioxidant status, as observed in the present study. One point worth noting is that the extent of decrease in LDH leakage in EMD/OA or IPC-pretreated hearts after I-R challenge did not correlate well with the increase in the degree of contractile force recovery. This inconsistent observation may be related to the differential mechanism involved in the development of I-R-induced LDH leakage and contractile dysfunction. While the LDH leakage is caused by the loss of structural and functional integrity of sarcoplasmic membranes, the contractile force impairment may probably be due to the dysfunction of intracellular sarcoplasmic reticulum that eventually leads to the excitation-contraction uncoupling of cardiac muscle [26]. In this regard, OA pretreatment and IPC may be more effective than EMD pretreatment in conferring protection on sarcoplasmic reticulum.

Ischemic damage to the mitochondrial electron transport chain increases the generation of oxy-radicals during reperfusion [29], which can produce additional mitochondrial and cardiomyocyte injury [29]. The maintenance of a good mitochondrial antioxidant capacity is therefore instrumental to reduce or limit the extent of I-R injury. Our results suggest that the cardioprotection afforded by pharmacological

preconditioning or IPC against I-R injury may at least in part be mediated by its ability to enhance mitochondrial antioxidant capacity. This postulation is supported by the observation that mitochondria isolated from EMD/OA-pretreated or IPC hearts were found to be less susceptible to peroxide-induced oxidation of lipids *in vitro* (unpublished data).

Results obtained for studies using combined EMD/OA and IPC pretreatment showed that the cardioprotective effects of these two modes of preconditioning are semi-additive. Given that, under the present experimental conditions, IPC afforded by 4 cycles of brief periods of ischemia and reperfusion produced an optimal cardioprotection against I-R injury (data not shown), EMD or OA pretreatment might offer additional protection via action mechanism(s) complementary to those of IPC. It is unlikely that the *in vivo* antioxidant potential of EMD and OA can abrogate the protective effect of IPC, which is mediated by cellular processes involving oxy-radicals. Alternatively, EMD/OA may be used as a pharmacological preconditioning agent to prevent the patients, particularly those with angina and chronic ischemic symptoms, from developing severe myocardial infarction. Such an agent may also reduce the extent of I-R damage that develops during a high-risk angioplasty procedure in which the occluded vessel supplies a very large risk area.

In conclusion, chronic EMD/OA pretreatment at low dose protected against myocardial I-R injury, as assessed by LDH leakage and contractile force recovery. The cardioprotection was associated with a differential enhancement in mitochondrial antioxidant components. The combined EMD/OA and IPC pretreatment produced cardioprotective action in a semi-additive manner. This suggested that EMD/OA pretreatment and IPC protected against myocardial I-R injury via a similar but not identical biochemical mechanism.

References

1. Baines CP, Zhang J, Wang GW, Zheng YT, Xiu JX, Cardwell EM, Bolli R, Ping P: Mitochondrial PKCε and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKCε-MAPK interactions and differential MAPK activation in PKCε-induced cardioprotection. Circ Res 90: 390–397, 2002
2. Ghosh S, Standen NB, Galinanes M: Preconditioning the human myocardium by simulated ischemia: Studies on the early and delayed protection. Cardiovasc Res 45: 339–350, 2000
3. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusche G, Cohen MV, Downey JM: Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. Circ Res 87: 460–466, 2000
4. Kloner R, Jennings R: Consequences of brief ischemia: Stunning, preconditioning and their clinical implications Part II. Circulation 104: 3158–3167, 2001
5. Huang KC: The Pharmacology of Chinese Herbs. CRC Press, Boca Raton, Ann Arbor, London, Tokyo, pp 101–103, 1993
6. Liu J: Pharmacology of oleanolic acid and ursolic acid. J Ethnopharmacol 49: 57–68, 1995
7. Shi HY, Cheng SH, Chen CC, Lee H: Emodin inhibits the mutagenicity and DNA adducts induced by 1-nitropyrene. Mut Res 329: 205–212, 1995
8. Huang HC, Chu SH, Chao-Lee PD: Vasorelexants from Chinese herbs, emodin and scoparone, possess immunosuppressive properties. Eur J Pharmacol 198: 211–213, 1991
9. Marquina S, Maldonado N, Garduno-Ramirez ML, Aranda E, Villareal ML, Navarro V *et al.*: Bioactive oleanolic acid saponins and other constituents from roots of Viguiera decurrens. Phytochemistry 56: 93–97, 2001
10. Yim TK, Wu WK, Pak WF, Ko KM: Hepatoprotective action of an oleanolic acid-enriched extract of *Ligustrum lucidum* fruits is mediated through an enhancement on hepatic glutathione regeneration capacity in mice. Phytother Res 15: 589–592, 2001
11. Yim TK, Wu WK, Mak DHF, Ko KM: Myocardial protective effect of an anthraquinone-containing extract of Polygonum multiflorum *ex vivo*. Planta Med 64: 607–611, 1998
12. Du Y, Ko KM: 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. Life Sci 77: 2770–2782, 2005
13. Du Y, Ko KM: Oleanolic acid protects against myocardial ischemia-reperfusion injury by enhancing mitochondrial antioxidant mechanism mediated by glutathione and α-tocopherol in rats. Planta Med (in press)
14. Li PC, Mak DHF, Poon MKT, Ip SP, Ko KM: Myocardial protective effect of Sheng Mai San (SMS) and a lignan-enriched extract of Fructus Schisandrae, in vivo and *ex vivo*. Phytomedicine III: 217–221, 1996
15. Evans WH: Isolation and characterization of membranes and cell organelles. In: Rickwood D (ed). Preparative Centrifugation: A practical approach. Oxford University Press, New York, 1992, pp. 233–270
16. Griffith OW: Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106: 207–12, 1980
17. Sadrzadeh SM, Nanji AA, Meydani M: Effect of chronic ethanol feeding on plasma and liver alpha- and gamma-tocopherol levels in normal and vitamin E-deficient rats. Relationship to lipid peroxidation. Biochem Pharmacol 47: 2005–2010, 1994
18. McCord JM, Fridovich I: Superoxide dismutase. An enzyme function for erythrocuprein (Hemocuprein). J Biol Chem 244: 6049–6055, 1969
19. Takemura G, Onodera T, Millard RW, Ashraf M: Demonstration of hydroxyl radical and its role in hydrogen peroxide-induced myocardial injury: hydroxyl radical dependent and independent mechanisms. Free Radic Biol Med 15: 13–25, 1993
20. Kaul N, Siveski-Iliskovic N, Hill M, Slezak J, Singal PK: Free radicals and the heart. J Pharmacol Toxicol Methods 30: 55–67, 1993
21. Pagliaro P, Mancardi D, Rastaldo R, Penna C, Gattullo D, Miranda KM, Feelisch M, Wink DA, Paolocci N: Nitroxyl affords thiol-sensitive myocardial protective effects akin to early preconditioning. Free Radic Biol Med 34: 33–43, 2003
22. Baker JE, Boerboom LE, Olinger GN: Age-related changes in the ability of hypothermia and cardioplegia to protect ischemic rabbit myocardium. J Thorac Cardiovasc Surg 96: 717–724, 1988
23. Maupoil V, Rochette L: Evalulation of free radical and lipid peroxide formation during global ischemia and reperfusion in isolated perfused rat heart. Cardiovasc Drugs Ther 2: 615–621, 1988
24. Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M, Chiariello M: Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J Biol Chem 268: 18532–18541, 1993
25. Venditti P, Masullo P, Di Meo S: Effects of myocardial ischemia and reperfusion on mitochondrial function and susceptibility to oxidative stress. Cell Mol Life Sci 58: 1528–1537, 2001

26. Kaneko M, Beamish RE, Dhalla NS: Reduction of calcium channel antagonist binding sites by oxygen free radicals in rat heart. J Mol Cell Cardiol 21: 935–943, 1989
27. Leedle RA, Aust SD: The effect of glutathione on the vitamin E requirement for inhibition of liver microsomal lipid peroxidation. Lipids 25: 241–245, 1990
28. Murry CE, Richard VJ, Reimer KA, Jennings RB: Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. Circ Res 66: 913–931, 1990
29. Otani H, Tanaka H, Inoue T, Umemoto M, Omoto K, Tanaka K, Sato T, Osako T, Masuda A, Nonoyama A *et al.*: (1984). *In vitro* study on contribution of oxidative metabolism of isolated rabbit heart mitochondria to myocardial reperfusion injury. Circ Res 55: 168–175, 1984
30. Ambrosio G., Zweier JL, Flaherty JT: The relationship between oxygen radical generation and impairment of myocardial energy metabolism following post-ischemic reperfusion. J Mol Cell Cardiol 23: 1359–74, 1991