

# Circulation

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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*Circulation* 2006;114;1713-1720; originally published online Oct 2, 2006;

DOI: 10.1161/CIRCULATIONAHA.106.632273

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 72514

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## Midkine Plays a Protective Role Against Cardiac Ischemia/Reperfusion Injury Through a Reduction of Apoptotic Reaction

Mitsuru Horiba, MD; Kenji Kadomatsu, MD; Kenji Yasui, MD; Jong-Kook Lee, MD; Hiroharu Takenaka, MD; Arihiro Sumida, MD; Kaichiro Kamiya, MD; Sen Chen, MD; Sadatoshi Sakuma, PhD; Takashi Muramatsu, PhD; Itsuo Kodama, MD

**Background**—Midkine (MK) is a heparin-binding growth factor involved in diverse biological phenomena, eg, neural survival, carcinogenesis, and tissue repair. MK could have a protective action against ischemia/reperfusion (I/R) injury in the heart, because MK was shown to have cytoprotective activity in cultured neurons and tumor cells. We investigated this hypothesis in mice with and without genetic MK deletion.

**Methods and Results**—Myocardial injury after I/R was produced by transient occlusion of coronary arteries. In wild-type ( $Mdk^{+/+}$ ) mice, MK expression was increased after I/R in the periinfarct area. Infarct size/area at risk 24 hours after I/R in MK-deficient ( $Mdk^{-/-}$ ) mice was larger than in  $Mdk^{+/+}$  mice ( $55.4 \pm 9.1\%$  versus  $32.1 \pm 5.3\%$ ,  $P < 0.05$ ). Terminal dUTP nick end-labeling–positive myocyte population in the periinfarct area in  $Mdk^{-/-}$  mice was higher than in  $Mdk^{+/+}$  mice ( $6.8 \pm 0.9\%$  versus  $3.2 \pm 0.6\%$ ,  $P < 0.05$ ). Left ventricular fractional shortening 24 hours after I/R in  $Mdk^{-/-}$  mice was significantly less than that in  $Mdk^{+/+}$  mice ( $34.3 \pm 4.4\%$  versus  $50.8 \pm 2.1\%$ ,  $P < 0.05$ ). Supplemental application of MK protein to left ventricle of  $Mdk^{-/-}$  mice at the time of I/R resulted in reduction of the infarct size. Application of exogenous MK to cultured cardiomyocytes resulted in increased Bcl-2 expression and decreased apoptosis after hypoxia/reoxygenation.

**Conclusions**—These results suggest that MK plays a protective role against I/R injury, most likely through a prevention of apoptotic reaction. MK is a potentially important new molecular target for treatment of ischemic heart disease. (*Circulation*. 2006;114:1713-1720.)

**Key Words:** apoptosis ■ myocardial infarction ■ reperfusion ■ signal transduction

Cardiomyocyte apoptosis is one of the major pathogenic mechanisms underlying myocardial ischemia and reperfusion (I/R) injury.<sup>1-3</sup> Apoptosis indicates cell death and removal without the activation of an inflammatory process, based on DNA and cellular fragmentation. Activation of caspases is supposed to play a pivotal role in the genesis of apoptosis via activation of the endonucleases responsible for DNA degradation. Necrosis is a faster process, with early membrane failure, cellular swelling, and the release of cellular debris, which activates inflammation.<sup>4</sup> This classification is becoming more obscure, however, because recent evidence has revealed that signaling processes for these 2 modes of cell death can switch with each other depending on the availability of high-energy phosphates.<sup>5,6</sup> There is a continuum in the mode of cell death, with apoptosis on 1 end and necrosis at the other, and reperfusion leads to an increase in the rate of apoptosis. Apoptosis, which appears within 24 hours after

I/R, would lead to decreased cardiac contractile performance and increased risk of heart failure through a direct massive loss of myocytes and induction of necrosis.<sup>7</sup> Therefore, cardiac myocyte apoptosis may be a good target for therapeutic modulation in the context of myocardial infarction and I/R injury. Several strategies have been used to reduce apoptotic cell damage, including pharmacological and genetic interventions to modulate ion channels, nitric oxide (NO), growth factors, and downstream signaling molecules.<sup>4</sup>

### Clinical Perspective p 1720

Midkine (MK) is a heparin-binding growth factor with a molecular weight of 13 kDa, first isolated as the product of a retinoic acid–responsive gene in an embryonic carcinoma cell differentiation system; it is rich in basic amino acids and cysteine.<sup>8</sup> Structurally, MK shares  $\approx 50\%$  sequence homology with pleiotrophin/heparin-binding growth-associated molecule

Received April 6, 2006; revision received August 3, 2006; accepted August 7, 2006.

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*Circulation* is available at <http://www.circulationaha.org>

DOI: 10.1161/CIRCULATIONAHA.106.632273

but is not related to other growth factors or neurotrophic factors. MK is intensely expressed in many tissues during midgestation embryonic periods, whereas its expression in adult tissues is generally weak except in the kidney. MK has various biological activities: It promotes neurite outgrowth, survival of embryonic neurons, fibrinolytic activity of endothelial cells, and migration of inflammatory leukocytes. MK is expressed strongly in both early and advanced stages of tumors and is involved in carcinogenesis and tumor progression.<sup>9</sup>

MK could have a protective action against I/R injury of the heart through its antiapoptotic activity, because MK was shown to exert cytoprotective activity in Wilms' tumor cells through enhancement of the expression of an antiapoptotic factor, Bcl-2,<sup>10</sup> and to inhibit caspase-dependent cell damage via the activation of extracellular signal-regulated kinase (ERK) in cultured neurons.<sup>11</sup> The present study was designed to test this hypothesis. We investigated the morphological and functional consequences of I/R insult in mice with and without genetic MK deletion, as well as the effects of supplementation with recombinant MK. In vitro experiments with cultured cardiomyocytes were also performed to shed light on the molecular signals involved. The data provide evidence that endogenous MK is cardioprotective and exogenous MK attenuates I/R injury, most likely by preventing the apoptotic reaction of cardiomyocytes.

## Methods

### Mouse Models

All animal experiments were performed in accordance with the regulations adopted by the National Institutes of Health and approved by the Animal Care and Use Committee of Nagoya University. Mice were supplied by the Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan. MK-deficient (*Mdk*<sup>-/-</sup>) mice were generated as described elsewhere.<sup>12</sup> Adult male wild-type C57BL/6 (*Mdk*<sup>+/+</sup>) and male *Mdk*<sup>-/-</sup> mice with the C57BL/6 genetic background were used in the I/R model (10 to 12 weeks old, weight 22 to 25 g) and were fed normal rodent chow. Mice were anesthetized with pentobarbital (100 mg/kg IP) and ventilated through a nose cone with a tidal volume of 0.2 mL at 120 breaths/min with a rodent respirator (model SN-480-7; Shinano, Tokyo, Japan). The extremity leads of the ECGs were monitored continuously. A thoracotomy was performed in the left third intercostal space, and the beating heart was exposed. An 8-0 polypropylene suture was passed under the left coronary artery at the inferior edge of the left atrium and tied with a slipknot to produce occlusion. Myocardial ischemia was verified by blanching of the left ventricle (LV) and ST elevation in ECGs. After 60 minutes of ischemia, the left coronary artery occlusion was released by pulling on the slipknot. Air was then evacuated from the chest cavity, and the chest was closed with the ends of the slip outside of the incision. The ventilator was then removed, and normal respiration was restored. After 6 to 48 hours of reperfusion, animals were euthanized. Serum creatine phosphokinase (CPK) levels were measured in blood samples from mice at 24 hours after reperfusion. Serum CPK assays were performed by an outside laboratory (SRL, Tokyo, Japan).

### Evaluation of Risk Area and Infarct Size

After 24 hours of reperfusion, mice were anesthetized and underwent a thoracotomy as described above. The heart was exposed, and the original suture, which remained in place, was retied. The heart was then perfused with 5% Evans blue. The presence of the blue dye indicates perfusion, and its absence indicates lack of perfusion. The heart was then dissected, and the LV, including the interventricular septum, was sectioned into 4 slices and further stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) to demarcate viable tissue.

The slices were then weighed, and both sides of each slice were photographed with a digital camera (Nikon Coolpix, Nikon Corp, Tokyo, Japan). For each picture, LV area excluding chamber, area lacking Evans blue (risk area), and area lacking TTC staining (infarct area) were measured by digital planimetry with a computer software program (Scion, Frederick, Md). Measurements from both sides of each slice were averaged. For each slice, the ratios of risk area to LV and infarct area to LV were determined and multiplied by the weight of the slice. These numbers of each slice were then summed over all slices, divided by the total weight of all slices, and multiplied by 100 to yield the risk area per LV and infarct area per LV for that heart as a percentage. Finally, the infarct area was calculated as a percentage of the risk area. All measurements and calculations were performed by a single individual who was blinded to genotype and treatment status.

### Echocardiography

Transthoracic echocardiography was performed with a Nemio 20 (Toshiba Medical, Tokyo, Japan) to evaluate global cardiac function before and after I/R insult. Mice were lightly anesthetized with diethyl ether and placed in the supine position on a heating pad. The level of anesthesia was kept very light to maintain regular spontaneous respiration and to avoid compromising hemodynamic conditions. A 12-MHz transducer was applied to the left hemithorax, and 2D targeted M-mode tracings were recorded. The data were analyzed by an observer blinded to mouse genotype.

### MK Protein and Antibodies

Human recombinant MK protein was generated and purified as described previously.<sup>13</sup> Monoclonal antibodies against mouse MK were raised by injection of the purified protein into rabbits and were refined by affinity chromatography on protein-A and MK columns. Antibodies were specific to MK and did not react with pleiotrophin/heparin-binding growth-associated molecule.

### Western Blotting Analysis

Western blot analysis was performed to evaluate MK protein levels in mouse LV tissue and to evaluate Bcl-2 and ERK-1/2 protein levels in cultured cardiomyocytes. For MK detection, LV tissue homogenates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on a 15% polyacrylamide gel, and proteins were electroblotted on polyvinylidene fluoride membranes (Atto, Tokyo, Japan). After blocking, the membrane was sequentially incubated with anti-mouse MK antibody, biotinylated anti-mouse immunoglobulin G (IgG; Sigma, St Louis, Mo), horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech UK, Little Chalfont, United Kingdom), and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech UK). To detect Bcl-2 and ERK-1/2 proteins, cultured myocytes were lysed in SDS sample buffer, the cell lysate was subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel, and proteins were electroblotted on polyvinylidene fluoride membrane. The membrane was subjected to overnight blocking and was sequentially immunoblotted with anti-mouse anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-ERK-1/2 antibody (Sigma), or anti-phosphorylated ERK-1/2 antibody (NEN Life Science Products, Boston, Mass). The intensity of bands was quantified by densitometry (Atto, Tokyo, Japan).

### Immunohistochemistry

Mouse hearts were embedded in paraffin after fixation with 4% paraformaldehyde and were cut into 5- $\mu$ m sections across the apex-base axis of the LV. Immunostaining of MK in paraffin sections was performed as described previously.<sup>13</sup> Exposure to secondary antibody conjugated with goat anti-rat IgG (Jackson Laboratory, Bar Harbor, Me) was followed by incubation with biotinyl-tyramide and streptavidin-horseradish peroxidase (NEN Life Science Products) to enhance the immunoreactive signals. The specificity of immunostaining for MK was confirmed by absorption of the anti-MK antibodies with recombinant MK, followed by

heparin-sepharose affinity chromatography as described previously.<sup>13</sup> For immunolabeling of inflammatory cells, the section was stained with anti-mouse CD45 (leukocyte common antigen) antibody (Laboratory Vision Corporation, Fremont, Calif). Exposure to secondary antibody conjugated with goat anti-rat IgG (Jackson Laboratory) was followed by incubation with biotiny-tyramide and streptavidin–horseradish peroxidase.

### Cell Culture

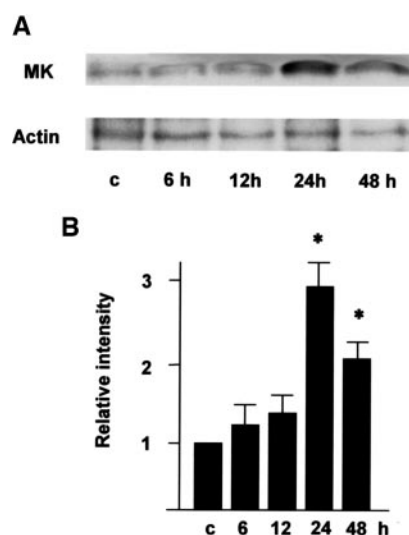
Neonatal mouse ventricular myocytes were prepared from 1-day-old Institute of Cancer Research mice according to the manual of the Neonatal Cardiomyocyte Isolation System (Worthington, Lakewood, NJ). Briefly, ventricular pieces were incubated and stirred in  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$  free Hank's balanced salt solution containing 100 U/mL collagenase at 37°C for 15 minutes. The cell suspension was collected, and the isolated cells were cultured in M199 medium (GIBCO BRL, Rockville, Md) with 10% fetal bovine serum, 5  $\mu\text{mol/L}$  cytosine arabinoside, 50 U/mL penicillin, and 50  $\mu\text{g/mL}$  streptomycin at 37°C in a humidified 5%  $\text{CO}_2$ /20%  $\text{O}_2$  incubator. For hypoxia-reoxygenation (H/R) experiments, the hypoxic condition was created by incubating the cardiomyocytes in an anaerobic chamber equilibrated with 95%  $\text{N}_2$  plus 5%  $\text{CO}_2$  at 37°C for 6 hours. The myocytes were then returned to the former  $\text{CO}_2$  incubator for reoxygenation. At the initiation of reoxygenation, the culture medium was changed to M199 with 0.5% fetal bovine serum, 5  $\mu\text{mol/L}$  cytosine arabinoside, 50 U/mL penicillin, and 50  $\mu\text{g/mL}$  streptomycin. Simultaneously, MK protein (100 ng/mL) was added to the altered culture medium for the MK treatment group. Cells were harvested at 18 hours after reoxygenation.<sup>14</sup> To study the signaling pathways involved in the antiapoptotic action of MK in H/R, 20  $\mu\text{mol/L}$  Bcl-2 inhibitor (Calbiochem, Darmstadt, Germany) or 50  $\mu\text{mol/L}$  PD 98059 (Calbiochem), an ERK inhibitor, was added together with MK protein at the time of reoxygenation. In experiments in which ERK activity was measured, cultured myocytes were treated with 100 ng/mL MK protein for 30 minutes after 24 hours of serum starvation.<sup>11</sup>

### Detection of Apoptosis

Apoptotic myocardial cells were identified by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) staining or by quantification of cytosolic oligonucleosome-bound DNA. For the TUNEL assay, deparaffinized LV tissue sections or cultured cardiomyocytes fixed by 4% paraformaldehyde in phosphate-buffered saline were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP with terminal deoxynucleotidyl transferase (Roche Diagnostics Corp, Indianapolis, Ind). The total cell population was estimated by counting the Hoechst 33342-stained nuclei in 5 fields of each specimen using the 40 $\times$  objective (total nuclei counted in each specimen were 5300 to 6100), and the ratio of TUNEL-positive cells to Hoechst 33342-stained nuclei was calculated. The DNA fragmentation of cultured cardiomyocytes was measured by quantification of cytosolic oligonucleosome-bound DNA with the Cell Death Detection ELISA kit (Roche Diagnostics Corp). Briefly, cardiomyocytes were rinsed with phosphate-buffered saline 3 times to remove nucleosomes that had leaked out from necrotic cells and then were incubated with the lysis buffer for 30 minutes. The supernatant containing nucleosomes from the cytosolic fraction of apoptotic cell was used as the antigen source in the sandwich ELISA with a primary antihistone antibody coated to the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase.<sup>15</sup>

### Statistic Analysis

All values are expressed as mean $\pm$ SEM. Statistical comparisons among the groups were performed by ANOVA with Bonferroni post hoc tests. Comparison between 2 groups were made with unpaired Student *t* test. Probability values of <0.05 were considered significant.



**Figure 1.** Increase of MK protein expression in *Mdk*<sup>+/+</sup> mouse hearts after I/R injury. A, Western blot of MK protein and actin in *Mdk*<sup>+/+</sup> mouse hearts in control conditions (c) and 6 to 48 hours after I/R. B, The intensity of MK bands was analyzed by densitometry. Values are normalized to control. n=5. \**P*<0.01 vs control.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

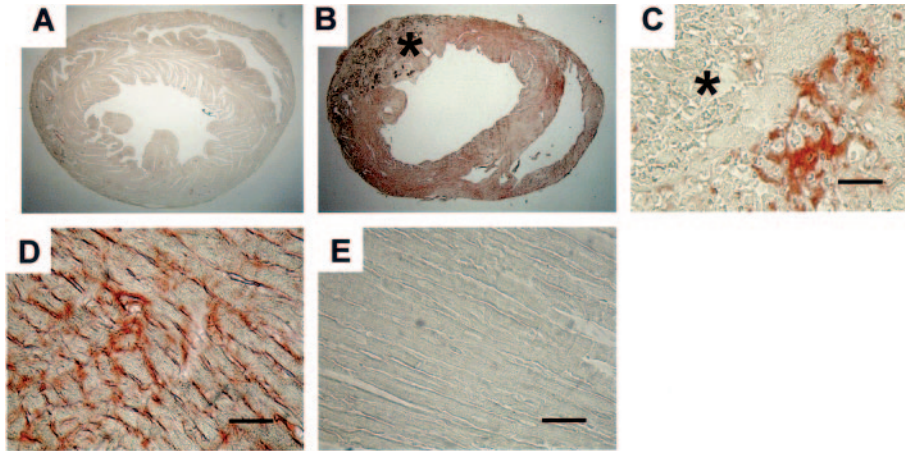
## Results

### MK Expression Is Increased in *Mdk*<sup>+/+</sup> Mice Hearts After I/R

To examine the MK expression pattern in *Mdk*<sup>+/+</sup> mice hearts after I/R, we subjected mice to 60 minutes of left coronary artery occlusion followed by 48 hours of reperfusion. We evaluated the time course of MK expression after I/R by Western blotting. Weak MK expression was recognized in control hearts, and the expression increased gradually through 6 to 12 hours. The strongest blotting band was shown after 24 hours and persisted as obviously expressed until 48 hours (Figure 1A and 1B). Next, we performed immunohistochemistry to detect localization of MK in heart sections of *Mdk*<sup>+/+</sup> mice. Although only faint MK expression was recognized diffusely in control conditions (Figure 2A), MK was labeled strongly in the periinfarct region after 24 hours of I/R insult (Figure 2B). MK expression was clearly detected in the border region between infarct and noninfarct areas (Figure 2C) and was found mainly in the extracellular space adjacent to the myocardial cell membrane (Figure 2D). The negative staining with preabsorbed antibodies indicated that the immunostaining was specific (Figure 2E).

### Myocardial Infarct Size Is Increased in *Mdk*<sup>-/-</sup> Mice

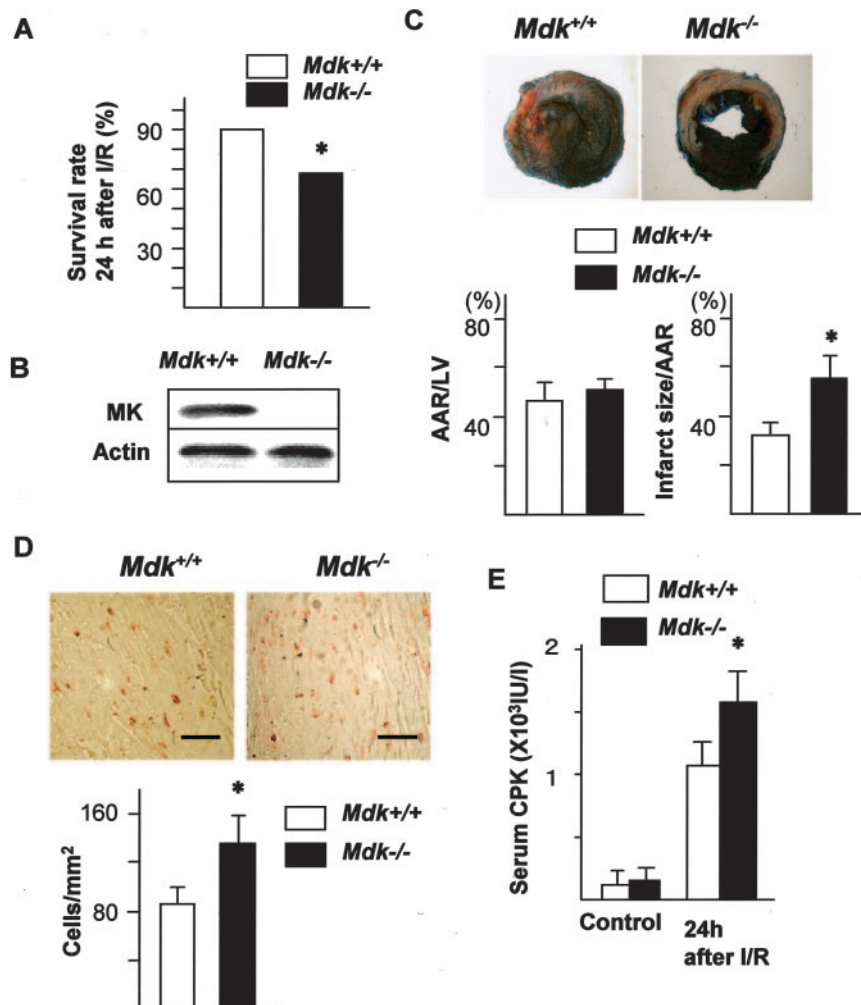
We compared myocardial damage in *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice. The age and body weight of *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice were set to be identical. At baseline, there were no morphological differences between *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> hearts, and heart rate was identical. Although all mice survived the surgical induction of I/R, 32% (8/25) of *Mdk*<sup>-/-</sup> mice died within 24 hours after I/R, whereas only 10% (4/40) of *Mdk*<sup>+/+</sup> mice died during the same period (Figure 3A). In Western blotting, there was no obvious MK expression in *Mdk*<sup>-/-</sup> mouse hearts 24 hours after



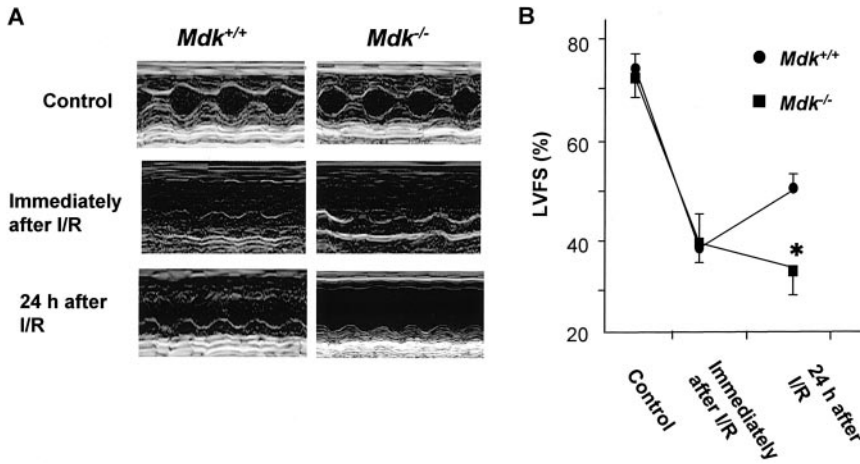
**Figure 2.** Immunohistochemistry of MK protein in *Mdk*<sup>+/+</sup> mouse hearts. A and B, Ventricular sections of *Mdk*<sup>+/+</sup> mouse hearts in control conditions (without I/R; A) and at 24 hours after I/R (B) at a low magnification. C, Border region between the infarct and noninfarct area at a higher magnification (×100). Scale bar=200 μm. D, MK immunopositive area at the highest magnification (×400). Immunoreactive MK protein was mainly localized in the extracellular space adjacent to the myocardial cell membrane. E, Negative staining with preabsorbed antibodies. Scale bar in D and E: 50 μm. Asterisks in B and C indicate the infarct region.

I/R (Figure 3B). To delineate area at risk (AAR) and infarct area, we stained the mouse hearts with Evans blue and TTC. Despite the fact that AAR was similar between *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice, the extent of white necrotic area (infarct area) within the AAR of

*Mdk*<sup>-/-</sup> mice was significantly larger than that of *Mdk*<sup>+/+</sup> mice. Representative photomicrographs showing AAR and infarct area in *Mdk*<sup>-/-</sup> and *Mdk*<sup>+/+</sup> mice 24 hours after I/R and summary data are presented in Figure 3C. We examined infiltration of inflam-



**Figure 3.** Consequences of I/R insult in *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice in terms of survival, MK expression, infarct size, inflammatory cell recruitment, and serum CPK. A, 32% of *Mdk*<sup>-/-</sup> mice died within 24 hours after I/R, whereas 10% of *Mdk*<sup>+/+</sup> mice died during the same period. n=25 *Mdk*<sup>-/-</sup> and n=40 *Mdk*<sup>+/+</sup> mice. \**P*<0.05 vs *Mdk*<sup>+/+</sup>. B, In Western blotting, there was no obvious MK expression in *Mdk*<sup>-/-</sup> mouse hearts 24 hours after I/R. C, Photomicrographs of LV sections from *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice at 24 hours after I/R insult. Tissue stained blue by Evans blue represents nonischemic area; tissue stained red by TTC within ischemic area indicates viable tissue. Tissue not stained by either Evans blue or TTC appears pale to white and represents infarct myocardium. Infarct size was measured as the percentage of LV area or that of total ischemic AAR. Graphs show mean±SEM of AAR/LV and infarct size/AAR (n=12). \**P*<0.05 vs *Mdk*<sup>+/+</sup>. D, Immunohistochemistry of CD45 24 hours after I/R in *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice. CD45-positive cells in the periinfarct area were counted in 5 fields of each specimen, and their number/mm<sup>2</sup> was obtained (mean±SEM, n=7 each). \**P*<0.05 vs *Mdk*<sup>+/+</sup>. Scale bar=100 μm. E, Serum CPK levels before and 24 hours after I/R in *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice (n=10 each). \**P*<0.05 vs *Mdk*<sup>+/+</sup>.



**Figure 4.** Echocardiographic assessment of LV function in *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice. A, Representative M-mode LV images of *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice at control, immediately after I/R, and 24 hours after I/R. B, Changes in LVFS after I/R. Values are mean ± SEM (n=10). *Mdk*<sup>+/+</sup> mice showed a partial recovery of LVFS at 24 hours after I/R, whereas *Mdk*<sup>-/-</sup> mice showed a further deterioration in LVFS. \*P<0.05 vs *Mdk*<sup>+/+</sup>.

matory cells in the periinfarct area by immunostaining for CD45. The number of CD45-positive cells in *Mdk*<sup>-/-</sup> mice was significantly larger than that in *Mdk*<sup>+/+</sup> (Figure 3D). Although there was no significant difference in serum CPK activity between the sham-operated *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice, serum CPK activity of *Mdk*<sup>-/-</sup> mice was significantly higher than that of *Mdk*<sup>+/+</sup> mice at 24 hours after I/R (Figure 3E).

**Cardiac Function After I/R Is Preserved in *Mdk*<sup>+/+</sup> Compared With *Mdk*<sup>-/-</sup> Mice**

We investigated the morphological changes and left ventricular function of *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice. We performed echocardiography under light anesthesia so as to not compromise respiration and hemodynamic conditions. Before operation, there were no significant changes between *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice. The LV cavity was dilated, and LV fractional shortening (LVFS) was reduced immediately after I/R insults in both *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice; there was no statistical difference between the 2 animal groups. LVFS of *Mdk*<sup>+/+</sup> mice was partially recovered, whereas LVFS of *Mdk*<sup>-/-</sup> mice was further deteriorated; LVFS 24 hours after I/R in *Mdk*<sup>-/-</sup> mice was significantly less than that in *Mdk*<sup>+/+</sup> mice (Figures 4A and 4B).

**Increased Myocardial Apoptosis in *Mdk*<sup>-/-</sup> Mice**

To investigate the extent of apoptosis in AAR, we performed TUNEL staining on the different experimental groups. The heart section obtained from the periinfarct area of *Mdk*<sup>-/-</sup> mice exhibited a significantly larger number of TUNEL-positive

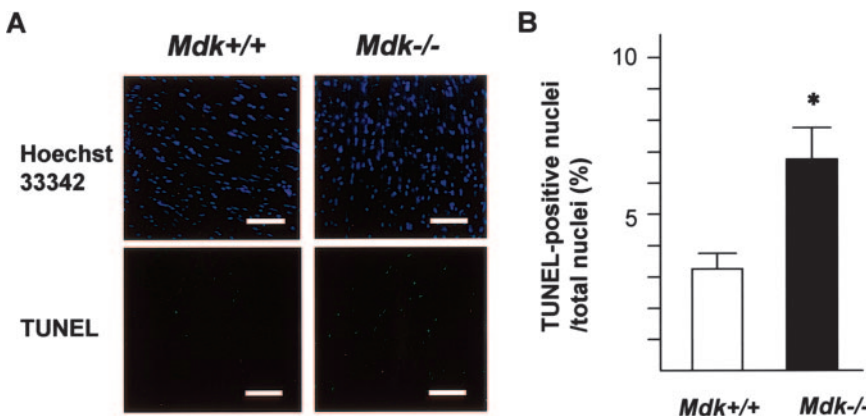
myocytes than that of *Mdk*<sup>+/+</sup> mice (Figures 5A and 5B). Autopsy results showed that 3 of 8 *Mdk*<sup>-/-</sup> mice that died within 24 hours after I/R had intramyocardial hemorrhage, which reflected massive tissue destruction, whereas none of the 4 *Mdk*<sup>+/+</sup> mice showed such myocardial tissue hemorrhage (data not shown).

**MK Protein Inhibits Apoptosis Through Bcl-2 and ERK Activation**

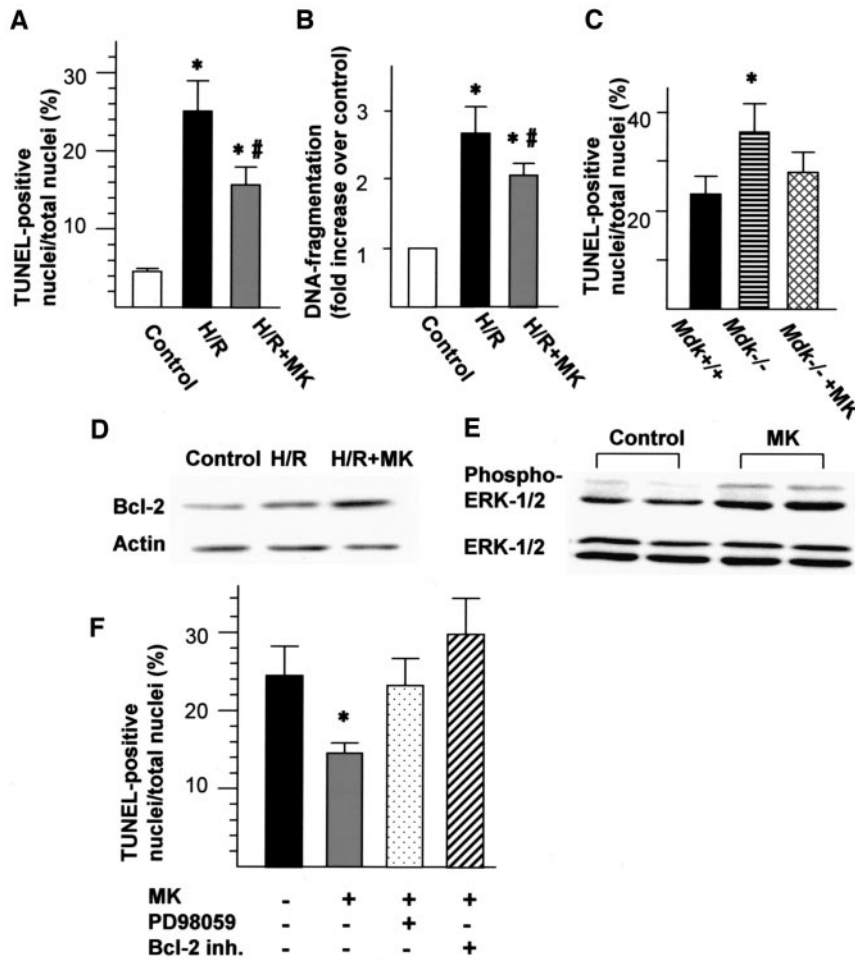
In experiments using cultured myocytes, we first investigated the cell-protective properties of exogenous MK using an H/R insult. To analyze DNA fragmentation, we examined nucleosomes measured by ELISA and TUNEL-positive cells. In myocytes isolated from *Mdk*<sup>+/+</sup> mice, both the amount of nucleosome and the TUNEL-positive cell population after H/R significantly decreased by treatment with 100 ng/mL MK protein (Figures 6A and 6B).

We next compared the H/R-induced apoptosis in cardiomyocytes from *Mdk*<sup>+/+</sup> and those from *Mdk*<sup>-/-</sup> mice (Figure 6C). The TUNEL-positive cell population after H/R was significantly greater in cardiomyocytes from *Mdk*<sup>-/-</sup> mice than in those from *Mdk*<sup>+/+</sup> mice. The increase of the H/R-induced TUNEL-positive cell population in *Mdk*<sup>-/-</sup> myocytes was reversed by application of exogenous MK protein (100 ng/mL). This indicates that both endogenous and exogenous MKs possess a considerable protective action against the H/R-induced apoptosis.

Because MK upregulates Bcl-2 activity in tumor cell lines,<sup>10</sup> we investigated the influence of Bcl-2 expression by treatment



**Figure 5.** TUNEL staining and quantification of apoptosis in the periinfarct area of *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> hearts. A, Representative Hoechst 33342-stained (top for nuclei) and TUNEL-stained (bottom) LV sections from *Mdk*<sup>+/+</sup> and *Mdk*<sup>-/-</sup> mice at 24 hours after I/R. Scale bars=100 μm. B, Apoptotic cell population was estimated by TUNEL-positive nuclei/total nuclei (%). Values are mean ± SEM (n=5, 5 fields of each specimen). \*P<0.05 vs *Mdk*<sup>+/+</sup>.



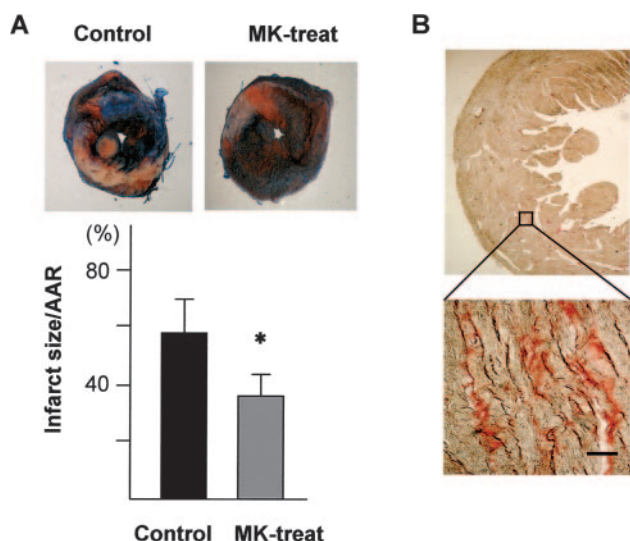
**Figure 6.** Antiapoptotic action of MK in cultured cardiomyocytes subjected to hypoxia/reoxygenation (H/R). A and B, H/R-induced apoptosis in cardiomyocytes from *Mdk*<sup>+/+</sup> mice and the effects of exogenous MK protein (100 ng/mL) application. Apoptosis was quantified by TUNEL staining (A) and by DNA fragmentation estimated with ELISA (B). TUNEL-stained nuclei were quantified as the percentage of total nuclei number (Hoechst 33342-stained; n=12). The values of DNA fragmentation (cytosolic oligonucleosome-bound DNA) were normalized to control (n=12). \*P<0.05 vs control, #P<0.05 vs H/R in the absence of MK protein. C, Quantification of H/R-induced apoptosis by TUNEL staining in *Mdk*<sup>+/+</sup> cardiomyocytes and *Mdk*<sup>-/-</sup> cardiomyocytes without and with exogenous MK protein (100 ng/mL; n=12). \*P<0.05 vs *Mdk*<sup>+/+</sup>. D, Bcl-2 protein expression in cultured cardiomyocytes from *Mdk*<sup>+/+</sup> mice. Western blots for Bcl-2 and actin in myocytes at control (left), after H/R in the absence of MK protein (middle), and after H/R in the presence of MK protein (100 ng/mL; right). E, Activation of ERK-1/2 by MK in cultured cardiomyocytes. Activated ERK-1/2 was assessed by Western blots with anti-phospho ERK-1/2 antibody. Cardiomyocytes were incubated for 30 minutes in the absence (control, left 2 lanes) or presence (right 2 lanes) of 100 ng/mL MK protein. Photographs in D and E are representative of 4 independent experiments. F, Effects of an ERK inhibitor, PD 98059, and a Bcl-2 inhibitor (Bcl-2 inh.) on the protective action of exogenous MK protein against H/R-induced apoptosis. H/R-induced apoptosis in *Mdk*<sup>+/+</sup> myocytes was quantified by TUNEL staining in the absence and presence of exogenous MK protein (100 ng/mL) without and with PD 98059 (50 μmol/L) or Bcl-2 inhibitor (20 μmol/L; n=10 under each condition). \*P<0.05 vs control (in the absence of MK protein).

of exogenous MK protein in the H/R insult on cardiomyocytes from *Mdk*<sup>+/+</sup> mice. Bcl-2 expression of ventricular myocytes after reoxygenation was upregulated with 100 ng/mL MK treatment, whereas there was no significant change between control and H/R without MK treatment (Figure 6D). Because activation of ERK is associated with cytoprotection in cardiomyocytes<sup>16</sup> and directly mediates the upregulation of Bcl-2,<sup>17</sup> we also analyzed ERK-1/2 phosphorylation in cultured cardiomyocytes. Under serum-starved conditions, the phosphorylation of ERK-1/2 was at a weak level, whereas the addition of 100 ng/mL MK significantly increased phosphorylated ERK-1/2 (Figure 6E). To verify the involvement of Bcl-2 and ERK in the signaling pathway for the antiapoptotic action of MK, we examined the effects of their respective inhibitors. In the presence of either an ERK inhibitor, PD 98059 (50 μmol/L), or a

Bcl-2 inhibitor (20 μmol/L), MK protein treatment (100 ng/mL) did not cause a significant reduction of the TUNEL-positive cell population (Figure 6F).

**Exogenous MK Reduces Infarct Size in *Mdk*<sup>-/-</sup> Mice**

We tested the therapeutic potential of MK for I/R injury. Using *Mdk*<sup>-/-</sup> mice, we injected 20 μL MK protein (10 μg/mL) directly into the periinfarct area of LV free wall immediately after coronary reperfusion (MK-treated mice). In the control group, vehicle was given at the indicated time. Although AAR was similar, the infarct area within AAR of MK-treated mice was significantly smaller than that of the control group 24 hours after I/R (Figure 7A). Immunohistochemistry showed that a substantial amount of MK protein remained in the injected region 24 hours after I/R (Figure 7B).



**Figure 7.** Reduction of infarct size by application (injection) of exogenous MK protein to *Mdk*<sup>-/-</sup> mice subjected to I/R. A, Representative photomicrographs of LV sections from *Mdk*<sup>-/-</sup> mice with and without injection of MK protein (10 μg/mL [20 μL]). Sections (24 hours after I/R insult) were stained with Evans blue (for non-ischemic area) and TTC (for viable tissue). Tissue not stained by either Evans blue or TTC appears pale to white and represents infarcted myocardium. Infarct size was measured as the percentage of total ischemic AAR. Values are mean ± SEM (n=15). \**P*<0.05 vs control (without MK injection). B, Immunohistochemistry of injected MK protein in *Mdk*<sup>-/-</sup> mouse heart. Exogenous MK protein remained in the injected site. Scale bar=50 μm.

## Discussion

The present study demonstrates that MK plays a crucial role in the cardioprotection in response to I/R. Specifically, I/R resulted in upregulation of MK in *Mdk*<sup>+/+</sup> mice, with the highest expression in the periinfarct area at 24 hours after the insult. Furthermore, infarct size after I/R was larger and LV function after I/R was lower in *Mdk*<sup>-/-</sup> mice than in *Mdk*<sup>+/+</sup> mice, and there was a greater degree of apoptosis in the periinfarct area in *Mdk*<sup>-/-</sup> mice than in *Mdk*<sup>+/+</sup> mice. Supplemental in vivo MK application to LV of *Mdk*<sup>-/-</sup> mice at the time of I/R resulted in a reduction of infarct size. In vitro MK application to cardiomyocytes exhibited significant protective action against H/R-induced apoptosis in association with an enhancement of Bcl-2 expression and ERK activation. The in vitro action of exogenous MK was prevented by specific inhibitors of Bcl-2 and ERK. Taken together, these data suggest that MK exerts a protective effect against ischemic or hypoxic stress in cardiomyocytes, most likely through prevention of apoptosis.

Catheter-based and pharmacological reperfusion therapies are currently used in clinical practice to minimize cardiac damage after acute myocardial infarction. Prevention of apoptotic reaction in cardiomyocytes in the periinfarct zone is an alternative strategy. Several new experimental approaches have been reported recently. In a rat model of I/R injury, Nakamura et al<sup>18</sup> provided evidence that endogenous hepatocyte growth factor is cardioprotective, and exogenous hepatocyte growth factor attenuates I/R injury through suppression of apoptosis in cardiomyocytes. Parsa et al<sup>19</sup> reported that erythropoietin protected isolated cardiomyocytes from oxidative and hypoxic stress and protected

in vivo rabbit hearts from myocardial infarction, possibly via its ability to activate cell survival pathways (Akt) and inhibit myocyte apoptosis. Other studies reported that adrenomedullin, a potent vasodilator, suppressed apoptosis of cardiomyocytes through activation of Bcl-2 signaling pathway, and its cardio-protective action against I/R injury was verified by adenoviral gene delivery to rat hearts.<sup>20</sup> The G-actin sequestering peptide, thymosin β<sub>4</sub>, promotes myocardial and endothelial cell migration in embryonic heart and retains this property in postnatal cardiomyocytes. Bock-Marquette et al<sup>21</sup> used a mouse model of myocardial infarction to demonstrate that thymosin β<sub>4</sub> activated cell survival pathways, enhanced early myocyte survival, and improved cardiac function.

Certain biological functions of MK reported to date are mediated by its antiapoptotic action. These include a promotion of cell growth and an induction of oncogenic transformation.<sup>9,10,22</sup> Thus, the antiapoptotic action of MK is widely involved in carcinogenesis, neurogenesis, and tissue repair. Candidates for the MK receptors are protein-tyrosine phosphatase-ζ, a chondroitin sulfate proteoglycan, and members of the low-density-lipoprotein receptor-related protein family.<sup>23,24</sup> The downstream signaling systems of these receptors include ERK,<sup>25</sup> which participates in the reduction of necrotic and apoptotic cell death.<sup>16</sup> Therefore, induction of ERK by MK in the present study indicates that MK may prevent cardiomyocyte cell death in conjunction with the antiapoptotic effect via Bcl-2 enhancement.

MK is also known to promote the chemotaxis of neutrophils and the migration of macrophages. We have shown that MK has a critical role in neointima formation by enhancing the recruitment of inflammatory cells.<sup>13</sup> In the present study, however, inflammatory cell numbers in the ischemic area of *Mdk*<sup>-/-</sup> mice were significantly greater than those of *Mdk*<sup>+/+</sup> mice. In the ischemic myocardium, the benefit of antiapoptotic action of MK leading to a reduction of secondary necrosis, which activates inflammation, could offset the intrinsic proinflammatory reaction. Apoptosis in cardiac muscle may be more prone to shift to the necrosis, because the beating heart has higher energy requirements than other organs.<sup>5</sup>

Substantial controversy exists as to the role of inflammatory reaction in the progress of I/R injury of the heart.<sup>26–28</sup> It is pointed out by some investigators that the evidence for its undesirable influence is equivocal.<sup>29,30</sup> Indeed, antiinflammatory substances or leukocyte filters do not consistently prevent I/R injury and limit infarct size.<sup>31,32</sup>

We conclude that endogenous MK may primarily play a protective role against I/R injury of the heart, most likely through activation of antiapoptotic signaling pathways. Beneficial effects of exogenous MK to minimize I/R injury would provide a new perspective for innovation in the treatment of acute myocardial infarction.

## Acknowledgments

We thank Drs H. Tsutsui and T. Murohara for critical advice and helpful discussion. We thank M. Hojo and T. Koike for technical assistance.

## Sources of Funding

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and from the Suzuken Memorial Foundation, Japan (to Dr Horiba).

## Disclosures

None.

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## CLINICAL PERSPECTIVE

Apoptosis of cardiomyocytes is one of the major contributors to myocardial ischemia and reperfusion (I/R) injury. Because apoptosis appears within 24 hours after I/R and induces massive loss of myocytes, it will jeopardize cardiac function in the acute phase of myocardial infarction. A sequence of deteriorating events in the heart in response to myocardial infarction and I/R is, therefore, expected to be minimized if the apoptotic reaction in cardiomyocytes is prevented efficiently. This study addresses a novel protective action of midkine (MK) in the heart subjected to I/R. MK is a heparin-binding growth factor involved in diverse biological phenomena, eg, neural survival, carcinogenesis, and tissue repair. The study has shown a potent antiapoptotic effect of MK via the activation of Bcl-2 and extracellular signal-regulated kinase in cardiomyocytes under ischemic and hypoxic stress in experiments with MK knockout mice and exogenous MK application. This novel cardioprotective effect of MK appears to limit infarct size by protecting myocardium in the ischemic zone, which leads to preservation of cardiac contractile function. Beneficial effects of exogenous MK to minimize I/R injury would provide a new perspective for innovation in the treatment of acute myocardial infarction.