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Therapeutic Potential of a Novel Necrosis Inhibitor, 7-Amino-Indole, in Myocardial Ischemia–Reperfusion Injury

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Abstract—Opening of mitochondrial permeability transition pore and Ca^{2+} overload are main contributors to myocardial ischemia–reperfusion injury, which paradoxically causes a wide variety of myocardial damage. We investigated the protective role of a novel necrosis inhibitor (NecroX-7; NecX) against myocardial ischemia–reperfusion injury using in vitro and in vivo models. H9C2 rat cardiomyoblasts and neonatal cardiomyocytes were exposed to hypoxia–reoxygenation stress after pre-treatment with NecX, vitamin C, a combination of vitamin C and E, N-acetylcysteine, an apoptosis inhibitor (Z-VAD-fmk), or cyclosporine A. The main mechanism of cell death after hypoxia–reoxygenation stress was not apoptosis but necrosis, which was prevented by NecX. Protective effect of NecX was based on its potent reactive oxygen species scavenging activity, especially on mitochondrial reactive oxygen species. NecX preserved mitochondrial membrane potential through prevention of Ca^{2+} influx and inhibition of mitochondrial permeability transition pore opening, which was more potent than that by cyclosporine A. Using Sprague-Dawley rats exposed to myocardial ischemia for 45 minutes followed by reperfusion, we compared therapeutic efficacies of NecX with cyclosporine A, vitamin C, a combination of vitamin C and E, and 5% dextrose, each administered 5 minutes before reperfusion. NecX markedly inhibited myocardial necrosis and reduced fibrotic area to a greater extent than did cyclosporine A and other treated groups. In addition, NecX preserved systolic function and prevented pathological dilatory remodeling of left ventricle. The novel necrosis inhibitor has a significant protective effect against myocardial ischemia–reperfusion injury through inhibition of mitochondrial permeability transition pore opening, indicating that it is a promising candidate for cardioprotective adjunctive measure on top of reperfusion therapy. (*Hypertension*. 2018;71:1143-1155. DOI: 10.1161/HYPERTENSIONAHA.117.09405.)
● **Online Data Supplement**

Key Words: myocardial reperfusion injury ■ necrosis ■ reactive oxygen species ■ reperfusion injury

Despite tremendous progress in the treatment of myocardial infarction (MI), the mortality and morbidity of MI is still one of the most important healthcare problems.[1] After appropriate revascularization therapy to treat MI, reperfusion itself may cause a wide variety of injuries to the myocardium.[2] Although the net effect of reperfusion is a reduction in infarct size, the introduction of blood flow into an ischemic zone generates reactive oxygen species (ROS), calcium ion (Ca^{2+}) influx, and a rapid correction of acidosis, all of which induce the opening of mitochondrial permeability transition pore (mPTP). Therefore, during reperfusion, a damaged myocardium experiences additional necrotic cell death and increase in infarct size, known as myocardial ischemia–reperfusion (I/R) injury.[3,4]

Given the fundamental evidence that mPTP opening is the key mechanism of myocardial I/R injury, a series of trials investigated the effect of cyclosporine A (CsA) in patients with MI undergoing revascularization. However, the Does CIRCUS (Cyclosporine Improve Clinical Outcome in ST Elevation Myocardial Infarction Patients trial; NCT01502774) and the CYCLE (CYCLosporinE A in Reperfused Acute Myocardial Infarction trial; NCT01650662) reported that the administration of CsA neither reduced the burden of reperfusion injury nor improved clinical outcomes.[5,6] Therefore, the interest and attention to the novel therapeutic measure against myocardial I/R injury are higher than ever.

The novel necrosis inhibitor (NecroX-7; NecX) has a broad spectrum of applications against necrotic insults.[7,8]

NecX scavenges mitochondrial ROS, thereby blocking necrotic cell death.[9–11] Another derivative of NecX inhibits a mitochondrial Ca^{2+} uniporter and protects mitochondria from hypoxia–reoxygenation stress.[12] The aim of this study was to investigate the protective role of this novel necrosis inhibitor against myocardial I/R injury using in vitro and in vivo models, in comparison with CsA, the established inhibitor of mPTP opening.

Materials and Methods

The data that support the findings of our study are available from the corresponding author, and the study material (NecX) is available from LG Chem Life Science R&D Campus (Daejeon, Korea; http://www.rnd.lgchem.com/global/main) on reasonable request. Detailed methods are described in online-only Data Supplement.

Material: Necrosis Inhibitor

The novel necrosis inhibitor, NecX, is a 7-amino-indole chemical developed by LG Chem (Korea). Based on preliminary experiments of various compounds in the NecX series, we chose NecroX-7 ((tetrahydropyran-4-yl)-[2-phenyl-5-(1,1-dioxothiomorpholin-4-yl) methyl-1H-indol-7-yl]amine; $C_{25}H_{32}N_4O_4S_2$) considering its efficacy, stability, and bioavailability (Figure 1A).

In Vitro Models

Cell Culture and Conditioning

H9C2 rat cardiomyoblasts and primary cardiomyocytes from neonates of Sprague-Dawley rats were used (Figure 1B; Figure S1 in the online-only Data Supplement). The cells were exposed to hypoxia for 24 hours. At 23.5 hours of hypoxia (1% oxygen), cells were pre-treated with one of the following: 0.01% dimethyl sulfoxide (vehicle); NecX, 20 µmol/L; vitamin C, 10 µmol/L or 20 µmol/L; vitamin E (trolox), 20 µmol/L; a combination of vitamin C, 10 µmol/L, and vitamin E, 20 µmol/L; N-acetylcysteine, 250 µmol/L; Z-VAD-fmk, a caspase inhibitor, 20 µmol/L; or CsA, 1.0 µmol/L. For the Western blot analysis and the luminescence assay for caspase-3 activity, H9C2 cells were pre-treated with vehicle, NecX, vitamin C, Z-VAD-fmk, or 5 µmol/L of staurosporine (S6942; Sigma-Aldrich, St Louis, MO), an apoptosis inducer. Then, the cells were exposed to H_2O_2 and incubated at 37°C for 90 minutes (20% oxygen), following the relevant method to simulate I/R injury.[13,14]

Comparison of Fluorescent Signal Intensity

To assess the potential influences of study drugs on the fluorescent assays, the fluorescent signal intensities of a common secondary antibody (R37114; ThermoFisher Scientific, Waltham, MA) were compared between NecX, vitamin C, a combination of vitamin C and vitamin E, N-acetylcysteine , Z-VAD-fmk, and the vehicle, using a Cary Eclipse fluorescence spectrophotometer (Varian, CA) with excitation wavelength at 485 nm and emission at 530 nm (Figure S2).

Analyses of ROS Scavenging Activity

With the use of dihydrorhodamine 123 (D1054, Sigma-Aldrich, St Louis, MO) which is not fluorescent until oxidized by ROS to the highly fluorescent product rhodamine 123, we assessed the levels of mitochondria-selective ROS in H9C2 rat cardiomyoblasts. A 50 mmol/L dihydrorhodamine 123 stock solution was prepared in dimethyl sulfoxide and used to each sample at the final concentration of 16.6 µmol/L. Formation of rhodamine 123 was monitored by confocal fluorescence microscopy using excitation and emission wavelengths of 488 and 555 nm, respectively.

Measurement of Mitochondrial Membrane Potential and Ca^{2+} Influx

Mitochondrial membrane potential ($\Delta\psi_m$) was measured by the use of tetramethylrhodamine ethyl ester (TMRE; I35103, ThermoFisher Scientific) fluorescence. H9C2 rat cardiomyoblasts were incubated with 100 nmol/L TMRE for 20 minutes after 24 hours of hypoxia and 90 minutes of oxidative stress. Levels of TMRE fluorescence at 555 nm were expressed as percentages of the value of control (no staining). Emission of fluorescence was recorded by confocal microscopy (LSM 710 META, Carl Zeiss, Peabody, MA).

We also measured $\Delta\psi_m$ by the use of JC-1 (MP03168; Molecular Probes, Invitrogen) in neonatal rat cardiomyocytes. J-aggregate (the predominant form at high $\Delta\psi_m$) emits orange fluorescence and JC-1 monomer (the predominant form at low $\Delta\psi_m$) emits green fluorescence. Neonatal rat cardiomyocytes were stained with 10 µmol/L JC-1 for 10 minutes at 37°C. Then, the cells were washed and placed in a perfusion chamber. Fluorescence was detected by laser scanning confocal microscopy, and the $\Delta\psi_m$ in isolated mitochondria was evaluated by flow cytometry FL1 and FL2 channels, detecting JC-1 monomer and J-aggregate forms, respectively.

Mitochondrial Ca^{2+} retention capacity, a sensitive and qualitative measure of the ability of mitochondria to open mPTP in response to accumulated Ca^{2+} influx, was assessed. After incubation in hypoxic chamber for 24 hours, cells were treated with MitoTracker Green FM (M7514; ThermoFisher Scientific) for 5 minutes, and then, the media was changed. NecX 20 µmol/L or 0.01% dimethyl sulfoxide was treated with Rhod-2 (R1244; ThermoFisher Scientific), a calcium indicator, for 5 minutes in room temperature. Immediately after the addition of 400 µmol/L of H_2O_2, mitochondrial calcium level (Rhod-2 fluorescence) and mitochondrial shape were examined by confocal microscopy.

Assessment of mPTP Opening

For the detection of mPTP opening in H9C2 rat cardiomyoblast, we used the calcein-AM release assay (MitoProbe Transition Pore Assay, Molecular Probes). H9C2 cells were incubated with 1 µmol/L calcein and 1 mmol/L of cobalt chloride for 30 minutes after 24 hours of hypoxia and pre-treatment of NecX or others. After 90 minutes of oxidative stress, the cells were washed with warm Hanks' balanced salt solution, and the medium was changed to calcein-free medium containing 1 mmol/L of cobalt chloride. For staining of mitochondria and nucleus, we used the 200 µmol/L MitoTracker Red CMXRos and 1 µmol/L Hoechst 33342 dye for 5 minutes. Fluorescence of calcein-AM was recorded by confocal microscopy (LSM 710 META, Carl Zeiss, Peabody, MA), and the calcein-stained area overlapped with MitoTraker red-stained area was used as an index of mitochondria with closed mPTP.

In Vivo Model

Animal Care

Male Sprague-Dawley rats (age, 8 weeks; weight, 270–360 g) were used (Figures 5A and 6A; online-only Data Supplement). All animal experiments were approved from the Biomedical Research Institute of Seoul National University Hospital and complied with the guide for the care and use of laboratory animals of the Institutional Animal Care and Use Committee.

Myocardial I/R Injury Model

The experimental myocardial I/R model was induced by temporary ligation of the left anterior descending coronary artery for 45 minutes, followed by releasing the ligature. After adequate anesthesia, the rats were intubated and ventilated with a mixture of 100% oxygen and room air (Harvard Apparatus Inc, Holliston, MA). Rats were randomly assigned to 6 groups: sham operation group, control group (0.5 mL of 5% dextrose), CsA-treated group (5 mg/kg diluted in 0.5 mL of 5% dextrose, intraperitoneally), NecX-treated group (1 mg/kg diluted in 0.5 mL of 5% dextrose, intravenously), vitamin C–treated group (50 mg/kg diluted in 0.5 mL of 5% dextrose, intraperitoneally), and a combination of vitamin C and vitamin E (50 mg/kg of vitamin C and 25 mg/kg of Trolox, intraperitoneally). Each drug was administered 5 minutes before reperfusion, considering the irreversible feature of cell death and the clinical feasibility.[15–17] Dosages of NecX, CsA, vitamin C, and a combination of vitamin C and vitamin E were selected to achieve physiological relevance based on in vitro and in vivo data.

In rats that underwent measurement of the necrotic area, anti–myosin heavy chain (MHC) antibody (Cat No. 3–48; Abcam, Cambridge,
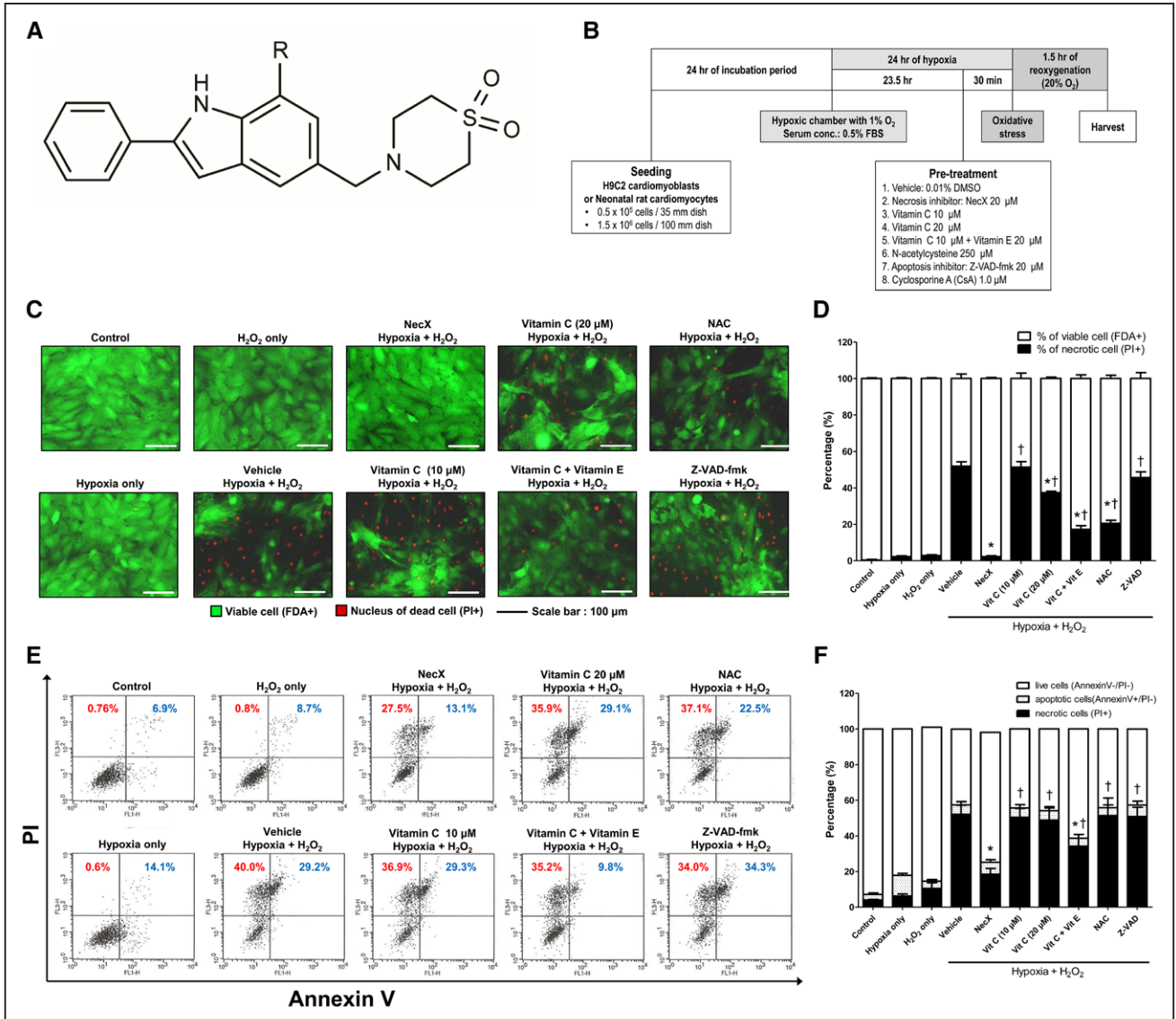
Figure 1. Protection of cardiomyocytes from necrosis after hypoxia–reoxygenation stress by NecX. **A**, Chemical structure of the novel necrosis inhibitor, 7-amino-indole (NecroX-7; NecX). **B**, Experimental protocol. **C**, Representative figures of H9C2 rat cardiomyoblasts stained with fluorescein diacetate (FDA)/propidium iodide (PI). **D**, Percent of dead cells by FDA/PI staining. **E**, Contour plots of annexin-V vs PI, showing necrotic cells in red color (annexin-V/PI –/+; upper left) and apoptotic cells in blue color (annexin-V/PI +/+; upper right) among H9C2 rat cardiomyoblasts. **F**, Percentages of viable, apoptotic, and necrotic cells calculated. *$P<0.05$ vs vehicle, and †$P<0.05$ vs NecX. Bars represent the mean±SEM from 3 independent experiments. NAC indicates N-acetylcysteine; NecX, necrosis inhibitor; Veh, vehicle; Vit C, vitamin C; and Vit E, vitamin E.

MA) was intravenously injected 30 minutes before left anterior descending coronary artery ligation. If sarcolemmal structures are damaged by I/R injury, anti-MHC antibody binds to the exposed myosin, enabling measurement of the necrotic area. At 12 hours after I/R injury, 30 rats (n=5 per group) were euthanized to measure the areas of myocardial necrosis. The investigators were blinded with respect to the treatment.

We also performed another set of in vivo experiments, following the rats until 14th days after the induction of myocardial I/R injury for assessment of myocardial fibrosis and hemodynamic parameters. In the 14-day follow-up experiment, rats were randomly assigned to 3 groups, which were blinded to the investigators before the measurements: control group, CsA-treated group, and NecX-treated group. The NecX-treated group was fed 30 mg/kg of oral NecX once a day for 3 consecutive days after I/R injury. Other groups were fed the same dose of water per os. Serial echocardiographic assessment was performed before LAD ligation, 3, 7, and 14 days after I/R injury (n=10 per group), and fibrotic areas were measured 14 days after I/R injury on Masson trichrome–stained slides (n=5 per group).

Statistical Analysis

All data were presented as means±SEM. Kruskal–Wallis test, Mann–Whitney test, or repeated-measures ANOVA was used when appropriate. Values of $P<0.05$ were considered significant, and all analyses were performed with SPSS version 20.0 (Chicago, IL).

Results

Necrosis After Hypoxia–Reoxygenation Stress Was Prevented by NecX

To understand the nature of cell death after myocardial I/R injury, we stained rat H9C2 cardiomyoblasts and neonatal cardiomyocytes with fluorescein diacetate and propidium iodide after exposure to hypoxia–reoxygenation stress (Figure 1; Figure S3). Dead cells were rarely observed after hypoxia or reoxygenation stress alone whereas were abundantly observed

after exposure to the sequence of hypoxia–reoxygenation stress in the vehicle-treated cells. Apoptosis inhibitor (Z-VAD-fmk) and other antioxidants suppressed hypoxia–reoxygenation stress but to a lesser degree than did necrosis inhibitor. NecX was the most effective drug against hypoxia–reoxygenation stress. Using flow cytometry analysis of annexin-V/PI staining, we compared the proportions of necrotic (annexin-V/ PI −/+) versus apoptotic cells (annexin-V/PI +/+; Figure 1E and 1F). In the NecX group, 14.5%±1.9% of total cells were necrotic, whereas 40.2%±7.8% were necrotic in the vehicle group ($P<0.01$). Other groups did not show a cytoprotective effect against necrosis. Apoptotic fractions did not significantly differ among the groups after hypoxia–reoxygenation stress. In neonatal rat cardiomyocytes, NecX had the same significant protective effect: the NecX-treated primary cardiomyocytes were protected from hypoxia–reoxygenation stress, but other treated groups were not (Figure S3).

We performed Western blots on H9C2 rat cardiomyoblasts, for apoptotic (89 kDa) or necrotic (55 kDa) fragments of cleaved-PARP1 (poly[ADP-ribose] polymerase 1; Figure 2A). The apoptotic fragment was significantly induced by staurosporine treatment but not by hypoxia–reoxygenation stress. In contrast, necrotic fragment of cleaved PARP1 was significantly induced by hypoxia–reoxygenation stress, which was markedly prevented by NecX. Another indicator of apoptosis, caspase-3, was assessed using Western blot and luminescence assay (Figure 2B and 2C). The expression of cleaved caspase-3 which indicates the activation of apoptotic pathway was noted in cells treated with staurosporine (apoptosis inducer) and was reduced by Z-VAD-fmk (apoptosis inhibitor; Figure 2B; Figure S4A). The caspase-3 activity by luminescence assay was also markedly reduced in the cells treated with Z-VAD-fmk (Figure 2C). The NecX-treated cells inhibited the cleavage of caspase-3 and showed a tendency for reduced caspase-3 activity.

We also performed Western blots for RIP1 (receptor-interacting protein 1) and RIP3, the molecular switches of cell death between necrosis and apoptosis. Hypoxia–reoxygenation stress in vehicle-treated cells significantly increased the levels of RIP1 and RIP3 (Figure 2D; Figure S4B), which was reduced by NecX. Specifically, RIP3 that is required for necrosis but does not affect RIP1-mediated apoptosis was significantly reduced by NecX than by vitamin C or Z-VAD-fmk.

The p38 MAPK (mitogen-activated protein kinase) pathway, known to be activated by extracellular stress and regulated by mitochondrial ROS, is important in many inflammatory diseases, such as myocardial I/R injury. The increased ratio of phospho-p38 MAPK/total p38 by hypoxia–reoxygenation stress was prevented by NecX treatment but not by vitamin C or Z-VAD-fmk (Figure 2D; Figure S4C). The ratio of phospho-c-Jun N-terminal kinase/total Jun N-terminal kinase was also significantly lower in the NecX-treated cells (Figure 2D; Figure S4D).

Novel Necrosis Inhibitor Was a Potent Mitochondrial ROS Scavenger

Given the ROS scavenging activity of NecX by DPPH assay (2,2-diphenyl-1-picrylhydrazyl; Table S1), we compared the mitochondrial ROS scavenging activity of NecX with various antioxidants in H9C2 cardiomyoblasts exposed to

hypoxia–reoxygenation stress, using dihydrorhodamine 123 assay (Figure 3A and 3B). NecX was the strongest one among antioxidants, such as vitamin C, vitamin E, their combination, and N-acetylcysteine. Fluorescence intensity of rhodamine 123, the oxidized form of dihydrorhodamine 123 by mitochondrial ROS, was significantly lower in the NecX-treated cells. DCF-DA assay (2',7'-dichlorofluorescein diacetate) also showed that the cells pre-treated with NecX had the lowest intracellular ROS (Figure S5).

Necrosis Inhibitor Protected Mitochondria Against Hypoxia–Reoxygenation Stress

Compared with the vehicle-treated H9C2 rat cardiomyoblasts, the NecX-treated cells showed significantly less mitochondrial swelling (Figures S6). Transmission electron microscopy revealed that NecX prevented swelling of mitochondria and preserved the ultrastructure of cells. Vehicle-treated cells had swollen and ruptured mitochondria with degenerated crista and electron dense bodies and intracytoplasmic vacuoles, which are typical features of necrosis. In the same manner, NecX inhibited mitochondrial swelling, and thus prevented cell death, in neonatal rat cardiomyocytes exposed to hypoxia–reoxygenation stress (Figure S7).

To detect the dissipation of mitochondrial membrane potential ($\Delta\psi_m$) with the morphological change of mitochondria in H9C2 cardiomyoblasts, we used TMRE fluorescent probes with MitoTracker (Figure 3C). Pre-treatment with NecX effectively preserved high $\Delta\psi_m$, orange fluorescence, with normal elongated shape of mitochondria, whereas other treated groups showed necrotic cells with low $\Delta\psi_m$ and globular-shaped mitochondria. Flow cytometry analysis confirmed the preserved high $\Delta\psi_m$ in the NecX-treated group (Figures 3D; Figure S8A). The dissipation of mitochondrial membrane potential ($\Delta\psi_m$) was also assessed with JC-1 fluorescent probes in neonatal rat cardiomyocytes (Figure 3E). Necrotic cells with low $\Delta\psi_m$ (JC-1 monomer, green) and globular-shaped mitochondria were observed in vehicle-treated cells. Cardiomyocytes pre-treated with vitamin C or Z-VAD-fmk showed similar dissipation of $\Delta\psi_m$. However, pre-treatment with NecX significantly preserved high $\Delta\psi_m$, orange fluorescence. Flow cytometry analysis confirmed the markedly preserved high $\Delta\psi_m$ in the NecX-treated group compared with the other groups (Figure 3E; Figure S8B).

Necrosis Inhibitor Inhibited mPTP Opening and Blocked Ca²⁺ Influx

The opening of mPTP in H9C2 rat cardiomyoblasts was directly assessed using calcein-AM assay (Figure 4A and 4B). After the sequence of hypoxia and reoxygenation stress, the green fluorescence of calcein was maintained in a significantly higher level in the NecX-treated cells than the other treated cells, indicating that NecX inhibited the opening of mPTP (Figure 4A). The elongated shape of mitochondria was noted in NecX-treated cells, as colocalized with red MitoTracker and green calcein (yellow fluorescence in the fourth column), suggesting the closed mPTP. However, the vehicle group rarely showed the colocalized signals of green calcein and red mitochondria, suggesting
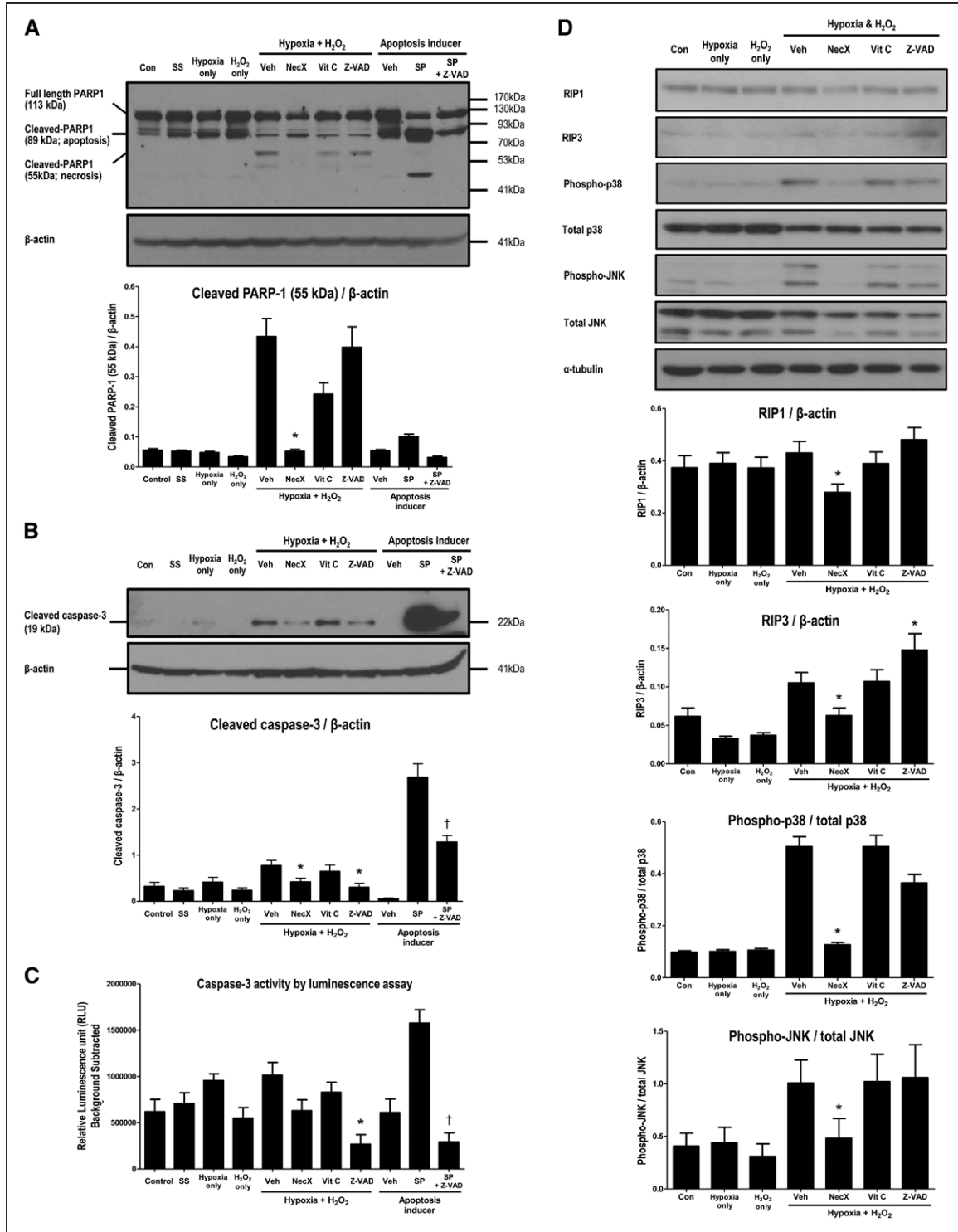
Figure 2. Activation of necrosis signaling pathways after hypoxia–reoxygenation stress in H9C2 rat cardiomyoblasts. **A**, Two cleaved forms of PARP1 (poly[ADP-ribose] polymerase 1); apoptotic fragment of cleaved PARP1 (89 kDa) and necrotic fragment of cleaved PARP1 (55 kDa). Activation of apoptosis pathways was assessed by (**B**) Western blot of cleaved caspase-3 and (**C**) the luminescence assay for caspase-3 activity. **D**, Western blot analyses of key molecules involved in necrosis; RIP1 (receptor-interacting protein 1), RIP3, phospho-p38 MAPK (mitogen-activated protein kinase), total p38 MAPK, phospho-JNK (Jun N-terminal kinase), and total JNK. *$P<0.05$ vs vehicle, and †$P<0.05$ vs SP (staurosporine; apoptosis inducer). Bar represents the mean±SEM from 4 independent experiments. Con indicates control; SP, staurosporine; and SS, serum starvation.

the opening of mPTP. And other groups could not inhibit the opening of mPTP when compared with the NecX group. The status of mPTP was quantitatively evaluated: the calcein fluorescence was maintained higher, suggesting the closed mPTP, in NecX-treated cells than other treated cells (Figure 4B).
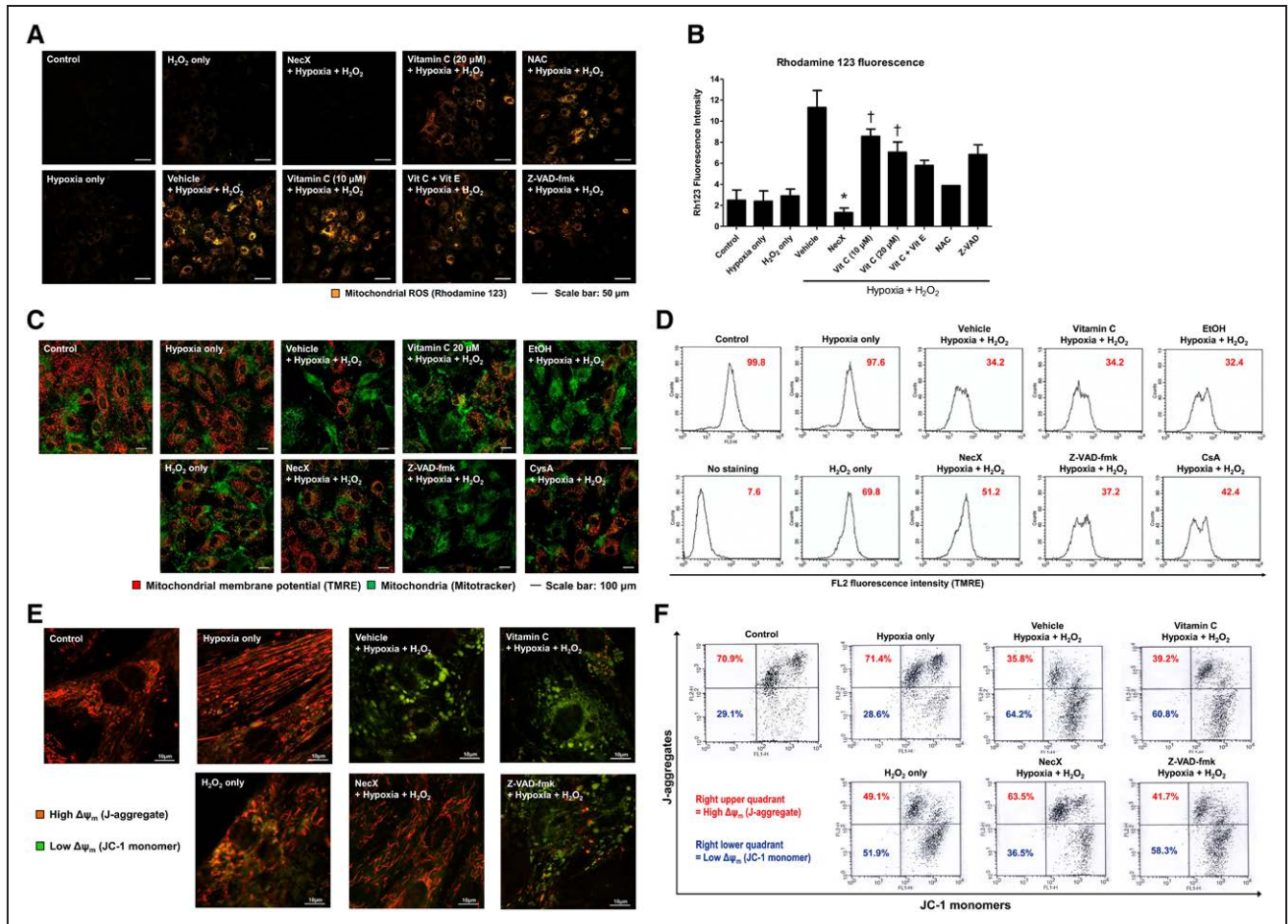
Figure 3. Mitochondrial effect of the necrosis inhibitor. **A** and **B**, Mitochondria-specific reactive oxygen species (orange fluorescence) was measured in H9C2 rat cardiomyoblasts under hypoxia–reoxygenation stress using dihydrorhodamine 123 assay. **C**, Mitochondrial membrane potential ($\Delta\psi_m$) was measured using tetramethylrhodamine ethyl ester (TMRE; orange fluorescence) probe. **D**, Flow cytometry histograms of TMRE fluorescence. **E**, Mitochondrial membrane potential ($\Delta\psi_m$) was measured in neonatal rat cardiomyocytes. The cells pre-treated with NecX showed significantly preserved high $\Delta\psi_m$ (orange fluorescence), whereas vehicle, vitamin C, or apoptosis inhibitor demonstrated low $\Delta\psi_m$. **F**, Flow cytometry dot plots of JC-1 monomers (low $\Delta\psi_m$; right lower) vs J-aggregates (high $\Delta\psi_m$; right upper). The NecX-treated cells demonstrated higher mitochondrial membrane potentials while vehicle, vitamin C, and Z-VAD-fmk did not maintain normal mitochondrial membranes. *$P<0.05$ vs vehicle, and †$P<0.05$ vs NecX. Bars represent the mean±SEM from 3 independent experiments.

Mitochondrial ROS generation contributes to the disruption of membrane potential, opening of mPTP, and Ca^{2+} influx into mitochondria. We compared the influx of Ca^{2+} into mitochondrial level (Figure 4C). The vehicle-treated cells showed a dynamic increase in Ca^{2+} level (Rhod-2 fluorescence) after hypoxia–reoxygenation stress along with mitochondrial swelling, indicating the opening of mPTP. The increase in Ca^{2+} level began in mitochondria, showing colocalization of green (mitochondria) and red (Ca^{2+}) fluorescence. Over time, Ca^{2+} levels increased in mitochondria as well as cytoplasm and nucleus, leading to necrosis. In contrast, increases in Ca^{2+} influx and changes in mitochondrial shape were minimal in the NecX-treated cells, even 90 minutes after reoxygenation. Reorganized movie files are provided in online-only Data Supplement(Movies S1 and S2).

Necrosis Inhibitor Reduced Infarct Size and Preserved Heart Function In Vivo

Cardioprotective effect of NecX was compared with those of CsA, vitamin C, and a combination of vitamin C and E in rat I/R injury models (Figure 5; Table S2). Necrotic areas were visualized by the use of anti-MHC antibody that bounds to MHC in the necrotic cardiomyocytes. We administered anti-MHC antibody systemically into rats before euthanize for the antibody to interact with MHC in the cytoplasm of necrotic cardiomyocytes whose cell membrane was disrupted and permitted antibody to access to cytoplasmic MHC. After euthanasia, we performed immunofluorescent staining to detect the anti-MHC antibody that interacted with MHC in necrotic cardiomyocytes. At 12 hours after myocardial I/R injury, necrotic area was the largest in the control group (17.7%±3.9%; Figure 5B and 5D). The cardioprotective effects of CsA and antioxidants were less than that of NecX (CsA, 11.5%±2.5%; vitamin C, 15.6%±1.4%; a combination of vitamin C and vitamin E, 15.9%±0.7%; versus NecX, 7.3%±2.8%). Also, the percentage of necrotic cardiomyocytes under confocal microscopy was lowest in the NecX group and lower in the CsA group than in the control or antioxidant groups (Figure 5C and 5E).

Serum concentrations of inflammatory cytokines 12 hours after I/R injury were higher in the control group but comparable
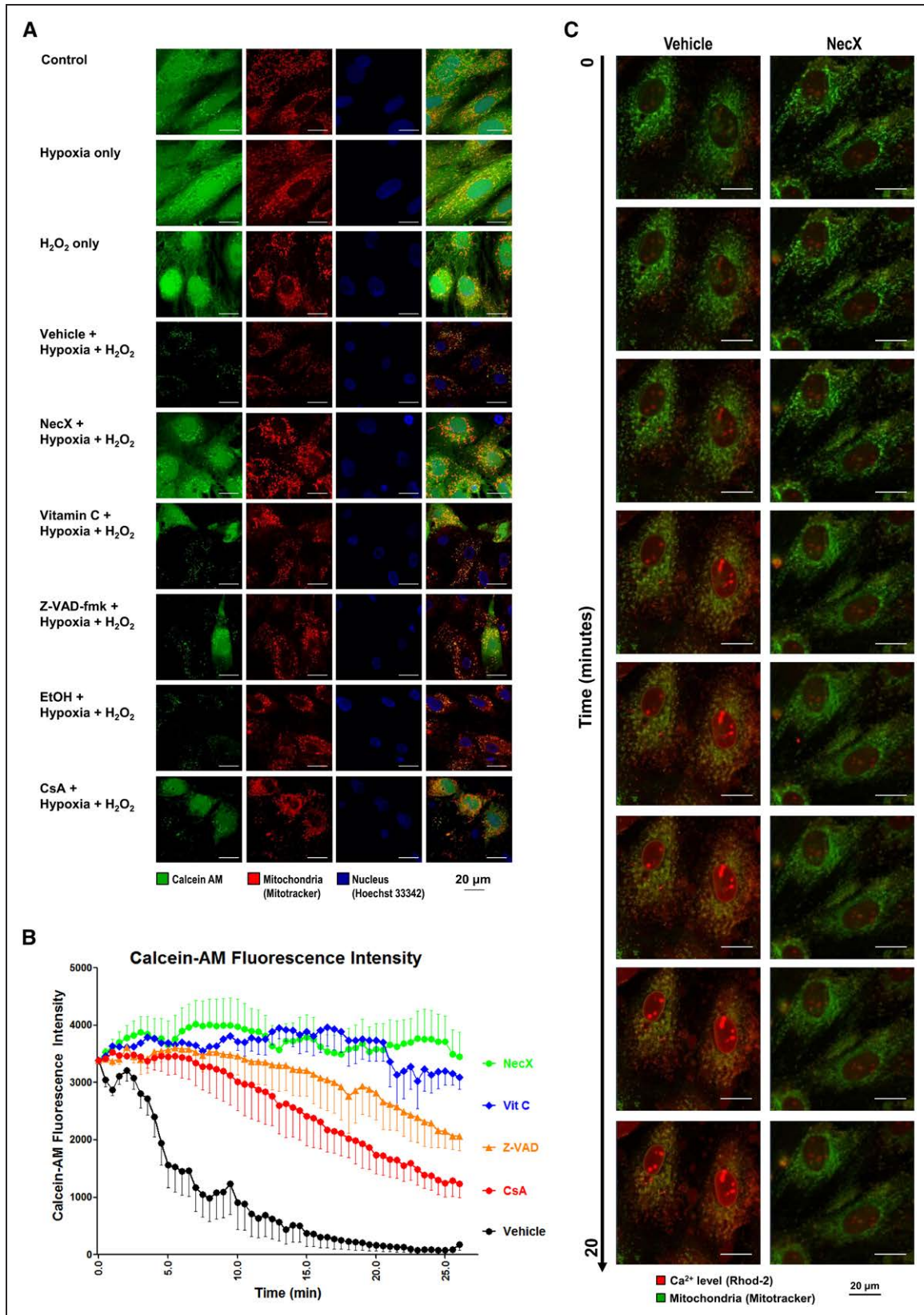
Figure 4. Inhibition of mitochondrial permeability transition pore (mPTP) opening and prevention of calcium influx into mitochondria. **A,** The opening of mPTP was directly assessed by using calcein-AM (green fluorescence) with addition of cobalt chloride for mitochondrial localization of calcein-AM fluorescence. The cells were costained with MitoTracker (red fluorescence) and Hoechst 33342 (blue fluorescence) to demonstrate the mitochondria and nucleus, respectively. **B,** Time courses of changes in calcein-AM fluorescence intensity. Bars represent the mean±SEM. **C,** Two vertical columns depict the changes of mitochondrial Ca^{2+} level (Rhod-2 fluorescence; red) and mitochondrial shapes (MitoTracker; green) according to time course. CsA indicates cyclosporine A.
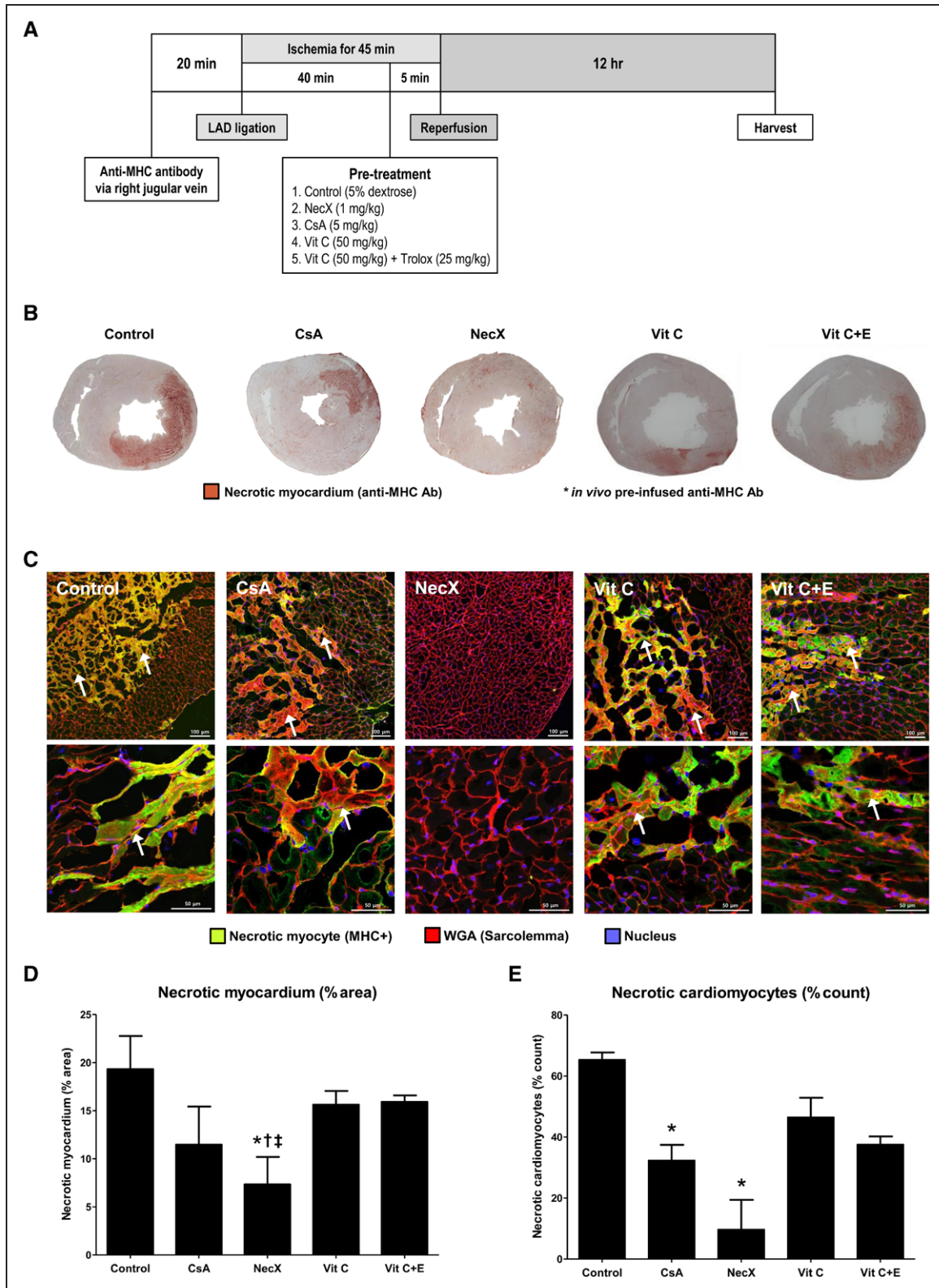
Figure 5. Myocardial necrosis after ischemia–reperfusion injury. **A**, Experimental protocol. **B**, Heart tissue sections showing necrotic zones stained in dark-brown color by the use of anti–myosin heavy chain (MHC) antibody. **C**, Results from immunohistochemistry of heart tissue sections showing necrotic cardiomyocytes in green fluorescence and intact cardiomyocytes in red fluorescence (staining the sarcolemma) and blue fluorescence (staining nucleus). **D**, Necrotic areas expressed as the percent of total left ventricular areas. **E**, Necrotic cardiomyocytes expressed as the percent of total myocytes. *$P<0.05$ vs control, †$P<0.05$ vs Vit C, and ‡$P<0.05$ vs Vit C+E; n=5 per group. Bars represent the mean±SEM. CsA indicates cyclosporine A; and Vit, vitamin.

between CsA and NecX groups, except for tumor necrosis factor-α that was significantly lower in the NecX-treated group (Figure S9). Releases of cardiac enzymes, such as lactate dehydrogenase, creatine kinase, troponin T, and troponin I, were also significantly lower in the NecX group. Furthermore, tissue expressions of HMGB1 (high mobility group box 1) were attenuated in the NecX-treated group (Figure S10).

We also evaluated the protein expression of signaling molecules involved in necrosis using Western blots of myocardial tissues obtained 12 hours after I/R injury (Figure S11). The levels of RIP1, RIP3, phospho-p38 MAPK, and phospho-Jun N-terminal kinase were lower in myocardial tissues from the NecX group than those from control or antioxidant groups. Levels of phospho-p38 MAPK were lower in NecX group and CsA group than the other groups, whereas the levels of cleaved caspase-3 were similar between the rat myocardium tissues from NecX, CsA, vitamin C, and a combination of vitamin C and E groups.

At 14 days after myocardial I/R injury, we observed significant differences in myocardial fibrosis among the 3 groups; NecX-treated group had a less myocardial fibrosis than did the CsA-treated group (Figure 6B–6D; Table S3). NecX preserved left ventricular systolic function and prevented pathological dilatory remodeling (Figure 6E and 6F; Table S4).

Discussion

In this study, we investigated the protective effect of NecX, a novel inhibitor of necrosis, from myocardial I/R injury using both in vitro and in vivo models. In H9C2 rat cardiomyoblasts and rat cardiomyocytes exposed to hypoxia–reoxygenation stress, which physiologically mimicked I/R injury to myocardium, the necrotic cell death was mainly inhibited by NecX. This protective effect of NecX was based on the inhibition of mPTP opening through its potent scavenging activity on mitochondrial ROS. Rats pre-treated with NecX had significantly smaller areas of myocardial necrosis and fibrosis after I/R injury than those pre-treated with CsA or antioxidants. NecX also preserved cardiac function and geometry. These findings strongly suggest the cardioprotective effect of NecX from I/R injury.

Novel Necrosis Inhibitor

NecX is a novel class of necrosis inhibitor that has strong ROS scavenging activity: NecX prevented acetaminophen-induced hepatotoxicity[10] and improved hepatic steatosis and fibrosis[18] through the inhibition of mitochondrial ROS formation.[9] Therapeutic effect of NecX as a ROS scavenger results in the attenuation of HMGB1 and the inhibition of a wide range of inflammatory responses, as demonstrated in models of renal I/R injury,[8] hepatic I/R injury,[19] and graft-versus-host disease.[11] Another derivative of NecX inhibited mitochondrial Ca^{2+} uniporter and protected mitochondria against hypoxia–reoxygenation stress.[12]

Based on these findings, NecX is increasingly recognized as a potent necrosis inhibitor through the mitochondria-targeting antioxidant activity and has been expected to be effective in various clinical conditions where necrosis plays an important role in pathogenesis.[7,8,20] Considering the discoveries that mitochondrial ROS is a critical early driver of mPTP opening and the resultant myocardial I/R injury,[1,7] we hypothesized that NecX would prevent myocardial I/R injury.

NecX Prevented I/R Injury Through Inhibition of Necrosis

Both apoptosis and necrosis are involved the pathophysiology of myocardial I/R injury, but more evidences support necrosis as the main mechanism.[3,21,22] In the present study, pre-treatment with NecX significantly reduced necrosis under in vitro and in vivo models of myocardial I/R injury. PARP1 is a 113 kDa nuclear enzyme that is cleaved into fragments of 89 and 24 kDa during apoptosis whereas into a 55 kDa fragment during necrosis.[23] In this study, 89 kDa of cleaved PARP1 was increased only in cells treated with staurosporine. In contrast, H9C2 cells exposed to hypoxia–reoxygenation stress produced high expression level of 55 kDa fragments of PARP1 indicative of necrosis, whereas NecX-treated cells expressed significantly lower level of the necrotic fragment.

Caspase-3, which is a critical executer of apoptosis,[24] was markedly activated with the apoptosis inducer staurosporine, but its activation was not prominent under hypoxia–reoxygenation stress, suggesting that hypoxia–reoxygenation stress mainly caused necrosis rather than apoptosis in H9C2 cells. Western blot analysis and the luminescence assay showed that the caspase-3 activity was inhibited with Z-VAD-fmk (apoptosis inhibitor) as well as with NecX treatment. In the in vivo experiment, the levels of cleaved caspase-3 significantly decreased in treatment groups compared with that of control group, but no significant differences were observed between the treatment groups. Considering the consistent inhibitory effect of necrosis by NecX in our study, the decreased level of cleaved caspase-3 in NecX-treated H9C2 cells indicates that necrosis and apoptosis have interactions in reperfused myocardium, and the treatment with NecX could provide overall cardioprotective effects against I/R injury through the potent inhibition of necrosis.[22]

The inhibition of necrosis by NecX was also revealed by the Western blot analyses of RIP1 and RIP3 for H9C2 rat cardiomyoblasts as well as rat myocardium. RIP3 is required for necrosis but does not affect RIP1-mediated apoptosis.[25] Although the levels of RIP1 and RIP3 increased in the control group, NecX-treated group demonstrated significant decrease in RIP3 expression. Thus, we concluded that the cytoprotective effect of NecX originated from inhibition of necrosis, not apoptosis.

Role of mPTP During Myocardial I/R Injury

Mitochondrial dysfunction by mPTP opening plays a pivotal role during the myocardial I/R injury.[1,4] The inner mitochondrial membrane is impermeable under normal physiological conditions. During myocardial ischemia, mPTP remains closed, but the cardiomyocyte undergoes several changes. The lack of oxygen inhibits oxidative phosphorylation, resulting in the depletion of ATP and cellular acidification, all of which contribute to the cytosolic Ca^{2+} overload. However, mitochondrial Ca^{2+} intake remains minimal because the mPTP is closed and also the $\Delta\psi_m$ is low. At myocardial reperfusion, re-energized electron transport chain in the mitochondria with re-established $\Delta\psi_m$ abruptly generates ROS, which contributes to the opening of mPTP and Ca^{2+} overload. Also with the rapid normalization of pH, these changes lead to the dissipation in $\Delta\psi_m$, swelling of mitochondria, and finally, cardiomyocyte deaths. Thus, myocardial reperfusion, which is necessary to protect ischemic myocardium from irreversible damage,
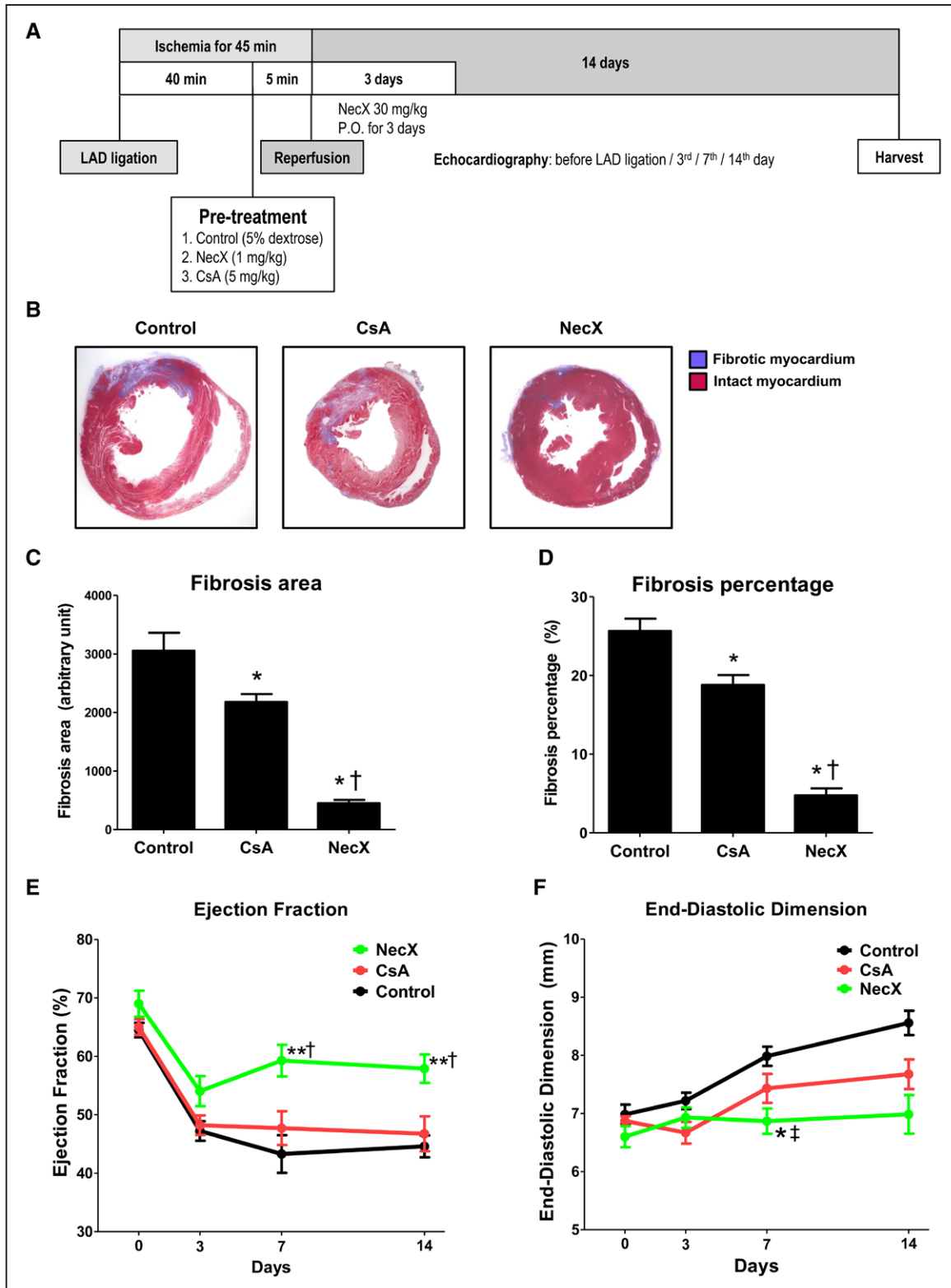
Figure 6. Myocardial fibrosis and echocardiographic parameters after ischemia–reperfusion (I/R) injury. **A**, Experimental protocol. **B**, Representative Masson trichrome–stained slides of the myocardium sections from each treated group obtained at 14 d after myocardial I/R injury. **C** and **D**, Comparison of the areas of fibrosis and the percentages of fibrotic myocardium to the total myocardium. *$P<0.05$ vs control, and †$P<0.05$ vs cyclosporine A (CsA); n=5 per group. **E** and **F**, Echocardiographic parameters including left ventricular ejection fraction and end-diastolic dimension. *$P<0.05$ vs control, and †$P<0.05$ vs CsA; n=10 per group. Bars represent the mean±SEM. LAD indicates left anterior descending coronary artery.

complicates the biochemical and metabolic changes generated during ischemia and paradoxically results in I/R injury.

Mechanism of Cardioprotection by the Novel Necrosis Inhibitor

As reported in the previous studies, NecX exerts ROS scavenging activity, attenuation of HMGB1 release, and suppression of inflammatory reaction.[7,8,11,12,18,26] Adding to these, our findings demonstrated that NecX has a potent cardioprotective effect through the inhibition of mPTP opening and preservation of $\Delta\psi_m$, based on its potent scavenging activity on mitochondrial ROS. These findings support the therapeutic potential of NecX as a mitochondria-targeting antioxidant and suggest that NecX can be a cardioprotective adjunctive measure in myocardial I/R injury.[20]

Cardiomyocyte damage during reperfusion is largely because of the generation of ROS.[1,3] The increase in ROS causes necrosis through the opening of mPTP and activation of necrotic pathways.[4,27,28] We demonstrated that the cytoprotective effects of NecX originated from the direct scavenging of mitochondrial ROS, which plays a key role in myocardial I/R injury (Figure S12).[29] The potent ROS scavenging activity of NecX inhibited the opening of mPTP and prevented the mitochondrial Ca^{2+} influx, thus inhibited mitochondrial swelling and maintained $\Delta\psi_m$, as shown in our results that NecX pre-treatment preserved calcein-AM fluorescence, reduced the increase in Rhod-2 fluorescence, preserved TMRE fluorescence, and maintained J-aggregate fluorescence, respectively. All of these finally led to the inhibition of necrosis against myocardial I/R injury. Our findings are concordant with the previous reports and confirmed the hypothesis that NecX would prevent myocardial I/R injury through inhibition of the mPTP opening.[9,12]

The p38 MAPK pathway, known to be activated by mitochondrial ROS, integrates extracellular stress, participates in various cellular responses including cytokine production, and plays an important role in myocardial I/R injury.[27,28] Inhibition of p38 MAPK blocks the production of inflammatory cytokines and thus attenuates I/R injury to myocardium.[27] We found a significantly reduced level of phospho-p38 MAPK by NecX pre-treatment both in vitro and in vivo myocardial I/R models. The level of phospho-Jun N-terminal kinase, which is activated by mitochondrial ROS, was also reduced in the NecX-treated H9C2 cells and rat myocardium.[28] The inhibition of the activations of MAPK pathways by NecX would be another evidence of cardioprotection by NecX against I/R injury.

HMGB1, an early mediator of I/R injury, is secreted from necrotic cells and activates MAPK pathways, which then signals the induction of inflammatory cytokines and stimulates necrosis in the reperfused myocardium.[30] NecX-treated rats showed relatively lower serum level of HMGB1 with dramatically lower tissue level. Moreover, cardiac enzymes revealed obvious differences between the 3 groups; the NecX-treated group had the lowest levels.

Clinical Implications

The lack of effective therapeutic strategies for myocardial I/R injury led to the introduction of many compounds.[1,2,7] Despite their effectiveness in animal models, most of the compounds failed to show efficacy in humans because of the low potency of the compounds, inadequate study design, and comorbidities or medications of patients. Considering the pathophysiology of myocardial I/R injury that occurs immediately after revascularization of ischemic myocardium, the expected effect of potential cardioprotective agents would be maximized when administered before or at the time of revascularization procedures. In this study, we compared the cardioprotective effects of NecX with other antioxidants and CsA, the suggested drug of choice for prevention of myocardial I/R injury, using in vitro and in vivo models where the study compounds were pre-treated to cardiomyocytes under ischemic condition, before reperfusion. Therefore, the results of our study strongly support the cardioprotective effect of NecX against I/R injury and also provide a translational rationale for the use of NecX in real-world practice. In addition to the consistent evidence that NecX scavenges the mitochondrial ROS and inhibits the mPTP opening, we expect NecX will have a marked cardioprotective effect against I/R injury in humans for the following reasons.

First, NecX demonstrated a potent inhibition of mPTP opening through mitochondrial ROS scavenging activity and therefore, reduced myocardial necrosis and fibrosis after I/R injury, preserved cardiac functions, and prevented dilatory remodeling. Consistent results of our study provide solid evidence for the cardioprotective effect of NecX. Second, the superiority of NecX over CsA should be emphasized. As an inhibitor of mPTP opening, CsA has been suggested to prevent I/R injury.[21,31] A pilot trial by Piot et al[17] showed a promising result of 40% reduction in infarct size. However, the statistical power was limited because of its small sample size, and more importantly, the administration of CsA failed to reduce the infarct size in patients with ST-segment–elevation MI undergoing thrombolysis[8] and those undergoing percutaneous coronary intervention as reported in the CIRCUS trial and the CYCLE trial.[5,6] Because of those disappointing results from the recent trials, the attention that previously focused on CsA has shifted to novel agents against myocardial I/R injury.[32] In our study, CsA protected about one third of reperfused myocardium, similar to the results of previous studies.[31] However, NecX protected >60% of reperfused myocardium, nearly twice the CsA effect. It seems attributable to a greater inhibition of mPTP opening by NecX than by CsA through its potent ROS scavenging activity with high affinity toward mitochondria. Taken together, NecX would have advantages over CsA against I/R injury because of its more pronounced inhibition of cardiomyocyte necrosis. Third, based on the consistent results from our in vitro and in vivo experiments as well as the favorable safety profile with reliable pharmacokinetics of NecX in a phase I clinical trial,[33] a phase II trial for patients with ST-segment–elevation MI (NexSTEMI trial, NecroX to reduced reperfusion injury after emergent percutaneous coronary intervention in patients with STEMI) was launched in 2014 after approval of the Investigational New Drug application by Korean Food and Drug Administration (NCT02070471).

Limitations

This study is subject to the following limitations. First, we applied H_2O_2 instead of the normoxic condition for

reoxygenation process. Some previous studies used hypoxia–reoxygenation without adding H_2O_2 to simulate I/R injury. But, the use of H_2O_2 is a relevant and widely used method.[13,14] Given that ROS is responsible for the lethal I/R injury in cardiomyocytes, the addition of H_2O_2 emphasized the cardioprotective effect of NecX. Second, we administered NecX to the in vivo myocardial I/R model 5 minutes before reperfusion but did not test the drug's effect when given simultaneously with reperfusion or later, which is an important issue for clinical translation. Considering the action mechanism of NecX which maintained mitochondrial integrity and thus prevented necrosis, its administration before reperfusion is appropriate and necessary to prevent the irreversible process of necrosis. Trials of another mPTP opening inhibitor also used the prereperfusion administration, for example, CsA and the other agents such as exenatide and metoprolol.[8,16,17] Third, we did not perform in vivo experiments in disease-specific models, such as rats with diabetes mellitus, hypertension, or metabolic syndrome. Given the prevalent cardiovascular risk factors in patients with ST-segment–elevation MI and the potential attenuation of cardioprotective effect by the presence of comorbidities, it would be of importance to investigate whether NecX could exert cardioprotective effects in disease-specific in vivo models.[10,34] However, the main scope of our translational study was to demonstrate the cardioprotective effects of NecX against I/R injury, as well as the action mechanism of NecX. The investigation of potential differences in the effects of NecX among disease-specific models would be another research topic.

Conclusions

The novel necrosis inhibitor, NecX, protected cardiomyocytes from myocardial I/R injury. NecX prevented mitochondrial swelling, maintained $\Delta\psi_m$, and protected the cells from necrosis through the inhibition of mPTP opening by scavenging of mitochondrial ROS and inhibition of Ca^{2+} influx. Administration of NecX reduced myocardial necrosis against I/R injury to a greater extent than did CsA or antioxidants. NecX is a promising candidate for cardioprotective adjunctive therapy with reperfusion in patients with MI.

Perspectives

During the reperfusion of ischemic myocardium, the opening of mPTP by abrupt mitochondrial ROS generation and Ca^{2+} overload causes paradoxical damage to myocardium called myocardial I/R injury. We demonstrated in vivo that the novel necrosis inhibitor (NecX) markedly reduced myocardial I/R injury in comparison with CsA, vitamin C, and a combination of vitamin C and E, with definitive supporting in vitro evidence. The necrosis inhibitor significantly prevented necrotic cell death through the inhibition of mPTP opening. The potent scavenging activity on mitochondrial ROS by this necrosis inhibitor prevented mitochondrial swelling, maintained the mitochondrial membrane potential ($\Delta\psi_m$), and blocked the Ca^{2+} influx into mitochondria. In addition, rat myocardial I/R injury model showed that pre-treatment with the necrosis inhibitor resulted in a significantly smaller myocardial infarct size than CsA or the antioxidants. These findings strongly suggest that NecX is a promising candidate for cardioprotective adjunctive therapy.

Disclosures

S.-H. Kim is an employee of LG Chem that synthesized the necrosis inhibitor, NecroX-7 (NecX; patent number, KR2008-0080519). The other authors report no conflicts.

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Novelty and Significance

What Is New?

- We investigated the protective effect of a novel necrosis inhibitor, 7-amino-indole ($C_{25}H_{32}N_4O_4S_2$; NecX), against myocardial ischemia–reperfusion (I/R) injury.
- In in vitro I/R injury model, NecX maintained $\Delta\psi_m$, inhibited Ca^{2+} influx, and protected the cells from necrosis through the inhibition of mitochondrial permeability transition pore opening by scavenging of mitochondrial reactive oxygen species.
- Administration of NecX reduced myocardial necrosis against I/R injury to a greater extent than did cyclosporine A and antioxidants.

What Is Relevant?

- During reperfusion of myocardial infarction, a damaged myocardium experiences additional necrotic cell death and increase in infarct size, known as myocardial I/R injury.
- Although numerous approaches have attempted, none has successfully inhibited necrotic signaling pathway directly, and the myocardial I/R injury still remains as a neglected therapeutic target.
- Cardioprotective effect of NecX against I/R injury was substantially superior to that of cyclosporine A, the established inhibitor of mitochondrial permeability transition pore opening.

Summary

We demonstrated in vivo that NecX markedly reduced myocardial I/R injury in comparison with cyclosporine A and several antioxidants, with definitive supporting in vitro evidence. These findings suggest that NecX is a promising candidate for cardioprotective adjunctive measure on top of reperfusion therapy.