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Identification and physiological activity of survival factor released from cardiomyocytes during ischaemia and reperfusion

Yoichi Mizukami[1]*, Kyoichi Ono[2], Cheng-Kun Du[3], Toshihiko Aki[1], Naoya Hatano[4], Yosuke Okamoto[2], Yasuhiro Ikeda[5], Hiroshi Ito[6], Kimikazu Hamano[6], and Sachio Morimoto[3]

[1]Center for Gene Research, Yamaguchi University, Yamaguchi 755-8505, Japan; [2]Department of Physiology, Akita University School of Medicine, Akita 010-8543, Japan; [3]Department of Clinical Pharmacology, Kyushu University Graduate School of Medicine, Fukuoka 812-8582, Japan; [4]Department of Cell Physiology, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan; [5]Department of Molecular Cardiovascular Biology, Yamaguchi University School of Medicine, Yamaguchi 755-8505, Japan; and [6]Department of Surgery and Clinical Science, Yamaguchi University School of Medicine, Yamaguchi 755-8505, Japan

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Aims We carried out a screening of survival factors released from cells exposed to simulated ischaemia and reperfusion (sI/R) using the embryonic rat heart-derived cell line, H9c2 cells, and examined the physiological role of the identified factor.

Method and results The culture medium supernatant of H9c2 cells exposed to sI/R was separated by column chromatography and the fractions examined for survival activity. The protein with survival activity was identified by mass spectrometry, and its physiological role was examined in the models of ischaemia. Cell survival activity was detected in at least three fractions of the cell supernatant collected during sI/R and subjected to a series of column chromatographic steps. Among the proteins measured by mass spectrometry and western blotting, a p36 protein identified as a glycolytic enzyme, lactate dehydrogenase muscle subunit (M-LDH), showed strong survival activity. H_2O_2-induced intracellular calcium overload in H9c2 cells and irregular Ca^{2+} transients in adult rat cardiomyocytes were both found to be inhibited by pretreatment with M-LDH. M-LDH also lowered the frequency and amplitude of early afterdepolarizations induced by H_2O_2 in adult rat cardiomyocytes and suppressed the ischaemia–reperfusion-induced reduction of cardiac output from mouse working heart preparations. M-LDH was found to increase the phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2), which plays a role in H9c2 cell survival.

Conclusion M-LDH released from cardiomyocytes after hypoxia and reoxygenation has a role in protecting the heart from oxidative stress-induced injury through an intracellular signal transduction pathway involving ERK1/2.

1. Introduction

Ischaemia–reperfusion of the heart is a major disease status arising from acute myocardial infarction, cardiopulmonary bypass surgery, and heart transplantation. After the recanalization of an infarct-damaged coronary artery, an expansion of the ischaemic region is observed as acute dilation and thinning of the infarcted area, hypertrophy of the remaining myocardium, induction of apoptotic cell death in the surrounding region of ischaemia, and an increase in cardiac fibrosis in the infarcted region.[1] These processes are referred to as remodelling and have been observed not only in cardiomyocytes directly exposed to ischaemia–reperfusion, but also in cells surrounding the ischaemic region. This suggests that cardiomyocytes exposed to ischaemia–reperfusion release a variety of factors that transmit some signal(s) to the surrounding cells.

It is well known that the heart functions as an endocrine tissue by secreting hormonal factors in addition to its role as a pump to propel blood to other tissues of the body.[2,3] Molecules released from heart, such as nitric oxide, prostaglandins, adenosine, and endothelin, are involved in the

* Corresponding author. The Center for Gene Research, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. Tel: +81 836 22 2183; fax: +81 836 22 2185.
E-mail address: mizukami@yamaguchi-u.ac.jp

regulation of the cardiovascular system after myocardial ischaemia.[4–8] In addition to these small molecules, inflammatory cytokines are also released from cardiomyocytes after ischaemia and reperfusion.[9] For example, tumour necrosis factor-α is produced in the ischaemic heart and involved in cardiac contractile depression through the inhibition of the sarcoplasmic ryanodine receptor.[10] The inflammatory cytokines interleukin (IL)-1β and IL-6 are also released from the myocardium after ischaemia and reperfusion and are secreted to the blood. These factors generated in the ischaemic heart not only aggravate cardiac contractility but also lead to dysfunction in intact cardiomyocytes.[11–13] Growth factors are reported to act as the survival factors released during myocardial ischaemia.[14–18] Most growth factors are involved in angiogenesis to the infarcted area as well as in inhibiting apoptotic cell death, thus leading to the protection of the myocardium from ischaemic injury through the activation of signal transduction molecules.[19] However, these factors have the ability to stimulate the proliferation of various cell types, including fibroblasts, hepatocytes, and lymphocytes, which may be a risk in heart disease.[20] During oxidative stress caused by ischaemia and reperfusion, we have demonstrated that the activation of extracellular signal-regulated kinase1/2 (ERK1/2) maintains contractility and survival in rat cardiomyocytes through the up-regulation of glycolytic α-enolase,[21–23] strongly suggesting that the activation of ERK1/2 might be required to restore heart function following ischaemic injury. Although Urocortin has been demonstrated as a substance that induces ERK1/2 activation and improves myocardial dysfunction during oxidative stress, a novel cardioprotective factor would be beneficial for patients with ischaemic heart disease.[24]

In the present studies, we identified a novel cardioprotective factor released from a rat heart-derived cell line, H9c2 cells, during simulated ischaemia (oxygen and glucose deprivation) and simulated reperfusion (oxygen and glucose addition) (sI/R). This factor was purified from polypeptides released from cells as a p36 protein having survival activity and identified as the lactate dehydrogenase muscle subunit (M-LDH). We found that the application of M-LDH to cardiomyocytes reduces the H_2O_2-induced early afterdepolarizations (EADs) closely related to arrhythmia by inhibiting intracellular Ca^{2+} ($[Ca^{2+}]_i$) overload. M-LDH was found to activate ERK1/2 and suppress the reduction of cardiac output by ischaemia–reperfusion in the isolated mouse heart. These findings suggest that M-LDH is released from cardiomyocytes during ischaemia–reperfusion, and that it plays a cardioprotective role against myocardial injury arising from oxidative stress in the ischaemic heart.

2. Methods

2.1 Materials

Anti-M-LDH, anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH), anti-phosphofructokinase (PFK), anti-phospho-tyrosine antibodies, M-LDH protein, taurin, creatine, 2,3-butanedione monoxime, insulin, and transferrin were purchased from Sigma (St Louis, MO, USA). Anti-PKCδ isoform antibody was purchased from Cell Signalling Technology Inc. (Beverly, MA, USA). DAPI nucleic acid stain (4′,6-diamino-2-phenylindol) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Collagenases B and D and laminin A were purchased from Roche Diagnostics (Mannheim, Germany). The concentrations of H_2O_2 were decided by measuring the activity after the addition of H_2O_2 in control cells. All other chemicals were commercially available.

2.2 Cell culture and ischaemic cell model (simulated ischaemia and reperfusion)

Cell culture and simulated ischaemia were achieved as described previously.[22,23,25,26] Briefly, an embryonic rat heart-derived cell line, H9c2 cells, was plated in dishes. After an incubation in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), the cells were cultured in serum-free DMEM and incubated in slightly hypotonic Hanks' balanced saline solution (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 69 mM NaCl, 4 mM $NaHCO_3$, and 0.3 mM NaH_2PO_4) without glucose and serum for 2 h at 37°C. Hypoxia was achieved using an air-tight incubator from which the oxygen was reduced to 1% by replacement with nitrogen. After incubation under conditions of simulated ischaemia, the cells were incubated in DMEM without FBS under normoxic conditions at 37°C.

2.3 Measurement of survival activity

H9c2 cells were grown in DMEM containing 10% FBS, and the medium was changed to DMEM without 10% FBS, and each of the eluate fractions was added to a well. The plates were then incubated for 24 h in a CO_2 incubator. Cell number was determined by a modified MTT assay as described previously (Dojindo Chemicals, Kumamoto, Japan).[27]

2.4 Purification of survival factors

Twenty litres of the supernatant of H9c2 cells (~2×10^{10} cells) exposed to simulated ischaemia for 2 h and simulated reperfusion for 24 h were used for the purification of survival factors. Ammonium sulfate was added to the supernatant to give a final concentration of 80% and the mixture was stirred for 24 h at 4°C. The sample was centrifuged for 30 min at 4°C (×18 000 *g*), the supernatant was removed, and the pellet was stored at −80°C for the purification of survival factors. The pellet was dissolved and dialysed against 10 mM Tris–HCl (pH 7.0) for 24 h at 4°C. The sample was loaded onto a column of HiTrap heparin Sepharose (5 mL) (GE Healthcare UK Ltd, Buckinghamshire, UK) equilibrated with the same buffer. Bound proteins were eluted with a linear NaCl gradient from 0 to 1000 mM for 30 min at 1 mL/min. A part of the pooled fractions was applied to gel filtration on Micro Bio-Spin 6 (Bio-Rad, Richmond, CA, USA) to determine the survival activity and to detect the proteins by SDS–PAGE and silver staining. After the active fractions were pooled and the salt was removed by gel filtration, the sample was applied to a Resourse Q Column (GE Healthcare, Milwaukee, WI, USA) equilibrated with 10 mM Tris–HCl, pH 8.0. The column was eluted with a stepwise NaCl gradient as shown in *Figure 1B* over 30 min at 1 mL/min, and 1 mL fractions were collected. The active fractions were desalted and concentrated to 0.05 mL by passing through a centrifugal filter device (Millipore, Bedford, MA, USA).

2.5 Identification of survival factors by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry

The sample was subjected to SDS–PAGE and stained with a mass spectrometry-compatible silver stain according to the instruction manual (Invitrogen, Carlsbad, CA, USA). In-gel digestion with trypsin was performed as described previously.[28,29] The tryptic peptides from the proteins of interest were dissolved in 0.1% trifluoroacetate and desalted using a Zip Tip C18 (Millipore, Bedford, MA, USA) according to the manufacturer's protocol. Peptides were eluted with matrix (α-cyano-4-hydroxycinnamic acid) prepared in 50% acetonitrile, 0.1% trifluoroacetate, and spotted onto a stainless

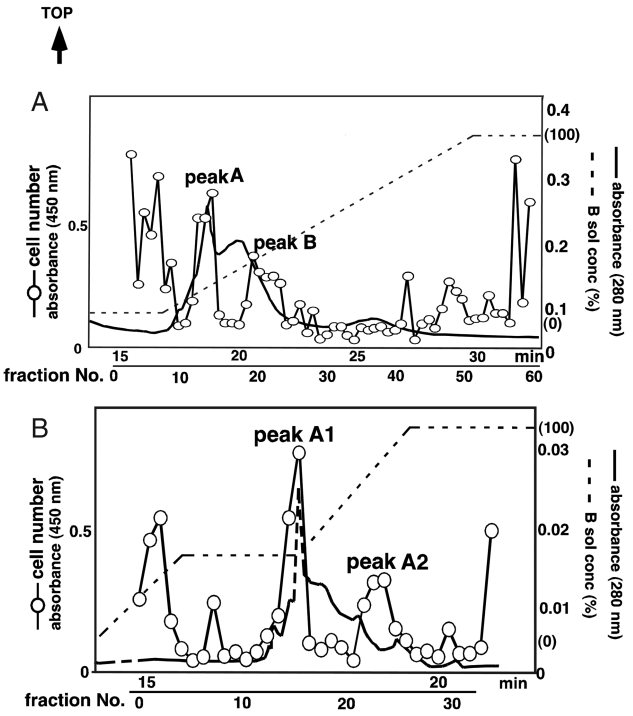


Figure 1 Identification of polypeptides released from H9c2 cells exposed to simulated ischaemia and reperfusion A–D, H9c2 cell supernatant (20 L) was concentrated by ammonium sulfate precipitation, and separated by heparin (*A*) and anion exchange column chromatographies (*B*). The survival activity and absorbance at 280 nm of each fraction were measured (*A* and *B*). The proteins in peak A1 of panel B were subjected to SDS–PAGE and silver staining (*C*). Purified p36 was digested with trypsin after the protocol described in 'Methods'. A portion of the resulting digest supernatant was used for matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis. The peptides were eluted from the C18 material (Zip Tips) with matrix (α-cyano 4-hydro cinnamic acid) prepared in 50% acetonitrile and 0.1% trifluoroacetate. The observed *m*/*z* values were submitted to ProFound (proteomics software package) for peptide mass fingerprint search (*D*). (*E*) H9c2 cells were grown for 2–3 days in 96-well plates in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, and then were treated without or with lactate dehydrogenase muscle subunit (M-LDH) at the indicated concentrations for 12 h. The cells were incubated for an additional 24 h in the presence of 200 μM H_2O_2. Cell number was determined by a modified MTT assay. Data represent the means $\pm$ SE of eight independent experiments. Statistical significance was determined by ANOVA followed by *post hoc* Dunnett's test. $^{\#}P < 0.05$ vs. 0 μM LDH. *F–H*, cell supernatants of H9c2 cells exposed to 2 h simulated ischaemia followed by 24–48 h simulated reperfusion (sI/R) or just exposed to 0–48 h serum starvation under normoxic conditions (serum (−)), broken cells were removed by centrifugation, and the supernatant was concentrated by ammonium sulfate precipitation. The samples were subjected to immunoblotting with anti-M-LDH antibody (F), anti-glyceraldehyde-3-phosphate dehydrogenase antibody (G), and anti-phosphofructokinase antibody (*H*). The blots show representative data obtained from three independent experiments. The protein levels were determined from the immunoblots by densitometric analysis. Data represent the means $\pm$ SE. Statistical significance was determined by ANOVA followed by *post hoc* Tukey's test. $^{*}P < 0.05$ vs. 0 h serum (−) (control), $^{\#}P < 0.05$ vs. 24 h serum (−), $^{+}P < 0.05$ vs. 48 h serum (−).

steel target plate. The protein digests were analysed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) using a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA, USA). Calibration was performed using the autodigestion peaks of trypsin (1045.5642 and 2211.1046). The observed *m*/*z* values were submitted to MS-Fit (available at http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) for mass fingerprint search. Search parameters were set as follows: Database, NCBInr; Digest, Trypsin; maximum number of missed cleavages, 1; Cys modified by, carbamidomethylation; Possible Modifications Mode, none; Min. number of peptides required to match, 4; Mass Tolerance, 25 ppm.

2.6 Electrophoresis and immunoblotting

Electrophoresis and immunoblotting were carried out as described previously.[21,30,31]

2.7 Immunofluorescent staining

Immunofluorescent staining was carried out as described previously.[22,23]

2.8 Preparation of adult cardiomyocytes

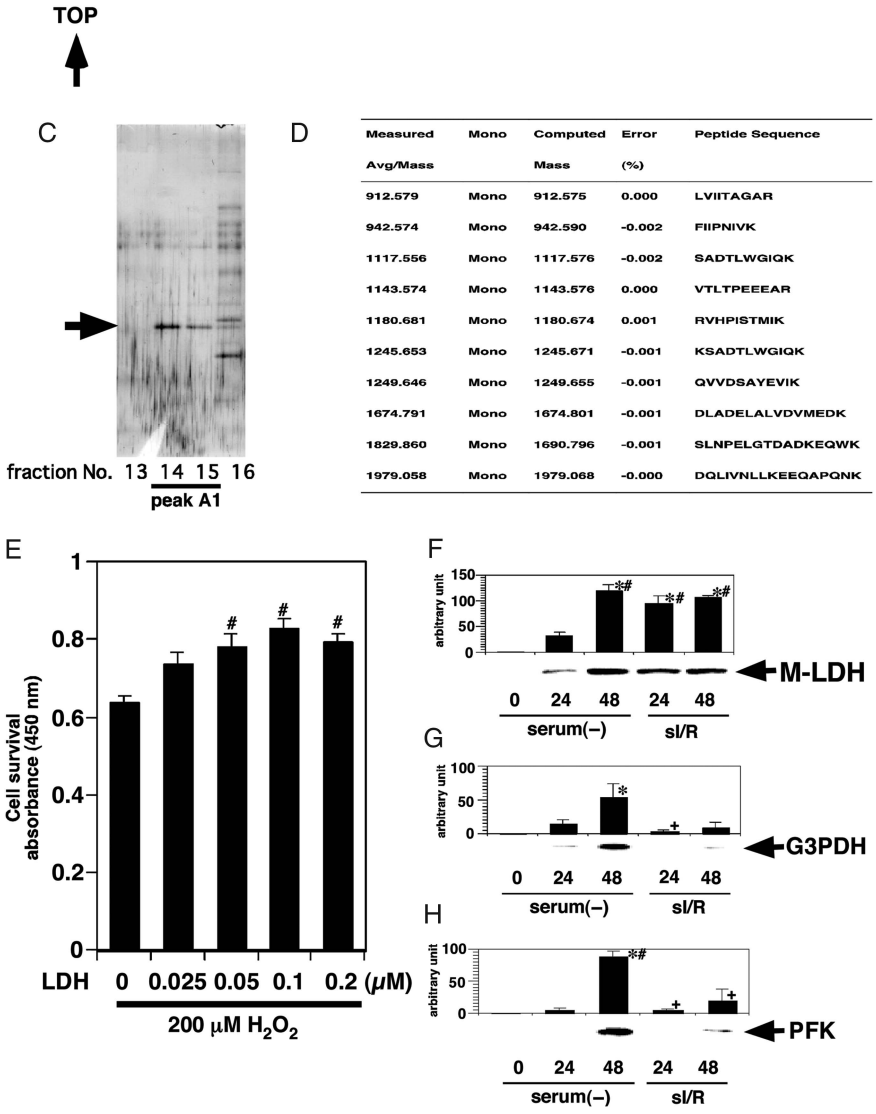
Ventricular cardiomyocytes were isolated from adult rat hearts as described previously.[32] In brief, hearts were perfused with perfusion buffer [MEM, pH 7.3 containing taurin (5 mM), 2,3-butanedione monoxime (10 mM), creatine (50 mg/100 mL), and insulin (2 U/100 mL)]. Enzymatic digestion was initiated by the addition of collagenase type B (0.5 mg/mL), collagenase type D (0.5 mg/mL), and protease type XIV (0.02 mg/mL) to the perfusion buffer. After 3 min of digestion, 50 μM $CaCl_2$ was added and the solution was incubated for 7 min. The ventricle was removed, cut into several pieces, and digested in a shaker (60–70 rpm) for 10 min at 37°C. The supernatant containing the cardiomyocytes was placed in a sterilized tube and gently centrifuged at 500 rpm for 1 min. The pellet was re-suspended in the perfusion buffer at a volume equal to that of the incubation buffer [MEM containing bovine serum albumin (500 mg/100 mL) and $CaCl_2$ (150 mM)] to stop the enzymatic reaction.

2.9 Digital imaging of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ transient in adult cardiomyocytes was measured by fura-2 fluorescence ratio digital imaging. Cardiomyocytes on glass-based dishes precoated with lamin B were loaded with 0.1 μM fura-2 AM (Dojin Chemical Co.) for 30 min at 37°C, placed on the stage of an inverted microscope (Nikon, Eclipse TS2000, Tokyo, Japan), and stimulated with 25 mA at a frequency of 0.5 Hz using a pair of platinum wires connected to a stimulator (IonOptix, Milton, MA, USA) in the presence of 40 μM H_2O_2. Digital imaging of the fura-2 fluorescence emitted at 510 nm was carried out during alternative excitation at 340 and 380 nm with a digital image system (Aquacosmos, Hamamatsu Photonics, Co., Hamamatsu, Japan). A pair of digital images was successively obtained at specific time intervals (21.0 ms for cardiomyocytes) and stored. The ratio of each image pair was calculated offline with background correction. H9c2 cells were also measured similarly without stimulation.

2.10 Measurement of action potentials in adult cardiomyocytes

Action potentials were recorded in isolated ventricular cells from Wistar rats (body weight, 200–300 g) by the Perforated Patch-Clamp Technique.[33] Pipettes had resistances in the range 1.5–3 MΩ when filled with the following solution (in mM) K-aspartate 110, KCl 30, NaCl 10, $CaCl_2$ 1.0 and HEPES 5.0 (pH 7.2 with KOH). Amphotericin B (Sigma), dissolved in dimethylsulfoxide as a stock solution (60 mg/mL), was added to the pipette solution to give a final concentration of 0.2 mg/mL. The bath solution was the control Tyrode solution containing (in mM) NaCl 136.9, KCl 5.4, $MgCl_2$ 0.53, $CaCl_2$ 1.8, NaH_2PO_4 0.33, HEPES 5.0, and glucose 5.5 (pH 7.4). The bath temperature was maintained at 36 $\pm$ 0.5°C. Action potentials were elicited by the brief (1–3 ms) current injections applied at 0.2 Hz. After stabilization of the resting potential and action potentials, selected concentrations of H_2O_2 were added to the cells. Voltage signals were stored in a microcomputer using PCLAMP software (Molecular Devices, Sunnyvale, CA, USA).



Measured Avg/Mass	Mono	Computed Mass	Error (%)	Peptide Sequence
912.579	Mono	912.575	0.000	LVIITAGAR
942.574	Mono	942.590	-0.002	FIIPNIVK
1117.556	Mono	1117.576	-0.002	SADTLWGIQK
1143.574	Mono	1143.576	0.000	VTLTPEEEAR
1180.681	Mono	1180.674	0.001	RVHPISTMIK
1245.653	Mono	1245.671	-0.001	KSADTLWGIQK
1249.646	Mono	1249.655	-0.001	QVVDSAYEVIK
1674.791	Mono	1674.801	-0.001	DLADELALVDVMEDK
1829.860	Mono	1690.796	-0.001	SLNPELGTDADKEQWK
1979.058	Mono	1979.068	-0.000	DQLIVNLLKEEQAPQNK

Figure 1 (Continued).

2.11 Working heart analysis

Hearts were excised from female ICR mice (8.5–12 weeks of age; weight ~30 g) after heparinization (15 U i.v.) under anaesthesia with 2,2,2-tribromoethanol (5 mg i.p.). After all pulmonary veins in a heart with lung preparation were tied with silk threads, the lung was trimmed away, and 18-gauge cannulae were tied onto the aortic stump and left auricle. The heart was mounted in an isolated heart perfusion apparatus (Model IPH-W2; Primetech, Japan). After several minutes of perfusion in Langendorff mode with a Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM EDTA-Na_2, 10 mM HEPES, and 11 mM D-glucose) oxygenated with 95% O_2 and 5% CO_2 at 37°C, an anterograde perfusion was achieved by applying a preload of 22 cm H_2O on the left atrium. Preload was then changed from 26 to 6 cm H_2O at a rate of 2 cm H_2O per 2 min at a constant afterload of 70 mmHg. The cardiac output was determined as the sum of the aortic and coronary flow during 1 min of perfusion just before changing the preload. The left ventricular (LV) pressure was simultaneously monitored with a blood pressure transducer DTXPlus (Nihon Koden, Japan) using a 26-gauge catheter inserted into the LV chamber from the apex.[34]

2.12 Measurement of the activation of signal transduction molecules by flow cytometry

The activation of signal transduction molecules was measured by cytometric bead array (CBA) using flow cytometry. Briefly, H9c2 cells were incubated in DMEM without FBS for 24 h and were stimulated with M-LDH for 15 min. After the stimulation, a denaturing buffer was added to stop the reaction, and the phosphorylations of ERK1/2 (T202/Y2004), c-Jnu N-terminal kinase (JNK1/2) (T183/Y185), p38 mitogen-activated protein kinase (MAPK) (T180/Y182), and phospholipase Cγ (Y783) were measured according to the instruction manual (BD Bioscience, San Diego, CA, USA). Standard curves based on the recombinant proteins were prepared before the measurement.

2.13 Determination of isozymes of lactate dehydrogenase activity in subjects

LDH isozyme assay was determined using LDH isozymes Test Wako (Wako Pure Chemical, Tokyo, Japan) according to the instruction manual.

2.14 Patients, anaesthesia, cardiopulmonary bypass, and myocardial protection

A total of five patients ranging from 54 to 75 years who were scheduled to undergo open heart surgery (coronary artery bypass grafting, two; aortic valve replacement, one; mitral valve replacement, one; double valve replacement, one) were included in this study with the approval by the local Ethics Committee. During surgery, anaesthetic induction and maintenance were achieved with a high dose of fentanyl. The extracorporeal circuit consisted of biopump (Bio-Console, Bio-Medics, Minneapolis, MI, USA), a cardiotomy reservoir, and a membrane oxgenator (Capiox-E, Terumo, Tokyo, Japan). The circuit was primed with 150–200 mL of Ringer's lactate. Heparin, 400 IU/kg body weight, was infused to maintain an activated clotting time of more than 400 s during bypass, and beta-methasone (4 mg/kg) was added to the priming solution. A flow rate of 2.4 L/min/m^2 was used to maintain a systemic perfusion pressure of 60–80 mmHg. After aortic cross-clamping, rapid blood cardioplegic solution (10 mL/kg body weight) was administered antegradely every 20 min at a pressure of 60–80 mmHg for myocardial protection. The cardioplegic solution consisted of the following: Na 85.3 mmol/L, K 25.0 mmol/L, Cl 85.5 mmol/L, Mg 10.0 mmol/L, and glucose 25 g/L; pH 7.38 (37°C), and osmolarity was 360 mOsm/L. One patient with OPCAB was not used extracorporeal circuit and aortic cross-clamping.

3. Results

3.1 Purification of survival factors released during simulated ischaemia and reperfusion

To purify the survival factors released from cardiomyocytes, the supernatant of cultured cells exposed to sI/R was loaded onto a heparin Sepharose column. The proteins bound to the affinity column were eluted with a linear salt gradient, with the survival activity of the eluate being monitored by cell number after serum removal from culture medium (*Figure 1A*). Strong survival activities to inhibit cell death caused by the removal of serum were detected in two peaks (A and B), and the fractions showing stronger survival activity (peak A) were subjected to further purification by anion exchange column chromatography (*Figure 1B*). The bound proteins were eluted with a step-wise salt gradient, and strong survival activities were detected in two peaks (A1 and A2). The survival activity profile of peak A1 was almost the same as the absorbance profile at 280 nm (*Figure 1B*), and active fractions of peak A1 (fraction numbers 14 and 15) contained a single protein with a molecular mass of ~36 kDa (p36) on SDS–PAGE (*Figure 1C*). Peak A2 is currently under investigation.

3.2 Survival factor released during simulated ischaemia and reperfusion is lactate dehydrogenase muscle subunit

The p36 protein was treated with trypsin under the conditions described in 'Methods', and subjected to peptide mass fingerprinting by MALDI-TOF-MS. The molecular weights of the peptide fragments derived from the p36 protein were MH+: 912.579, 942.574, 1117.556, 1143.574, 1180.681, 1245.653, 1249.646, 1674.791, 1829.860, and 1979.058, which are almost identical to the theoretical molecular weights of the peptide fragments of mouse M-LDH (also designated as LDH-A) digested with trypsin (*Figure 1D*). The calculated molecular mass of M-LDH from its amino acid sequence is 36 367, which is in approximate agreement with the apparent molecular mass of p36 estimated by SDS–PAGE. Furthermore, an anti-M-LDH antibody recognized p36 (data not shown). Purified M-LDH inhibited cell death induced by the addition of 200 μM H_2O_2 in a dose-dependent manner (0.025–0.2 μM) when added to cell line H9c2 derived from the embryonic heart (*Figure 1E*). Taken together, we conclude that the p36 protein is M-LDH.

3.3 Lactate dehydrogenase muscle subunit is released during simulated ischaemia and reperfusion

To confirm the release of M-LDH from cultured cells during sI/R, we carried out immunoblotting with an anti-M-LDH antibody using the supernatant of cells exposed to sI/R. The amount of M-LDH protein in the supernatant increased after 24 h of simulated reperfusion following simulated ischaemia for 2 h (*Figure 1F*); exposure to medium without serum for a long-time damages the cells so that no significant difference could not be detected between 48 h serum (−) and sI/R. In contrast, the releases of G3PDH and PFK, other glycolytic enzymes in the cytosol, during sI/R, were reduced compared with the control cells, indicating that M-LDH is released relatively specifically during sI/R (*Figure 1G* and *H*).

3.4 Lactate dehydrogenase muscle subunit has survival activity by protecting cardiomyocytes from oxidative stress-induced damage

Since oxidative stress induced by ischaemia and reperfusion plays an important role in heart injury, we examined the effects of H_2O_2 on adult rat cardiomyocytes in the presence of M-LDH. After 20 min of stimulation with 40 μM H_2O_2 in the absence of M-LDH, the cardiomyocytes changed to a round shape and lost the ability to contract in response to electrical stimulation (*Figure 2A*, *B*, and *D*). The rounded cells bound to an initial apoptosis marker annexin V-enhanced green fluorescence protein after 60 min of stimulation with H_2O_2 (data not shown). Pretreatment with M-LDH significantly reduced the morphological change of the cardiomyocytes to a round shape (*Figure 2A*, *C*, and *D*) and preserved cardiomyocyte contractility in response to electric stimulation (data not shown), indicating that M-LDH protects cardiomyocytes from oxidative stress-induced damage.

3.5 Lactate dehydrogenase muscle subunit inhibits $[Ca^{2+}]_i$ overload induced by an oxidative stress

To explore the mechanism involved in the survival activity of M-LDH, we examined the effects of M-LDH and H_2O_2 on $[Ca^{2+}]_i$ using H9c2 cells loaded with fura-2. Because the excess contraction of cardiomyocytes by H_2O_2 was inhibited by M-LDH treatment, Ca^{2+} influx might be involved in the excess contraction. Acute exposure to H_2O_2 caused a rapid increase in $[Ca^{2+}]_i$ within 4 min followed by a gradual increase in $[Ca^{2+}]_i$ up to 40 min after stimulation (see Supplementary material online, *Figure S1*). Pretreatment with M-LDH significantly delayed the initial rise in $[Ca^{2+}]_i$ of H9c2 cells exposed to H_2O_2 up to 20 min after stimulation and reduced the final level of the $[Ca^{2+}]_i$ increase (see Supplementary material online, *Figure S1*). We also examined the effects of M-LDH and H_2O_2 on $[Ca^{2+}]_i$ transients evoked

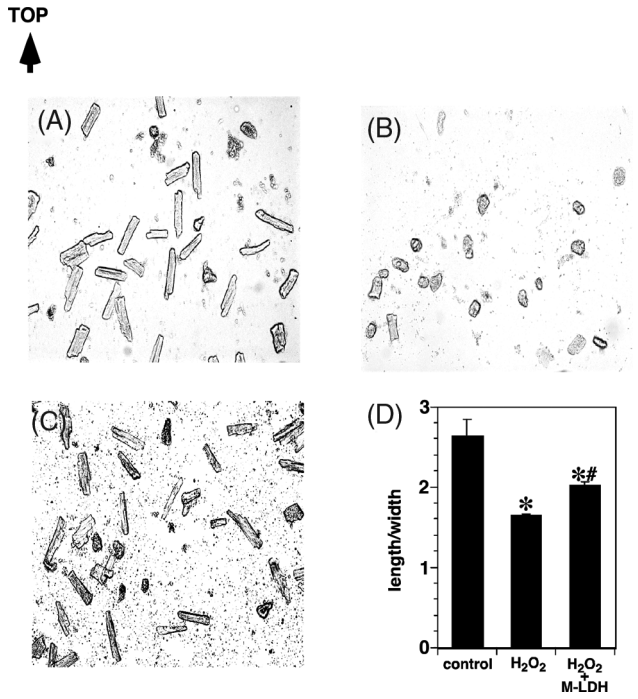


Figure 2 Effect of lactate dehydrogenase muscle subunit (M-LDH) on morphological changes that take place in adult cardiomyocytes in response to H_2O_2. Adult cardiomyocytes were prepared from rat heart, and the cells were treated without (*A* and *B*) or with (*C*) M-LDH (final 0.5 μM) for 12 h. Morphological changes to the cells were observed under bright field microscopy at 0 min (*A*) and 40 min (*B* and *C*) after the addition of H_2O_2 (final 40 μM), and the length and breadth of the cells were analysed by Metamorph Imaging Software (*D*). Data represent the means ± SE determined for cells prepared from the six independent experiments (control, $n = 42$ cells; H_2O_2, $n = 152$ cells; H_2O_2+M-LDH, $n = 173$ cells). Statistical significance was determined by ANOVA followed by *post hoc* Tukey's test. *$P < 0.05$ vs. control. Scale bar = 100 μm.

by the electric stimulation of adult cardiomyocytes. Under control conditions, cardiomyocytes underwent normal contractions with a transient increase in $[Ca^{2+}]_i$ in response to electrical stimulation (*Figure 3A* and *C*; Supplementary material online, *Movie 1A* and *C*). Treatment of cardiomyocytes with H_2O_2 for 20 min resulted in making the cell shape round, with Ca^{2+} transients being irregular and finally disappearing (*Figure 3B* and Supplementary material online, *Movie 1B*). However, pretreatment of cardiomyocytes with M-LDH preserved their normal morphology and contractility with regular Ca^{2+} transients even after 20 min of treatment with H_2O_2 (*Figure 3D* and Supplementary material online, *Movie 1D*). These findings strongly suggest that M-LDH suppresses the $[Ca^{2+}]_i$ overload caused by oxidative stress allowing cardiomyocytes to survive and maintain their contractile function intact even under severe oxidative conditions.

3.6 Lactate dehydrogenase muscle subunit suppresses early afterdepolarizations induced by oxidative stress in cardiomyocytes

$[Ca^{2+}]_i$ overload and a spontaneous rise in intracellular-free Ca^{2+} levels are implicated in EADs and ventricular tachycardia as mechanisms that trigger the long QT syndrome.[35–37] We observed the action potential of cardiomyocytes treated with H_2O_2 in the presence of M-LDH. H_2O_2 induced EADs in ventricular cardiomyocytes within 6 min after treatment (*Figure 4A–C*). The frequency and amplitude of the EADs increased up to 10 and 8 min after treatment, respectively, and then decreased with the elapse of time (*Figure 4B* and *C*). M-LDH inhibited the frequency and amplitude of EADs induced by H_2O_2 significantly (*Figure 4F–H*). H_2O_2 also prolonged the action potential duration; however, M-LDH had no effect on the prolongation of action potential duration by H_2O_2 (*Figure 4D* and *I*).

3.7 Lactate dehydrogenase muscle subunit suppresses oxidative stress- or ischaemia–reperfusion-induced decrease in contractility of isolated working heart

The effects of M-LDH on the intact heart were examined using isolated working heart preparations (*Figure 5*). H_2O_2 reduced the cardiac output from the working heart preparation significantly and M-LDH suppressed the reduction of cardiac output caused by H_2O_2; there were no significant differences in the cardiac output between hearts treated with H_2O_2 in the presence of M-LDH and controls (*Figure 5A*). A brief ischaemia–reperfusion reduced the cardiac output and systolic function (dP/dt_{max}) significantly and M-LDH suppressed the reduction of cardiac output and systolic function caused by this brief ischaemia–reperfusion; there were no significant differences in the cardiac output and systolic function between hearts exposed to ischaemia–reperfusion with M-LDH and controls (*Figure 5B* and C). M-LDH had no effects on the function of the heart exposed to no ischaemia (data not shown). The release of M-LDH was increased in the perfusion medium of intact heart exposed to ischaemia and reperfusion as compared with that of the control (*Figure 5D*). These observations indicated that M-LDH was involved in the recovery of the heart function from ischaemia.

3.8 Lactate dehydrogenase muscle subunit alters the phosphorylation levels of signal transduction molecules

To examine whether the survival activity of M-LDH involves any intracellular signalling pathways, the phosphorylation levels of signal transduction molecules ERK1/2, JNK1/2, p38MAPK, and PLCγ were determined by CBA using flow cytometry (*Figure 6A–D*). CBA is an assay that carries out particle-based immunoassay using flow cytometry, and beads with different positions can be combined to create a multiplex assay. Incubation of H9c2 cells with M-LDH increased the phosphorylation of ERK1/2, but the phosphorylation levels of JNK1/2, p38MAPK, and PLCγ remained unchanged. ERK1/2 activity was dependent on the M-LDH concentration (0.025–1.0 μM) in H9c2 cells (*Figure 6E*), consistent with western blotting data obtained using an anti-phospho-ERK1/2 antibody (data not shown). M-LDH inactivated by boiling did not induce ERK activity in H9c2 cells. These results suggest that M-LDH exhibits survival activity through some membrane receptors involving signal transduction molecules ERK1/2.

3.9 Lactate dehydrogenase muscle subunit is released into blood of patients exposed to ischaemia–reperfusion

We examined the release of M-LDH in patients exposed to ischaemia by pump-oxygenator during open heart surgery.

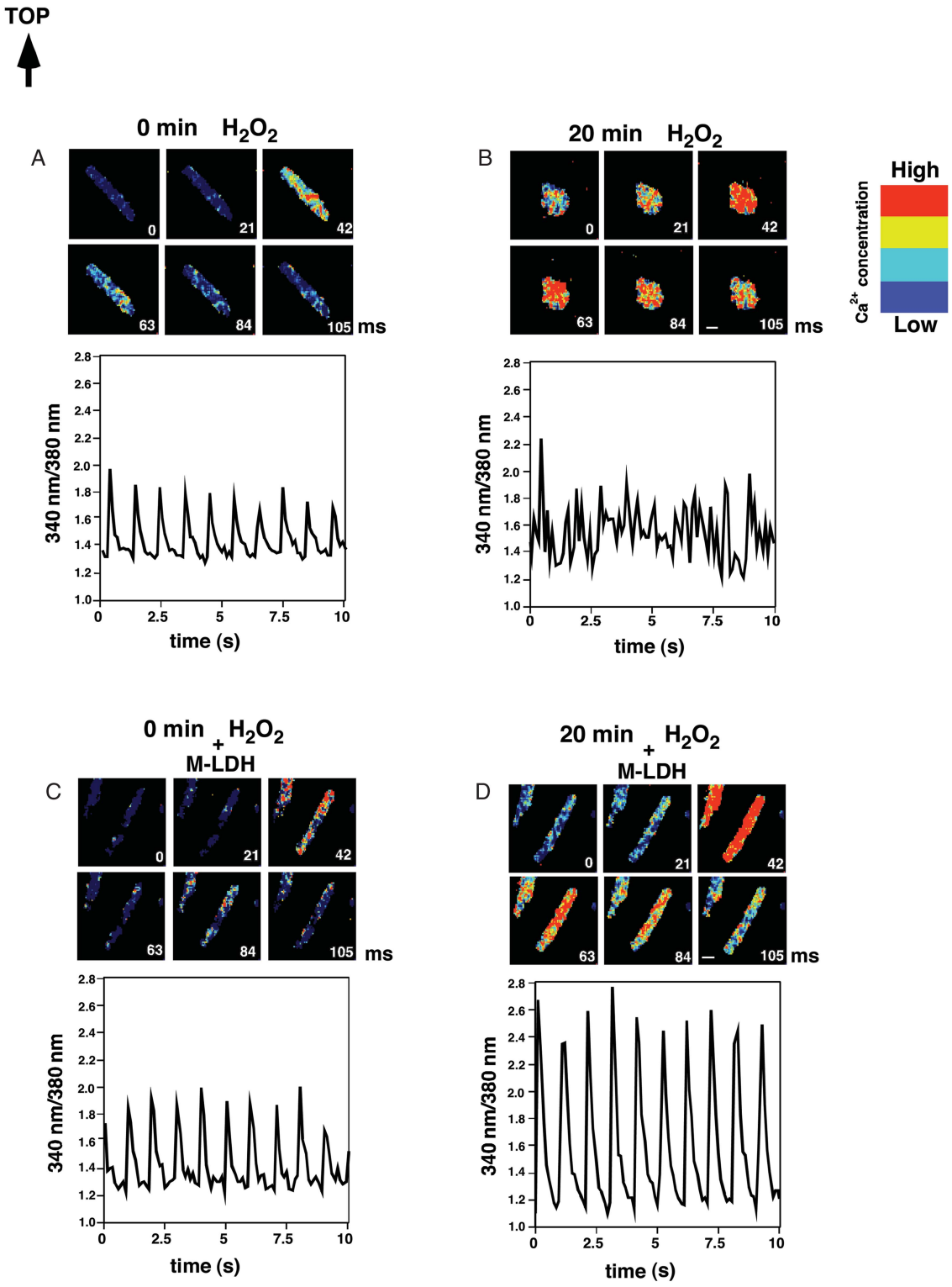


Figure 3 Effect of lactate dehydrogenase muscle subunit (M-LDH) on $[Ca^{2+}]_i$ transients in rat adult cardiomyocytes stimulated with H_2O_2. Rat adult cardiomyocytes were incubated without (*A*, *B*) or with (*C*, *D*) M-LDH (final 0.5 μM) for 12 h, and then loaded with fura-2AM (final 0.1 μM) for an additional 30 min. The cells were stimulated with 25 mA at a frequency of 0.5 Hz using the pairs of platinum wires connected to a stimulator in the absence (*A* and *C*) or presence of H_2O_2 (final 40 μM) (*B* and *D*), and pairs of digital images of fura-2 fluorescence at 510 nm with excitation at 340 and 380 nm were successively obtained at time intervals of 21.0 ms. The figure shows representative data obtained from three independent experiments. Scale bar = 20 μm.

Total LDH activity in serum was significantly increased in patients subjected to the surgery with pump-oxygenator at 30 min after reperfusion and continued to increase for 72 h (data not shown). Next, two LDH isozymes, the activities of M-LDH and LDH heart-type subunit (H-LDH) were determined in the serum of patients, because H-LDH was also known to be released from the heart in response to various injuries. H-LDH was rapidly released into the blood at 0.5 h after reperfusion, and the release of H-LDH continued to increase at least for 72 h after reperfusion (see

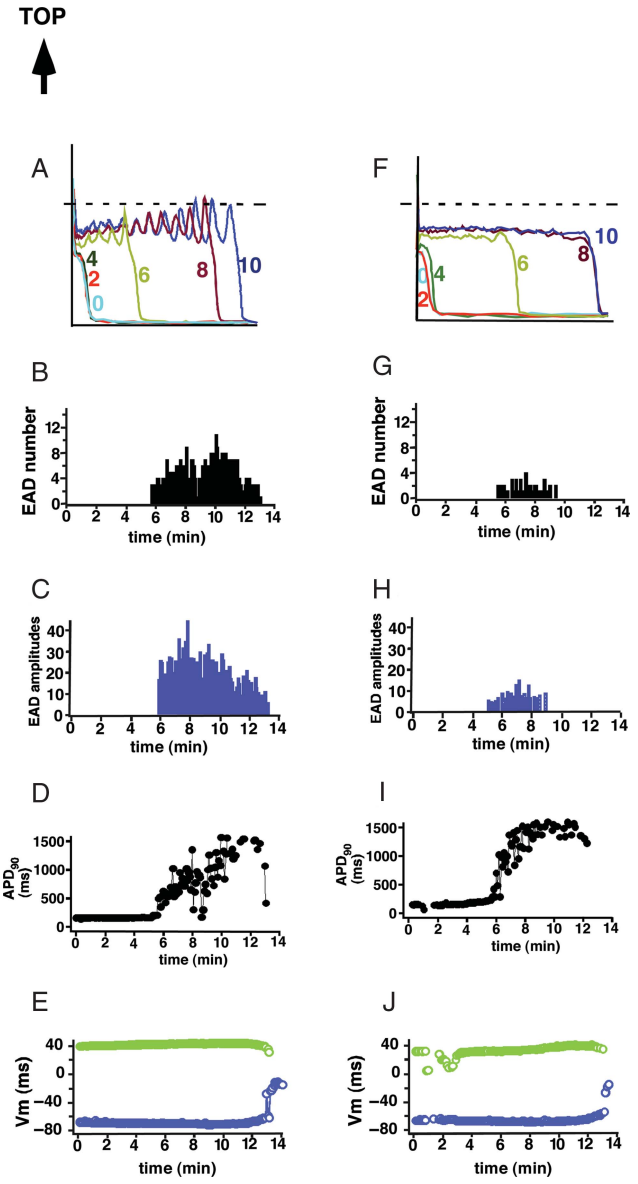


Figure 4 Effect of lactate dehydrogenase muscle subunit (M-LDH) on the membrane action potential of rat adult cardiomyocytes in the presence of H_2O_2. Rat adult cardiomyocytes were incubated without (*A*–*E*) or with (*F*–*J*) M-LDH (final 0.05 μM) for 6 h, and the current and action potential were determined using a clamp amplifier for the indicated times (*A* and *F*) after H_2O_2 stimulation (final 50 μM) as described in Experimental Procedures. The figure shows representative data obtained from independent experiments (*A* and *F*) (control; $n = 13$, M-LDH; $n = 6$). Early afterdepolarizations (EADs) frequency (*B* and *G*), EADs amplitude (*E* and *J*), APD_{90} (*D* and *I*), and V_m (*E* and *J*) were determined from the membrane potential data.

Supplementary material online, *Figure S2*). In contrast, much less amount of M-LDH was rapidly released into blood at 0.5 h after reperfusion and the release was decreased within 48 h after reperfusion (see Supplementary material online, *Figure S2*), suggesting that M-LDH rather than H-LDH may play an important role in the recovery from ischaemia of the human heart.

4. Discussion

As shown in this study, many polypeptides are released into the supernatant of cells exposed to sI/R, a cell model of ischaemia. Among these polypeptides, at least three have survival activities for cells exposed to the stress by serum removal. Strong survival activities were found in an eluate containing a nearly pure single protein at the second step of purification by anion exchange column chromatography, suggesting that the major protein in this eluate is responsible for the survival activity. The protein was identified as M-LDH by molecular peptide mass fingerprinting and a specific antibody.

Pretreatment with M-LDH significantly inhibited the morphological change of cardiomyocytes to a round shape induced by H_2O_2, suggesting that M-LDH inhibits $[Ca^{2+}]_i$ overload caused by oxidative stress. Cardiomyocytes contract through a myosin–actin interaction in response to an increase in $[Ca^{2+}]_i$, and a transient overload of $[Ca^{2+}]_i$ is thought to induce excess irreversible cell shortening, leading to the morphological change to a round shape. Indeed, pretreatment with M-LDH efficiently blocked the abnormal increase in $[Ca^{2+}]_i$ caused by H_2O_2. H_2O_2 has been reported to cause a biphasic pattern of $[Ca^{2+}]_i$ overload in a variety of cells including insulin-secreting cells[38] and smooth muscle cells,[39] consistent with the results obtained using the cardiac myocyte-derived cell line as shown in Supplementary material online, *Figure S1*. The spontaneous release of Ca^{2+} from the sarcoplasmic reticulum (SR) triggers EADs, oscillations in the membrane potential of the cells, which are generally thought to be a cause for arrhythmia associated with Q–T interval prolongation, including Torsades de Pointes.[40–43] M-LDH inhibits the increase in $[Ca^{2+}]_i$ in H9c2 cells exposed to H_2O_2 and the EADs induced by H_2O_2 in rat adult cardiomyocytes, suggesting that M-LDH may block $[Ca^{2+}]_i$ overload due to spontaneous Ca^{2+} release from SR. Consistent with these data, pretreatment with M-LDH significantly improves the cardiac pump function of the isolated mouse heart exposed to H_2O_2 or ischaemia–reperfusion, indicating that M-LDH has survival activity that protects the heart from oxidative stress and ischaemic injury.

It is well known that M-LDH reversibly controls the formation of lactate according to the intracellular environment. Under anaerobic conditions such as ischaemia, pyruvate, NAD, and H^+ are not further metabolized, leading to LDH-catalysed lactate accumulation. Intracellular lactate is reported to open sarcolemmal ATP-sensitive K channels in cells despite the presence of millimolar concentrations of ATP, and protect cells against Ca^{2+} overload and cell death.[44] Crawford *et al.* showed that M-LDH binds to ATP-sensitive K channels, leading to an effective inhibition of the harmful effects of $[Ca^{2+}]_i$ through the metabolic product lactate.[45] However, it is unlikely that lactate, a metabolic product of M-LDH, was acting as a survival factor in the present study, because the addition of lactate had no effect on the Ca^{2+} overload induced by oxidative stress (data not shown). H-LDH, a heart subunit of M-LDH, also catalyses the production of lactate, and large amounts of H-LDH are released from cardiomyocytes exposed to ischaemia and reperfusion; however, no cell survival activity was detected in the fractions containing H-LDH. In addition, the coenzyme NADH was not required for the survival activity of M-LDH under our experimental conditions, indicating that M-LDH does not exert its survival activity as an enzyme lactate dehydrogenase.

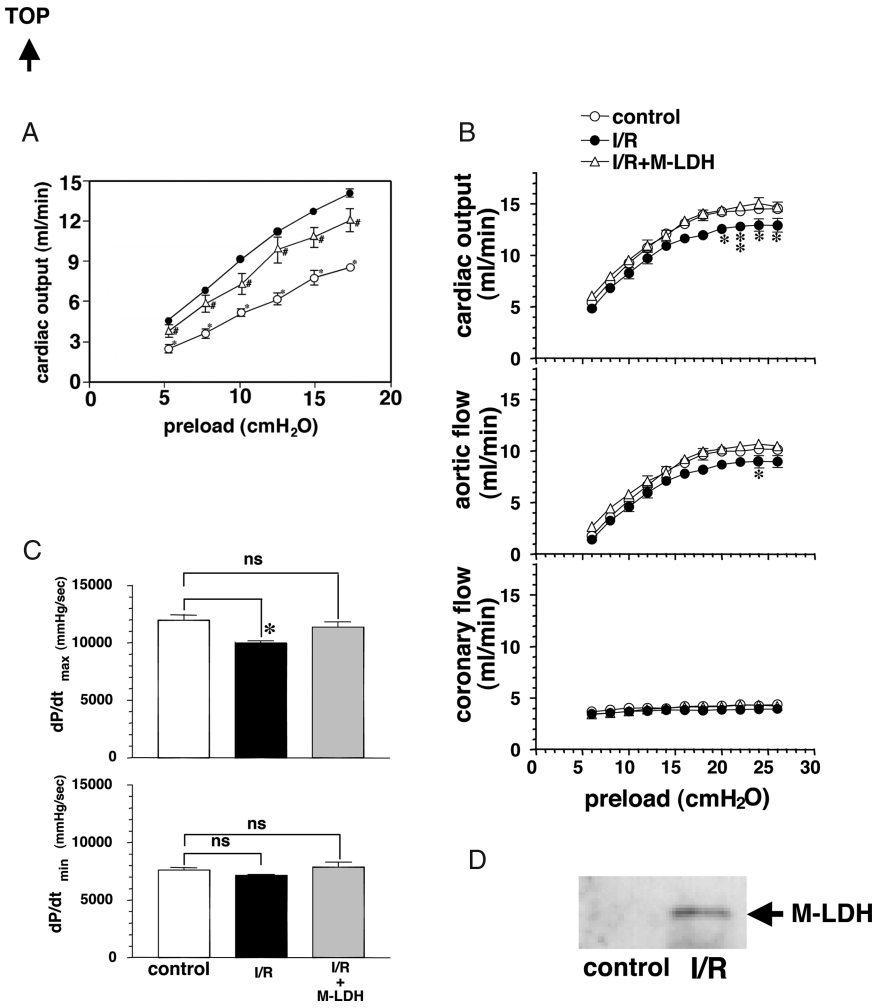


Figure 5 Effects of lactate dehydrogenase muscle subunit (M-LDH) on the heart function during H_2O_2 perfusion or ischaemia/reperfusion of isolated work-performing heart A, Cardiac outputs from working heart preparations were determined 10 min after perfusion with H_2O_2 (final 50 μM) (open circle and open triangle) or without H_2O_2 (closed circle), and the effect of M-LDH was determined in the presence of 25 nM M-LDH throughout the experiment (open triangle). Data were obtained in four independent experiments in each group, and the results are expressed as means ± SE. (*$P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. +H_2O_2-M-LDH, Tukey's Multiple Comparison Test). (*B–C*) After working heart preparations were exposed to ischaemia for 5 min followed by reperfusion for 10 min at a preload of 22 cm H_2O, cardiac output (*B*) and the maximum and minimum rates of LV pressure change (dP/dt_{max} and dP/dt_{min}, respectively) (*C*) were determined simultaneously. The effects of M-LDH on the heart function during ischaemia–reperfusion were determined by adding 0.025 μM M-LDH into the perfusate from 5 min before exposure to ischaemia. Control data were obtained from hearts neither pretreated with M-LDH nor exposed to ischaemia–reperfusion. Data were obtained in four independent experiments in each group, and the results are expressed as means ± SE (*$P < 0.05$, **$P < 0.01$ vs. control, Dunnett's Multiple Comparison Test, I/R, ischaemia–reperfusion). (*D*) Perfusates of mouse hearts exposed to ischaemia–reperfusion were collected during reperfusion, and were concentrated by size-exclusion columns. The samples were subjected to immunoblotting with anti-M-LDH antibody. The figure shows representative blots obtained from three independent experiments.

In this study, the mechanism of M-LDH release during sI/R remains to be clarified, but we could speculate that a mechanism other than release by a damaged membrane is involved, because if M-LDH was released from the damaged membrane, G3PDH and PFK, also present in the cytosol, should also be detected during sI/R. However, the amounts of G3PDH and PFK released into the extracellular spaces decreased during sI/R. In addition, the rate of cell death among cells exposed to sI/R was 10% or less, under our experimental conditions, and was almost equal to that of control cells, suggesting that cell injury was not very severe (data not shown). LDH activity is often determined as a biomarker of cell damage. However, we found that more than 90% of the activity released into the blood in a human ischaemia heart model is due to the H-LDH isoform (see Supplementary material online, *Figure S2*), suggesting that LDH activity measured in cell death assay might not be necessarily corresponding to the M-LDH activity.

In conclusion, the present study provides strong evidence for a novel role of M-LDH in the recovery of heart function following ischaemia. Two lines of evidence indicate that M-LDH acts through an intracellular signal transduction system: (i) pretreatment with M-LDH inhibits H_2O_2-induced $[Ca^{2+}]_i$ overload and death of cardiomyocytes; however, the simultaneous application of M-LDH and H_2O_2 does not inhibit them sufficiently strongly suggesting that M-LDH does not exert its survival activity through direct action, such as ion channel damage, on the membrane protein; (ii) M-LDH increases the phosphorylation of ERK1/2, which is involved in cell survival,[23] without changing the phosphorylation of JNK1/2, which is involved in apoptosis pathways. These observations suggest that a novel receptor for M-LDH may be present in cardiomyocytes. Further studies

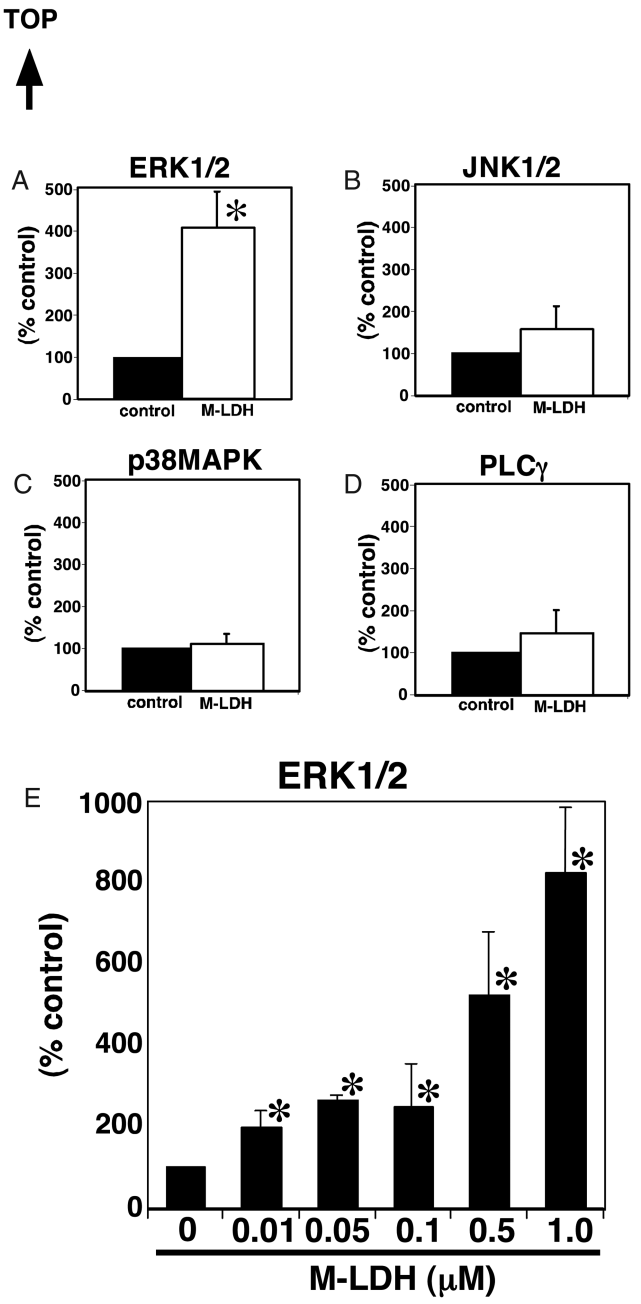


Figure 6 Phosphorylation of signal transduction molecules in H9c2 cells after treatment with lactate dehydrogenase muscle subunit (M-LDH) Cell extracts was prepared from H9c2 cells incubated without or with M-LDH (final 0.5 μM for *A*–*D* and the indicated concentrations for *E*) for 15 min, and subjected to cytometric beads array using anti-phospho antibodies for signal transduction molecules (ERK1/2, JNK1/2, p38MAPK, and PLCγ). Phosphorylation levels of ERK1/2 (*A* and *E*), JNK1/2 (*B*), p38MAPK (*C*), and PLCγ (*D*) were determined from standard curves based on recombinant proteins. Data were obtained from three independent experiments in each group. (*$P < 0.05$ vs. control, Student's *t*-test, means ± SE).

are required to clarify the detailed molecular mechanism involved in the intracellular signal transduction system and potential membrane receptors through which M-LDH exhibits its survival activity against ischaemia–reperfusion of the heart. The present study demonstrated that the protective effects of M-LDH was observed at very low concentrations (25–50 nM), strongly suggesting that even a small amount of M-LDH released from the cells damaged by ischaemia–reperfusion *in vivo* could activate these signaling pathways and protect surrounding cells.

Supplementary material

Supplementary Material is available at *Cardiovascular Research* Online.

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Conflict of interest: none declared.

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References

1. Sabbah HN. Apoptotic cell death in heart failure. *Cardiovasc Res* 2000; **45**:704–712.
2. Iglesias MJ, Pineiro R, Blanco M, Gallego R, Dieguez C, Gualillo O *et al.* Growth hormone releasing peptide (ghrelin) is synthesized and secreted by cardiomyocytes. *Cardiovasc Res* 2004;**62**:481–488.
3. Katwa LC. Cardiac myofibroblasts isolated from the site of myocardial infarction express endothelin de novo. *Am J Physiol Heart Circ Physiol* 2003;**285**:H1132–H1139.
4. Stangl V, Baumann G, Stangl K, Felix SB. Negative inotropic mediators released from the heart after myocardial ischaemia-reperfusion. *Cardiovasc Res* 2002;**53**:12–30.
5. Bing RJ. Myocardial ischemia and infarction: growth of ideas. *Cardiovasc Res* 2001;**51**:13–20.
6. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc Natl Acad Sci USA* 2004;**101**: 13683–13688.
7. Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R. Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res* 2000;**87**:241–247.
8. Xiao CY, Hara A, Yuhki K, Fujino T, Ma H, Okada Y *et al.* Roles of prostaglandin I(2) and thromboxane A(2) in cardiac ischemia-reperfusion injury: a study using mice lacking their respective receptors. *Circulation* 2001; **104**:2210–2215.
9. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res* 2002;**53**:31–47.
10. Herskowitz A, Choi S, Ansari AA, Wesselingh S. Cytokine mRNA expression in postischemic/reperfused myocardium. *Am J Pathol* 1995;**146**: 419–428.
11. Thaik CM, Calderone A, Takahashi N, Colucci WS. Interleukin-1 beta modulates the growth and phenotype of neonatal rat cardiac myocytes. *J Clin Invest* 1995;**96**:1093–1099.
12. Craig R, Larkin A, Mingo AM, Thuerauf DJ, Andrews C, McDonough PM *et al.* p38 MAPK and NF-kappa B collaborate to induce interleukin-6 gene expression and release. Evidence for a cytoprotective autocrine signaling pathway in a cardiac myocyte model system. *J Biol Chem* 2000;**275**: 23814–23824.
13. Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, Simmons RL. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 1992;**257**:387–389.
14. Li Y, Takemura G, Kosai K, Yuge K, Nagano S, Esaki M *et al.* Postinfarction treatment with an adenoviral vector expressing hepatocyte growth

factor relieves chronic left ventricular remodeling and dysfunction in mice. *Circulation* 2003;**107**:2499–2506.
15. Jiang ZS, Padua RR, Ju H, Doble BW, Jin Y, Hao J *et al*. Acute protection of ischemic heart by FGF-2: involvement of FGF-2 receptors and protein kinase C. *Am J Physiol Heart Circ Physiol* 2002;**282**:H1071–H1080.
16. Xaymardan M, Tang L, Zagreda L, Pallante B, Zheng J, Chazen JL *et al*. Multimedia article Platelet-derived growth factor-AB promotes the generation of adult bone marrow-derived cardiac myocytes. *Circ Res* 2004; **94**:E39–E45.
17. Parsa CJ, Kim J, Riel RU, Pascal LS, Thompson RB, Petrofski JA *et al*. Cardioprotective effects of erythropoietin in the reperfused ischemic heart: a potential role for cardiac fibroblasts. *J Biol Chem* 2004;**279**: 20655–20662.
18. Tanaka N, Masamura K, Yoshida M, Kato M, Kawai Y, Miyamori I. A role of heparin-binding epidermal growth factor-like growth factor in cardiac remodeling after myocardial infarction. *Biochem Biophys Res Commun* 2002;**297**:375–381.
19. Detillieux KA, Sheikh F, Kardami E, Cattini PA. Biological activities of fibroblast growth factor-2 in the adult myocardium. *Cardiovasc Res* 2003;**57**:8–19.
20. Meij JT, Sheikh F, Jimenez SK, Nickerson PW, Kardami E, Cattini PA. Exacerbation of myocardial injury in transgenic mice overexpressing FGF-2 is T cell dependent. *Am J Physiol Heart Circ Physiol* 2002;**282**: H547–H555.
21. Mizukami Y, Yoshida K. Mitogen-activated protein kinase translocates to the nucleus during ischaemia and is activated during reperfusion. *Biochem J* 1997;**323**:785–790.
22. Mizukami Y, Kobayashi S, Uberall F, Hellbert K, Kobayashi N, Yoshida K. Nuclear mitogen-activated protein kinase activation by protein kinase czeta during reoxygenation after ischemic hypoxia. *J Biol Chem* 2000; **275**:19921–19927.
23. Mizukami Y, Iwamatsu A, Aki T, Kimura M, Nakamura K, Nao T *et al*. ERK1/2 regulates intracellular ATP levels through alpha-enolase expression in cardiomyocytes exposed to ischemic hypoxia and reoxygenation. *J Biol Chem* 2004;**279**:50120–50131.
24. Schulman D, Latchman DS, Yellon DM. Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. *Am J Physiol Heart Circ Physiol* 2002;**283**:H1481–H1488.
25. Kimura M, Mizukami Y, Miura T, Fujimoto K, Kobayashi S, Matsuzaki M. Orphan G protein-coupled receptor, GPR41, induces apoptosis via a p53/Bax pathway during ischemic hypoxia and reoxygenation. *J Biol Chem* 2001;**276**:26453–26460.
26. Fujimoto K, Mizukami Y, Kimura M, Mogami K, Todoroki-Ikeda N, Kobayashi S *et al*. Molecular cloning of rat transmembrane domain protein of 40 kDa regulated in adipocytes and its expression in H9c2 cells exposed to ischemic hypoxia and reoxygenation. *Biochim Biophys Acta* 2001;**1518**:173–177.
27. Kawata Y, Mizukami Y, Fujii Z, Sakumura T, Yoshida K, Matsuzaki M. Applied pressure enhances cell proliferation through mitogen-activated protein kinase activation in mesangial cells. *J Biol Chem* 1998;**273**: 16905–16912.
28. Kikuchi M, Hatano N, Yokota S, Shimozawa N, Imanaka T, Taniguchi H. Proteomic analysis of rat liver peroxisome: presence of peroxisome-specific isozyme of Lon protease. *J Biol Chem* 2004;**279**:421–428.
29. Tokumitsu H, Hatano N, Inuzuka H, Yokokura S, Nozaki N, Kobayashi R. Mechanism of the Generation of Autonomous Activity of Ca2+/Calmodulin-dependent Protein Kinase IV. *J Biol Chem* 2004;**279**: 40296–40302.
30. Mizukami Y, Yoshioka K, Morimoto S, Yoshida K. A novel mechanism of JNK1 activation. Nuclear translocation and activation of JNK1 during ischemia and reperfusion. *J Biol Chem* 1997;**272**:16657–16662.
31. Mizukami Y, Hirata T, Yoshida K. Nuclear translocation of PKC zeta during ischemia and its inhibition by wortmannin, an inhibitor of phosphatidylinositol 3-kinase. *FEBS Lett* 1997;**401**:247–251.
32. Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B *et al*. Culture and adenoviral infection of adult mouse cardiac myocytes: methods for cellular genetic physiology. *Am J Physiol Heart Circ Physiol* 2000;**279**:H429–H436.
33. Ward CA, Giles WR. Ionic mechanism of the effects of hydrogen peroxide in rat ventricular myocytes. *J Physiol* 1997;**500**:631–642.
34. Grupp IL, Subramaniam A, Hewett TE, Robbins J, Grupp G. Comparison of normal, hypodynamic, and hyperdynamic mouse hearts using isolated work-performing heart preparations. *Am J Physiol* 1993;**265**: H1401–H1410.
35. Brachmann J, Scherlag BJ, Rosenshtraukh LV, Lazzara R. Bradycardia-dependent triggered activity: relevance to drug-induced multiform ventricular tachycardia. *Circulation* 1983;**68**:846–856.
36. Gilmour RF Jr, Moise NS. Triggered activity as a mechanism for inherited ventricular arrhythmias in German shepherd Dogs. *J Am Coll Cardiol* 1996;**27**:1526–1533.
37. Shimizu W, Ohe T, Kurita T, Takaki H, Aihara N, Kamakura S *et al*. Early afterdepolarizations induced by isoproterenol in patients with congenital long QT syndrome. *Circulation* 1991;**84**:1915–1923.
38. Herson PS, Lee K, Pinnock RD, Hughes J, Ashford ML. Hydrogen peroxide induces intracellular calcium overload by activation of a non-selective cation channel in an insulin-secreting cell line. *J Biol Chem* 1999;**274**: 833–841.
39. Lehtinen M, Hibma MH, Stellato G, Kuoppala T, Paavonen J. Human T helper cell epitopes overlap B cell and putative cytotoxic T cell epitopes in the E2 protein of human papillomavirus type 16. *Biochem Biophys Res Commun* 1995;**209**:541–546.
40. Janse MJ. Electrophysiological changes in heart failure and their relationship to arrhythmogenesis. *Cardiovasc Res* 2004;**61**:208–217.
41. Choi BR, Burton F, Salama G. Cytosolic Ca2+ triggers early afterdepolarizations and Torsade de Pointes in rabbit hearts with type 2 long QT syndrome. *J Physiol* 2002;**543**:615–631.
42. Yan GX, Wu Y, Liu T, Wang J, Marinchak RA, Kowey PR. Phase 2 early afterdepolarization as a trigger of polymorphic ventricular tachycardia in acquired long-QT syndrome: direct evidence from intracellular recordings in the intact left ventricular wall. *Circulation* 2001;**103**:2851–2856.
43. Nuss HB, Kaab S, Kass DA, Tomaselli GF, Marban E. Cellular basis of ventricular arrhythmias and abnormal automaticity in heart failure. *Am J Physiol* 1999;**277**:H80–H91.
44. Han J, So I, Kim EY, Earm YE. ATP-sensitive potassium channels are modulated by intracellular lactate in rabbit ventricular myocytes. *Pflugers Arch* 1993;**425**:546–548.
45. Crawford RM, Budas GR, Jovanovic S, Ranki HJ, Wilson TJ, Davies AM *et al*. M-LDH serves as a sarcolemmal K(ATP) channel subunit essential for cell protection against ischemia. *EMBO J* 2002;**21**:3936–3948.