Protective Effects of Cyclo(L-Leu-L-Tyr) against Postischemic Myocardial Dysfunction in Guinea-Pig Hearts

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The protective effects of cyclic dipeptides in alcoholic beverages were investigated in the perfused guinea-pig hearts subjected to ischemia and reperfusion. Subsequently, in order to determine the importance of cyclic dipeptide structure, the effects of cyclo(L-Leu-L-Tyr) (cLY) were compared with those of the newly synthesized non-cyclic dipeptides, L-Leu-L-Tyr (LY) and L-Tyr-L-Leu (YL). After reperfusion, pressure recovery (%) in the left ventricle reached a peak of over 90% in the presence of cLY (10^{-6} M and 10^{-5} M) (control: 22.9%). The recovery by LY and YL was significantly lower than that by cLY, and ATP levels simultaneously monitored using ³¹P-NMR were already lower during the ischemic end period than those observed with cLY treatment. In perfused mitochondrial preparations, cLY significantly inhibited mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_{\text{m}}$) elevation in a similar way to that of the mitochondrial permeability transition pore (MPTP) inhibitor cyclosporin A. *In vitro* electron paramagnetic resonance (EPR) revealed that the active oxygen radicals quenching activity of cLY was greater than those of non-cyclic dipeptides. cLY inhibited caspase-3-induced apoptosis. The cyclic dipeptide structure inhibits opening of the MPTP by preventing $[\text{Ca}^{2+}]_{\text{m}}$ overload-induced apoptosis related to mitochondrial active oxygen radical accumulation in ischemia-reperfusion hearts.

Key words cyclic dipeptide; ischemia-reperfusion injury; ³¹P-NMR; electron paramagnetic resonance; mitochondrial permeability transition pore; apoptosis

Cyclic dipeptides have been detected in a variety of natural products as well as in processed foods, beverages, and food and beverage ingredients. A number of bitter proline-based cyclic dipeptides, namely cyclo(Ala-Pro), cyclo(Ile-Pro), cyclo(Leu-Pro), cyclo(Met-Pro), cyclo(Phe-Pro), and cyclo(Pro-Pro), have now been identified in beer¹⁾ or aged sake.²⁾ Cyclic dipeptides, which are composed of other amino acids than proline, have been identified in the distilled residue of millet brandy (awamori), and many other cyclic dipeptide have been newly synthesized in studies on structure–activity interactions.³⁾ The ability of natural and synthesized cyclic dipeptides to scavenge active oxygen radicals has been measured by electron paramagnetic resonance (EPR), and the cardioprotective effects of these compounds have been investigated in perfused guinea-pig hearts subjected to ischemia and reperfusion. Pretreatment with cyclo(L-Leu-L-Tyr) (cLY) promoted optimal recovery after injury.

In order to determine the importance of the cyclic dipeptide in cardioprotection, the effects of cLY in guinea-pig hearts were compared with those of the newly synthesized non-cyclic dipeptides L-Leu-L-Tyr (LY) and L-Tyr-L-Leu (YL) (Fig. 1). We studied the effects of cLY, LY, and YL on sequential changes in left ventricular contractility as well as the cellular metabolism of high-energy phosphorous in isolated guinea-pig Langendorff hearts subjected to normothermic global ischemia and subsequent reperfusion. Additionally, mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_{\text{m}}$) uptake following changes in the perfusate acidity or Ca^{2+} content similar to those occurring at the end of ischemia or reperfusion after global is-

chemia in Langendorff hearts were investigated prior to the administration of cyclic dipeptide. The role of $[\text{Ca}^{2+}]_{\text{m}}$ mediated injury was determined by investigating the mitochondrial permeability transition pore (MPTP) using openers and inhibitors of the MPTP.^{4,5)}

Our results suggest that cyclic dipeptides inhibit the opening of the MPTP by preventing $[\text{Ca}^{2+}]_{\text{m}}$ overload-induced apoptosis in ischemic-reperfusion hearts.⁶⁾ There is now evi-

Fig. 1. Structures of Cyclic Dipeptide, cLY, and Non-cyclic Dipeptides, LY and YL

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dence that apoptosis, or programmed cell death, is an important response of the myocardium to ischemia that precedes cell necrosis and appears to contribute to the overall sequelae of cardiac injury.^{7–9} Therefore, we examined the protective activities of cyclic dipeptides and non-cyclic dipeptides with respect to their reduction of caspase-3 activity-induced apoptosis.

MATERIALS AND METHODS

Chemicals Fura-2 AM was obtained from Dojindo Laboratories (Kumamoto, Japan). Methylenediphosphonic acid and cremophore EL were purchased from Molecular Probes (Eugene, OR, U.S.A.). 5,5-Dimethyl-1-pyrroline-1-oxide (DMPO) was obtained from Labotec (Tokyo, Japan). Hypoxanthine, atractyloside potassium salt (Atr), cyclosporin A (CsA), diethylene triamine penta acetic acid (DETAPAC), and xanthine oxidase (XOD) were from Sigma (St. Louis, MO, U.S.A.). FK506 was generously provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). The CPP32/caspase-3 fluorometric protease assay kit was purchased from MBL (Nagoya, Japan). All other chemicals were of reagent grade.

Preparation of Cyclo(L-Leu-L-Tyr) To a solution of L-tyrosine methyl ester hydrochloride in dichloromethane at -15°C was added one equivalent triethylamine, *N*-tert-butoxycarbonyl (BOC)-L-leucine, and a solution of dicyclohexylcarbodiimide (DCC) in dichloromethane at 0°C . The reaction mixture was stirred at 0°C for 1 h. The reaction solution was stirred at room temperature for 3 h. The dicyclohexylurea was filtered from the reaction mixture. Dichloromethane was added to reaction mixture, and the reaction mixture was washed with 5% NaHCO_3 , water, 1 M HCl, water, and saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO_4 , filtrated, and evaporated. The residue was purified by silica gel column chromatography to afford *N*-BOC-L-Leu-L-Tyr methyl ester.

N-BOC-L-Leu-L-Tyr methyl ester was treated with 4 M HCl–1,4-dioxane at room temperature for 30 min. The reaction mixture was evaporated. The residue was dissolved in 0.1 M acetic acid–2-butanol, and to this solution was added one equivalent *N*-methyl-morpholine. The reaction mixture was refluxed for 3 h under N_2 . The reaction mixture was concentrated until crystal of cyclic dipeptide appeared. The crystal was collected by filtration, and was washed with small amounts of 2-butanol followed by silica gel column chromatography.

Cyclo(L-Leu-L-Tyr) Colorless powder. $[\alpha]_{\text{D}}^{25}$: +222.3 ($c=0.10$, methanol). IR ν_{max} (KBr): 3312, 3204, 3084, 2953, 1676, 1661 cm^{-1} . $^1\text{H-NMR}$ ($\text{DMSO}-d_6$, 400 MHz) δ_{H} : 0.14 (ddd, $J=5.0, 9.2, 13.8$ Hz), 0.63 (3H, d, $J=6.6$ Hz), 0.65 (3H, d, $J=6.6$ Hz), 0.76 (ddd, $J=5.0, 11.9, 13.8$ Hz), 1.42 (m), 2.68 (dd, $J=4.6, 13.3$ Hz), 3.01 (dd, $J=3.7, 13.8$ Hz), 3.45 (brdd, $J=4.1, 8.3$ Hz), 4.06 (brs), 6.63 (2H, d, $J=8.3$ Hz), 6.89 (2H, d, $J=8.3$ Hz), 8.03 (s), 8.04 (s), 9.20 (s). $^{13}\text{C-NMR}$ ($\text{DMSO}-d_6$, 125 MHz) δ_{C} : 21.3, 22.8, 23.0, 37.8, 43.7, 52.3, 55.7, 114.9, 125.9, 131.3, 156.4, 166.3, 167.5. High-resolution FAB-MS: $\text{C}_{15}\text{H}_{21}\text{N}_2\text{O}_3$ 277.1516 [(M+H)⁺, Calcd 277.1552]. Positive FAB-MS m/z : 277 (M+H)⁺.

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.

Langendorff Guinea-Pig Hearts—Heart Preparation and Examination Procedures Hartley strain guinea pigs of either gender weighing 300–350 g were anesthetized with diethyl ether and heparinized (250 IU, intraperitoneally (i.p.)). The heart was rapidly excised and the aorta was cannulated as described previously.^{10–12} The Langendorff heart preparations perfused spontaneous beating conditions 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 fluorometry. The perfused hearts were exposed to 40 min of global ischemia by clamping the perfusion flow line, and were then reperfused for 40 min. In the treatment groups, each concentration of dipeptide was introduced into the perfusate 4 min before the start of ischemia, and each drug was also added to the perfusate during reperfusion. Hearts used for examination of apoptotic cells and caspase-3 activity were subjected to 1 h of ischemia and 5 h of reperfusion. cLY or LY was administered for 4 min before ischemia and for 5 h throughout reperfusion.

Measurement of Myocardial Function Coronary flow (FR) during NMR analysis was measured continuously with an in-line flow probe connected to an ultrasonic flow meter (transonic T101, Advance, Ithaca, NY, U.S.A.). A latex balloon (Hirokawa, Niigata, Japan) was introduced into the left ventricle *via* the left atrium and connected to a strain-gauge transducer (MIP-5100, Baxer, Tokyo, Japan) for the measurement of the isovolumic left ventricular pressure. The left ventricular end-diastolic pressure (LVEDP) 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) was calculated by subtracting the LVEDP from the left ventricular systolic pressure.

$^{31}\text{P-NMR}$ Measurement and Data Analysis $^{31}\text{P-NMR}$ spectra were monitored along with simultaneous recordings of ventricular pressure in the ischemia-reperfusion experiments. $^{31}\text{P-NMR}$ spectra were obtained at 161.8 MHz on a GSX 400 spectrometer (JEOL, Tokyo, Japan) equipped with a 9.4-Tesla vertical-bore magnet. For each spectrum, 90 free-induction decays (4 min) were accumulated after 45-degree flip-angle pulses (18 μs) using 4k data points and a 15.015 kHz spectral width with a repetition time of 2 s. Accumulated free-induction decays (FID) were filtered exponentially, resulting in a broadening of 30 Hz.

Phosphocreatine (PCr), inorganic phosphate (Pi) and β -ATP were quantified by comparison with a capillary tube of standard methylenediphosphonic acid (0.25 M) fixed inside the NMR tube. Phosphate peaks expressed as percentages of control values were determined by measuring the area under each resonance peak. The subsequent relative intensities of each peak were used for quantitative analysis. Datum Station ALICE software (JEOL DATUM, Tokyo, Japan) was used to determine the area under each peak. The intracellular pH (pH_i) was calculated from the chemical shift between the PCr and Pi resonances.

Mitochondrial Fluorometric Measurements after Changes in the Ca^{2+} Concentration or pH in the Perfusate

Mitochondria were isolated from guinea-pig hearts as previously described.^{13,14} The suspensions were incubated at 24 °C in a normal medium containing respiratory substrates (composition in mM: sucrose 250, MgCl_2 1, KH_2PO_4 1, succinate 10, malate 5 and 3-morpholinopropanesulfonic acid (MOPS) 20 adjusted to pH 7.4 with KOH) and intracellular ionic compositions (100 nM Ca^{2+} , 10 mM Na^+ , and 110 mM K^+) containing the Ca^{2+} fluoroprobe fura-2AM (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. The coverslip was treated with poly-L-lysine to promote mitochondrial adhesion. Fura-2 Ca^{2+} signals were measured at 500 nm as the ratio of emitted fluorescence (R340/380) after excitation at 340 nm (F340) and 380 nm (F380). After the loading of fura-2 for a period of 30 min, the amplitude of F340 from treated mitochondria increased by 5–7-fold compared with that of unloaded mitochondria. The mitochondrial Ca^{2+} signal after preloading with high Ca^{2+} concentrations (10 μM) for over 5 min was markedly increased by lowering the Ca^{2+} concentration from a high to a physiologically low level (100 nM) or by acidifying the perfusate (pH 7.5 to 6.5). We then determined whether each dipeptide (10⁻⁶ M) suppressed the Ca^{2+} increase induced by these manipulations. MPTP related compounds also were tested in the same manner. The final mitochondrial protein concentration was adjusted to 30–35 mg/ml by dilution.

Electron Paramagnetic Resonance (EPR) Spectrometry—Measurement of the Quenching Effect of Superoxide Anion Radicals and Hydroxyl Radicals

The effect of each peptide on production of the superoxide anion radical (O_2^-) and hydroxyl radical ($\cdot\text{OH}$) was determined using EPR spectrometry as reported previously¹³ with some modifications. The final concentration of each peptide for testing of radical quenching was 2.5×10^{-6} – 2.5×10^{-3} M. The conditions of EPR spectrometry (JES-RE, JEOL Co., Ltd., Tokyo, Japan) used to estimate the concentration of O_2^- radicals and $\cdot\text{OH}$ were as follows: magnetic field: 335.7 ± 5 mT; power: 4 mW 9.414 GHz; modulation: 100 KHz $\cdot 1 \times 0.079$ mT; response: 0.1 s; temperature: 25 °C; amplitude: 160; sweep time: 2 min.

For the analysis of O_2^- radicals, hypoxanthine, DETAPAC, each peptide or vehicle (DMSO), DMPO, and XOD 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 measurement error.

For the analysis of $\cdot\text{OH}$, FeSO_4 solution, hydrogen peroxide (H_2O_2), each peptide or vehicle (0.5% methyl cellulose), and DMPO were mixed in a test tube. The amount of DMPO- $\cdot\text{OH}$ spin adducts that formed was estimated exactly 45 s after DMPO was added. The signal intensities were evaluated from the peak height of the second signal of the quartet of the DMPO- $\cdot\text{OH}$ spin adduct relative to the intensity of the Mn^{2+} signal.

Examination Procedures for the Detection of Apoptosis—Histological Examination and Assessment of Internucleosomal DNA-cleavage by the Terminal Deoxy-Nucleotidyl Transferase-Mediated Deoxyuridine Triphosphate (dUTP) Nick-End Labeling (TUNEL) Method and Measurement of Caspase-3 Activity The Langendorff heart preparations were perfused with normal Krebs–Henseleit solution as previously described for ³¹P-NMR measurement. Subsequent to an equilibration period of 30 min required to stabilize mechanical function, the perfusate was replaced with a modified KH solution in which 10 mM glucose was replaced with 5 mM 2-deoxy-D-glucose and 5 mM glucose 4 min before 1 h ischemia.¹⁵ After reperfusion for 5 h the left ventricle was cut into several pieces. Each peptide (10⁻⁶ M) was introduced into the modified perfusate for 4 min before the start of ischemia, and then reperused with the drug-containing medium of the perfusate. The changes in cellular levels of high phosphorous energy in the heart were monitored with ³¹P-NMR together with simultaneous recordings of LVDP, LVEDP and FR. The proximal portions of the isolated Langendorff hearts were fixed for 2 h at room temperature with phosphate-buffered solution containing 2% paraformaldehyde and embedded in paraffin for subsequent routine histological examination and the TUNEL staining.^{15,16}

Other sections of each heart were stored in liquid nitrogen for the measurement of the activity of caspase-3 using a CPP32/caspase-3 fluorometric protease assay kit.¹⁷ 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 ($\lambda_{\text{max}}=400$ nm); upon cleavage of the substrate by CPP32 or related caspases, free AFC emits yellow-green fluorescence ($\lambda_{\text{max}}=505$ nm), which can be quantified using a fluorometer. Comparison of the fluorescence of AFC from an apoptotic sample with an uninduced control allows the determination of the fold-increase in CPP32 activity. The uninduced control was a heart preparation, which was perfused at 30 min with normal solution (control-1).

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

RESULTS

Beneficial Effects of Cyclic Dipeptide (cLY) and Newly Synthesized Non-cyclic Dipeptides (LY and YL) during Ischemia-Reperfusion in Langendorff Heart Preparations—Post-Ischemic Recovery of Contraction (LVDP), End-Diastolic Pressure (LVEDP), and Coronary Flow Rate (FR), and Heart Rate (HR) Figure 2A shows sequential changes in the contractility of the drug-free group (control) and pretreated group during ischemia-reperfusion. Actual values of LVDP (mmHg), LVEDP (mmHg) and FR (ml/min), and HR (times/min) are shown in Table 1.

In the control hearts, LVDP recovered to less than 22.9% of the preischemic level (as 100%) at 40 min of reperfusion. In the cLY (10⁻⁶ M)-pretreated hearts during ischemia-reperfusion, there was a more marked recovery of LVDP to 95.8%

Fig. 2. (A) Beneficial Effects of cLY (10^{-6} M) During Ischemia-Reperfusion in Langendorff Heart Preparations

Treatment with cLY enhanced the recovery of LVDP after reperfusion compared to the controls, LY and YL.

(B) Post-Ischemic Recovery of LVDP by Reperfusion in Untreated Heart (Control), and Hearts Treated with cLY, LY or YL (Left) and the Changes in High Phosphorous Energy (ATP) during 40 min of Ischemia and a Subsequent 40 min of Reperfusion (Right)

The level of ATP at the end of ischemia and reperfusion was significantly higher for cLY-treated hearts than in the control. $**p < 0.01$; significantly different from the control value. Data are expressed as the mean values of 6 preparations. Vertical lines represent S.E.M.

of the preischemic value ($p < 0.01$ compared to control). The recovery of LVDP to preischemic values in cLY-pretreated hearts (Fig. 2B) was greater than that in LY- and YL-treated hearts (42.8, 21.6%, respectively).

LVEDP at the end of 40-min ischemia (ischemic contracture) was significantly lower in hearts pretreated with cLY (10^{-6} M) during ischemia and reperfusion than in untreated, LY-, or YL-treated hearts, as shown in 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.

At the end of reperfusion in the control hearts, FR was significantly lower than the preischemic level. However, compared to the control, hearts pretreated with cLY showed significant differences at the end of reperfusion. There was no significant difference between the control, LY, and YL. The rate of HR recovery in pre- and posttreated groups of Langendorff heart preparations exposed to cLY (10^{-6} M) was 94 and 70% at the end of reperfusion, respectively ($p < 0.05$ compared to control).

In posttreated group of Langendorff heart preparations exposed to cLY (10^{-6} M) during reperfusion, the rate of recovery of LVDP was 75% ($p < 0.05$ compared to control). The administration of cLY during reperfusion increased LVDP gradually, as shown at Na^+/H^+ exchange inhibitors in our previous paper.¹⁸⁾

Effect of cLY on High Phosphorous Energy during Ischemia and Reperfusion Determined by ^{31}P -NMR Spectroscopy High phosphorous energy, as represented by β -ATP levels, is presented graphically as percentages relative to the preischemic baseline with the post-ischemic LVDP in Fig. 2B. At the end of 40-min ischemia, significant differences in the preservation of ATP were obtained in hearts pretreated with cLY when compared to the control, LY, and YL groups. During reperfusion, there was a resynthesis of ATP in the pretreated hearts. The level of ATP at the end of 40-min reperfusion in the cLY groups was significantly higher

Table 1. Left Ventricular Developed Pressure (LVDP), Ventricular End Diastolic Pressure (LVEDP), Coronary Flow Rate (FR), and Heart Rate (HR) Changes during Ischemia-Reperfusion of Langendorff Hearts

		<i>n</i>	LVDP (mmHg)			LVEDP (mmHg)		
			Pre	Isch	Rep	Pre	Isch	Rep
Control		6	63.3 $\pm$ 7.8	0	24.7 $\pm$ 5.3	10	39.3 $\pm$ 4.1	41.5 $\pm$ 1.9
Pre-cLY	10^{-6} M	6	65.2 $\pm$ 4.2	0	62.5 $\pm$ 7.4*	10	14.8 $\pm$ 1.8**	13.7 $\pm$ 4.3**
LY	10^{-6} M	6	62.8 $\pm$ 4.5	0	26.2 $\pm$ 4.9	10	36.2 $\pm$ 1.8	36.2 $\pm$ 1.8
YL	10^{-6} M	6	61.5 $\pm$ 13.8	0	13.3 $\pm$ 4.3	10	36.4 $\pm$ 4.9	36.4 $\pm$ 4.9
Post-cLY	10^{-6} M	5	58.1 $\pm$ 4.7	0	43.6 $\pm$ 4.2**	10	37.0 $\pm$ 5.7	26.7 $\pm$ 4.8**

		<i>n</i>	FR (ml/min)			HR (times/min)		
			Pre	Isch	Rep	Pre	Isch	Rep
Control		6	10.4 $\pm$ 0.5	0	4.8 $\pm$ 0.7	180 $\pm$ 10.2	0	65.2 $\pm$ 7.2
Pre-cLY	10^{-6} M	6	10.5 $\pm$ 1.0	0	8.4 $\pm$ 0.7**	191 $\pm$ 11.0	0	180 $\pm$ 10.0*
LY	10^{-6} M	6	9.4 $\pm$ 0.9	0	5.4 $\pm$ 0.9	183 $\pm$ 9.5	0	73.2 $\pm$ 8.7
YL	10^{-6} M	6	9.8 $\pm$ 0.5	0	4.8 $\pm$ 0.9	189 $\pm$ 12.0	0	59.0 $\pm$ 11.0
Post-cLY	10^{-6} M	5	10.4 $\pm$ 1.2	0	5.8 $\pm$ 0.7*	186 $\pm$ 9.7	0	130 $\pm$ 6.7**

Changes during 40 min of ischemia and a subsequent 40 min of reperfusion. Values are the mean $\pm$ S.E.M. * $p < 0.01$, ** $p < 0.05$; significantly different from control values. Pre, preischemia; Isch, ischemia; Rep, reperfusion. Drug was introduced into the perfusion on-line 4 min before the start of ischemia (Pre-). Drug was introduced into the perfusate for reperfusion (Post-).

Fig. 3. (A) Recordings of Mitochondrial Ca^{2+} from Fura-2-Loaded Mitochondria Preparations Affected by Changing the Ca^{2+} Concentrations or pH in the Perfusate

The mitochondrial Ca^{2+} signal ($[\text{Ca}^{2+}]_m$) gradually increased with extremely high Ca^{2+} ($10 \mu\text{M}$), and perfusion with a low Ca^{2+} -content solution (100 nM) also rapidly increased the mitochondrial 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 observed on changing Ca^{2+} content (right, top). cLY (10^{-5} M) but not LY and YL at 10^{-5} M reduced the increase in mitochondrial Ca^{2+} induced signals by the displacement of perfusate.

(B) Comparison of the Inhibition of Ca^{2+} -Uptake into Mitochondria in the Presence of cLY, LY, or YL

The mitochondrial Ca^{2+} level was elevated by an external Ca^{2+} concentration change (left) or external acidification (right). The $[\text{Ca}^{2+}]_m$ elevation was reduced by cLY. $**p < 0.01$, significantly different from the control (drug-free) values. Values are expressed as the mean values of 3–5 preparations. Vertical lines represent the S.E.M.

than in the control, LY, and YL groups. A rapid decrease in PCr was observed during ischemia in all groups. However, pH_i values in these hearts at 40 min of reperfusion were not significantly different from the control value.

Mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_m$) Measurements with Fura-2 AM The mitochondrial fura-2 Ca^{2+} signal ($[\text{Ca}^{2+}]_m$) increased steadily and linearly but remained at only 10% excess at extremely high Ca^{2+} concentrations ($1 \mu\text{M}$ – 1 mM) of the perfusate (Fig. 3A). The displacement of the normal matrix Ca^{2+} concentration (100 nM) with perfusate led to a rapid and marked increase of up to about 9.2-fold. Pretreatment of mitochondria with cLY (10^{-6} and 10^{-5} M) markedly suppressed the increase in $[\text{Ca}^{2+}]_m$, that occurred in the drug-free perfusate by 72.0 and 50.0%, respectively. However, the non-cyclic dipeptides LY and YL (10^{-5} M) did not suppress the increase in $[\text{Ca}^{2+}]_m$ (Fig. 3B).

Changing the pH from 7.4 to 6.5 in a low- Ca^{2+} perfusate (100 nM) maximally elevated the $[\text{Ca}^{2+}]_m$ up to about ten-fold relative to the pH 7.4 perfusate (Fig. 3A). The appreciable $[\text{Ca}^{2+}]_m$ elevation induced by acidification (0.13 to 1.25) was reduced by pretreatment with cLY at 10^{-6} M and 10^{-5} M by 75 and 28.8%, respectively. LY and YL (10^{-5} M) did not suppress the increase in $[\text{Ca}^{2+}]_m$ (Fig. 3B). The acid-induced

Fig. 4. (A) The $[\text{Ca}^{2+}]_m$ Elevation That Was Brought about by Perfusate Acidification Was Reduced by the Infusion of cLY (10^{-5} M). (B) The Decrease in $[\text{Ca}^{2+}]_m$ Caused by the Administration of cLY Was Greater Than That Induced LY or YL. (C) The $[\text{Ca}^{2+}]_m$ Elevation Brought about by Perfusate Acidification Decreased after Addition of the MPTP Inhibitor Cyclosporin A (CsA) ($3 \times 10^{-4} \text{ M}$), but Was Unaffected by FK 506 ($3 \times 10^{-4} \text{ M}$) Which Does Not Inhibit MPTP Opening

Treatment with the mitochondrial PTP opener atractiroside (Atr) ($3 \times 10^{-3} \text{ M}$) resulted essentially in the same $[\text{Ca}^{2+}]_m$ elevation brought about by perfusate acidification and an external Ca^{2+} concentration change.

increase in $[\text{Ca}^{2+}]_m$ was greater and more rapid than that caused by perfusion with the low- Ca^{2+} perfusate.

As shown in Fig. 4A, the addition of cLY (10^{-5} M) to the perfusate released Ca^{2+} from the mitochondria. The effect of cLY and CsA (10^{-4} M), which inhibits MPTP opening, was greater than that of LY and YL (Fig. 4B). In contrast, Atr (10^{-3} M), which opens the MPTP, caused increases in $[\text{Ca}^{2+}]_m$ similar to those observed on acidifying the perfusate or changing its Ca^{2+} concentration. Treatment with cLY and CsA released mitochondrial Ca^{2+} that had been increased by changes in the perfusates Ca^{2+} content, acidification, or treatment with Atr (Fig. 4C) but not FK506, which inhibits the phosphatase calcineurin without inhibiting MPTP opening. The pretreatment with 10^{-4} M CsA attenuated mitochondrial Ca^{2+} release. The addition of Ru360, ryanosine, verapamil, diazoxide, and chlonazepam to perfusate increased steadily and constantly the change in concentration of Ca^{2+} released from mitochondria (Fig. 5). These effects were similar to those seen with cLY or CsA which inhibits MPTP.

In Vitro Free Radical Quenching Activity of cLY, LY, and YL In the experiment on quenching activity, cLY, LY, and YL at final concentrations from 2.5×10^{-6} to $2.5 \times 10^{-3} \text{ M}$ dose-dependently quenched the generation of $\cdot\text{OH}$. The IC_{50} (concentration causing a 50% scavenging effect) of cLY, LY and YL was $1.8 \times 10^{-4} \text{ M}$, 2.0×10^{-3} , and 1.3×10^{-3} , respectively. cLY also showed quenching activity against O_2^- radicals at $2.5 \times 10^{-3} \text{ M}$ with a 30% decrease, but LY and YL produced almost no change. However, the IC_{50} value of cLY could not be measured because it did not cause 50% inhibition. The IC_{50} of vitamin C was $2.4 \times 10^{-3} \text{ M}$ ($\cdot\text{OH}$) and $6.3 \times 10^{-5} \text{ M}$ (O_2^- radicals); the radical quenching activity of cLY against $\cdot\text{OH}$ was stronger than that of vitamin C.

Detection of Apoptosis Examples of apoptotic tissues obtained after the various treatments are shown in our previous report.¹⁸⁾ Apoptotic cells were first observed in the ven-

Table 2. Left Ventricular Developed Pressure (LVDP), Ventricular End Diastolic Pressure (LVEDP), and Coronary Flow Rate (FR) and High Phosphorous Energy (PCr, β -ATP) Changes during Ischemia-Reperfusion of Langendorff Hearts for the TUNEL and Caspase-3-Activity Measurements

	<i>n</i>	LVDP (mmHg)			LVEDP (mmHg)			FR (ml/min)		
		Pre	Isch	Rep	Pre	Isch	Rep	Pre	Isch	Rep
Control	5	62.3 $\pm$ 8.6	0	2.0 $\pm$ 1.2	10	34.2 $\pm$ 4.1	41.6 $\pm$ 2.0	10.4 $\pm$ 1.0	0	0.5 $\pm$ 0.1
cLY 10 ⁻⁶ M	5	60.5 $\pm$ 8.5	0	6.8 $\pm$ 1.7	10	24.7 $\pm$ 2.3*	35.7 $\pm$ 2.8*	10.8 $\pm$ 2.3	0	2.8 $\pm$ 1.0
LY 10 ⁻⁶ M	5	61.7 $\pm$ 7.9	0	3.4 $\pm$ 1.5	10	31.8 $\pm$ 3.5	40.7 $\pm$ 4.0	10.5 $\pm$ 1.7	0	0.7 $\pm$ 0.8

	<i>n</i>	ATP (%)			PCr (%)		
		Pre	Isch	Rep	Pre	Isch	Rep
Control	5	100	12.6 $\pm$ 2.0	12.4 $\pm$ 2.2	100	0	4.8 $\pm$ 2.3
cLY 10 ⁻⁶ M	5	100	18.9 $\pm$ 3.2	20.7 $\pm$ 2.7	100	0	38.7 $\pm$ 12.7*
LY 10 ⁻⁶ M	5	100	15.7 $\pm$ 3.5	15.7 $\pm$ 3.0	100	0	10.8 $\pm$ 8.7

Changes during 1 h of ischemia and a subsequent 5 h of reperfusion. Values are the mean $\pm$ S.E.M. * p <0.05; significantly different from control values. Pre, preischemia; Isch, ischemia; Rep, reperfusion.

Fig. 5. Mitochondrial Ca²⁺ Uptake by Ca²⁺-Content Changes of the Perfusate Decreased by Treatment with Verapamil (Ver), Ryanosine (Rya), Ru360, and Chlonezapam (Clo) at 10⁻⁴ M as Similar with Cyclosporin A, Which Inhibits MPTP as Shown in Fig. 4

tricle after 5 h of reperfusion following 1 h of ischemia with KH solution containing 2-deoxy-D-glucose.¹⁵⁾ As shown in Fig. 6A, 5 h of reperfusion resulted in an average of 27.7 $\pm$ 2.4 apoptotic cells per 100 microscopic fields, but 4 h of reperfusion did not produce apoptotic cells. cLY and LY treatment (10⁻⁶ M) reduced the incidence of apoptosis in the ischemic and reperfused myocardium to an average of 4.5 $\pm$ 1.1 (p <0.05) and 23.6 $\pm$ 2.6 (not significant) cells per 100 microscopic fields, respectively, compared with the control. The effect of cLY on apoptosis was greater than that of LY (p <0.05).

Caspase-3 activity in the control and apoptotic samples was measured in the same frozen tissues using the TUNEL method. Control-1 was heart preparations perfused for 30 min with normal KH solution, while control-2 was hearts subjected to 1 h of ischemia and 5 h of reperfusion with a drug-free solution (control). The decrease of caspase-3 activity in hearts pretreated with cLY (Control-1: 1.0, Control-2: 6.5 $\pm$ 1.0, LY: 5.9 $\pm$ 0.9, cLY: 2.0 $\pm$ 0.6, p <0.05 for control-2, p <0.05 for LY-treated heart) was similar to the results of TUNEL staining (Fig. 6B).

Fig. 6. (A) The Incidence of Apoptosis Observed Using the TUNEL Staining

Values are expressed as the mean of 5 preparations $\pm$ S.E.M. and indicate the number of positively stained cells identified per 100 microscopic fields. * p <0.05 significantly different compared with control (drug-free) values. # p <0.05 significantly different between LY and cLY-treated groups.

(B) Control-1: The Uninduced Control Was a Heart Preparation Perfused at 30 min with Normal KH Solution

Values are expressed as the mean of 5 preparations $\pm$ S.E.M. and indicate caspase-3 activity. * p <0.05 significantly different compared with the control (drug-free) values. # p <0.05 significantly different between the cLY and LY-treated groups.

As shown in Table 2, which lists the changes in mechanical function and high phosphorous energy, the LVEDP and PCr were significantly different in hearts treated with cLY at 10⁻⁶ M after 5 h reperfusion compared with the control (p <0.05), but LY at 10⁻⁶ M generated no appreciable difference. The changes in the coronary flow, LVDP, and ATP signals were also not significantly different in hearts pretreated with cLY compared to the control or LY-treated hearts. From these results, the effects of cLY on the recovery of mechanical function and tissue levels of high-energy phosphate were greater than those of LY.

DISCUSSION

In the pretreated hearts, cLY improved the outcome after ischemic injury more markedly than the newly synthesized non-cyclic dipeptides (LY and YL). cLY had most important effect on the recovery of LVDP and a significant preserving effect on tissue levels of high-energy phosphate (ATP) measured at the final stage of ischemia and after reperfusion when

compared to the drug-free hearts. These findings strongly indicate that the mitochondrial function, in close association with the synthesis of high-energy phosphate in the form of ATP, constituted an important part of the regulatory mechanism involved in myocardial ischemic injury. Therefore, the cyclic dipeptide is likely to play an important role in its beneficial effects.

On reperfusion, the cytosolic Ca^{2+} concentration that had increased during global ischemia rapidly returned to the control level, as previously reported.^{10,13,19)} The fura-2 Ca^{2+} signals ($[\text{Ca}^{2+}]_m$) in isolated myocardial mitochondria preloaded with abnormally high Ca^{2+} levels using a superfusion technique increased rapidly, reaching a maximum during displacement with a perfusate containing cytosolic Ca^{2+} (ca. 100 nM). In addition, the elevation of $[\text{Ca}^{2+}]_m$ was quickly detected on acidification of the perfusate that represented the final stage of global ischemia in the Langendorff hearts. Both elevations in the perfused mitochondrial preparation were attenuated by pretreatment with cLY.^{10,11,13)} From these findings, it is likely that, during reperfusion, mitochondria play an essential role in the cellular homeostasis of Ca^{2+} for the maintenance of cellular functions of the heart by acting as a scavenger of cytosolic Ca^{2+} .^{5,20,21)}

The MPTP opener atractyloside (Atr, 10^{-3} M) also generated increases in $[\text{Ca}^{2+}]_m$ similar to those obtained after changing the Ca^{2+} concentration or acidification of the perfusate.^{6,22)} In contrast to Atr-induced MPTP opening, pretreatment with cyclosporin A (CsA), which inhibits MPTP, depressed $[\text{Ca}^{2+}]_m$ elevation by changing the Ca^{2+} concentration and acidification, but not by treatment with FK506, which inhibits the phosphatase calcineurin without inhibiting MPTP opening.^{23,24)} Furthermore, posttreatment with CsA resulted in a release of Ca^{2+} from mitochondria after $[\text{Ca}^{2+}]_m$ was increased by changing the Ca^{2+} concentration or acidification of the perfusate. The release of accumulated Ca^{2+} was also affected by alkalization (pH 8.5) or changing the Mg^{2+} concentration of the perfusate, as a well-known inhibitor of MPTP.^{25–27)} These effects were similar to those noted for cLY, and were greater than those observed for YL or LY. Treatment with Ru360, ryanosine, verapamil, diazoxide, and chlonazepam, which do not transport Ca^{2+} to mitochondria, also led to the release of Ca^{2+} from the mitochondria.^{18,28)} Detailed data will be presented in another report. Therefore, mitochondrial fura-2 Ca^{2+} signals resulting from changes in the Ca^{2+} concentration or acidification of the perfusate were clearly observed during the time course of MPTP opening or closing. The increases in $[\text{Ca}^{2+}]_m$ were suppressed by infusion of the Na^+/H^+ exchange (NHE) inhibitor SM198110¹⁸⁾ and many other drugs that also promote favorable LVDP recovery in the Langendorff ischemia-reperfusion injury model.^{19,27,29,30)} These results suggest that MPTP is closely involved in postischemic myocardial dysfunction.

Irrespective of its exact composition, the MPTP complex contains multiple targets for pharmacological investigations⁸⁾ and is involved in different pathways of apoptosis induction as a sensor for stress and damage. There is now increasing evidence that apoptosis is an important response of the myocardium to ischemia and reperfusion, which is rapid, precedes cell necrosis, and appears to contribute to the overall sequelae of cardiac injury.⁷⁾ We have demonstrated that the administration of cLY reduced the number of TUNEL-positive

cardiomyocytes when compared with drug-free or LY-treated hearts. In the present study, cLY treatment reduced the number of TUNEL-positive cardiomyocytes and caspase-3 activity after ischemia and reperfusion when compared to LY. cLY treatment also significantly improved the recovery of the left ventricular function (LVDP) and coronary flow (FR) with accompanying increases in the levels of high-energy phosphates (ATP, PCr) when compared to drug-free hearts. cLY provided greater protection than LY of the ischemic myocardium as well as hearts subjected to reperfusion procedures. The role of cLY in this response has been shown to be similar to that of the NHE inhibitor SM198110.¹⁸⁾ These results suggest that apoptosis may be a major manifestation of ischemia-reperfusion injury and that MPTP is closely involved in postischemic myocardial dysfunction.

Reactive oxygen species (ROS) also promote apoptosis.³¹⁾ The production of ROS within the first few minutes of reperfusion may mediate cell damage due to ischemia-reperfusion injury.³²⁾ ROS action on mitochondria results in MPTP opening,³³⁾ and this triggers the mitochondria-related apoptotic pathway. It is also possible that peroxidative attacks may directly damage the outer mitochondrial membrane, resulting in the nonspecific liberation of intermembrane proteins, including a fraction of cytochrome *c*, during apoptotic signaling. On *in vitro* EPR spectrometry, the quenching activity (IC_{50}) of the cyclic peptide cLY for the $\cdot\text{OH}$ was found to be 1.8×10^{-4} M, which was greater than that of vitamin C (2.4×10^{-3} M), the non-cyclic peptides LY (2.0×10^{-3} M), or YL (3×10^{-3} M), while quenching of the O_2^- radical was 70% at cLY 2.5×10^{-3} M in contrast to LY and YL at 2.5×10^{-3} M, which showed no quenching. The role of superoxide (O_2^-), NO, and peroxynitrite (ONOO^-) in myocardial ischemia-reperfusion injury and preconditioning was reviewed by Freidary and Schulz.³⁴⁾ In another of our studies on guinea-pig hearts, NO supplied by the NO donor FK409,³⁵⁾ serotonin derivatives from safflower oil,¹⁹⁾ or the strongest antioxidative tea catechins (EGCg and GCG),^{26,27)} had cardioprotective actions, presumably by scavenging cytosolic superoxide anions. Immunoblotting analysis showed that endothelial nitric oxide synthase ((e)-NOS) was present in mitochondria and ONOO^- itself also inhibited $[\text{Ca}^{2+}]_m$ influx. Based on these findings, we concluded that e-NOS exists in mitochondria and that NO may play an important protective role in perfusion cardiac injury after ischemia by inhibiting $[\text{Ca}^{2+}]_m$ influx into mitochondria, which are otherwise damaged by O_2^- . Further research on ONOO^- generation in hearts will lead to a better understanding of how to protect the heart from oxidant stress in ischemia and reperfusion injury. Additional studies of *in vivo* whole body EPR using nitroxyl radicals³⁶⁾ are required to more adequately resolve the relationships between oxygen radicals generated in biological systems and the rate of redox reactions of spin probes during ischemia-reperfusion.

In conclusion, in ischemia-reperfusion injury hearts, we found that the cyclic dipeptide, cLY, showed greater cardioprotective effects *via* the inhibition of cardiomyocyte apoptosis (caspase-3 activity) than that of the non-cyclic peptides, LY and YL, utilized as a control. These findings strongly indicate that the cyclic dipeptide skeleton, in close association with the synthesis of high-energy phosphates, plays an important role in its protection against myocardial ischemic in-

jury. Further investigation of the cardiac effects of purified cyclic dipeptides synthesized with another peptide should provide valuable information regarding the normal physiology of cardiomyocyte apoptosis and help in the design of cardioprotective drugs.

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