NecroX-5 prevents hypoxia/reoxygenation injury by inhibiting the mitochondrial calcium uniporter

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Aims
Preservation of mitochondrial function is essential to limit myocardial damage in ischaemic heart disease. We examined the protective effects and mechanism of a new compound, NecroX-5, on rat heart mitochondria in a hypoxia/reoxygenation (HR) model.

Methods and results
NecroX-5 reduced mitochondrial oxidative stress, prevented the collapse in mitochondrial membrane potential, improved mitochondrial oxygen consumption, and suppressed mitochondrial Ca^{2+} overload during reoxygenation in an in vitro rat heart HR model. Furthermore, NecroX-5 reduced the ouabain- or histamine-induced increase in mitochondrial Ca^{2+}.

Conclusion
These findings suggest that NecroX-5 may act as a mitochondrial Ca^{2+} uniporter inhibitor to protect cardiac mitochondria against HR damage.

Keywords
NecroX-5 • Mitochondria • Hypoxia/reoxygenation • Calcium uniporter

1. Introduction
The structure and biochemical functions of cardiac mitochondria are altered during ischaemia–reperfusion (IR).1,2 Impaired mitochondrial function after ischaemia are disastrous for the heart and are manifested as depressed respiratory chain complex activity;3 decreased NADH dehydrogenase activity;4 lower mitochondrial membrane potential (ΔΨm);5 loss of adenosine triphosphate (ATP) synthesis;6 increased ATP hydrolysis;6 and Ca^{2+} overload-induced mitochondrial permeability transition pore (mPTP) opening.7 Mitochondrial oxidative stress has been also reported to suppress tricarboxylic acid cycle (TCA) enzymes, modify mitochondrial proteins;5,8 trigger mPTP opening;9 induce mitochondrial malfunction;10 and promote the release of pro-apoptotic proteins;11 and subsequently leading to cell death via apoptosis or necrosis.12

In this context, mitochondria are a major target of IR injury.6 Thus, the preservation of mitochondrial function is essential to limit myocardial damage in ischaemic heart disease. Among the possible mechanisms of cardioprotection, ischaemic post-conditioning (IPost) is one of the most effective mechanisms for protecting mitochondrial function against IR damage. IPost during the early reperfusion period prevents the opening of mPTPs by changing the pH.13,14 IPost also attenuates mitochondrial Ca^{2+} accumulation15 and mitochondrial reactive oxygen species (ROS) generation during reoxygenation.16 Additionally, the activation of the mitochondrial ATP-sensitive potassium channel in IPost is thought to play a protective role in limiting reperfusion injury.17

Pharmacological approaches to studying mitochondrial regulation have been focused either clinically or experimentally. Recently, we have developed NecroX compounds, which are cell permeable necrosis inhibitors with antioxidant activity that localize mostly in the mitochondria.18 NecroX-5 is one of the derivatives of NecroX series compounds, whose chemical composition is C_{25}H_{31}N_{3}O_{3}S_{2}CH_{4}O_{3}S with molecular weight 453.61 (see Supplementary material online, Figure S1A). We found that the cytoprotective effect of NecroX-5 could be due to the mitochondrial ROS scavenging activity.18 NecroX-5 effectively inhibited the generation of oxidized low-density lipoprotein cholesterol, tertiary-butyl hydroperoxide-induced...
mitochondrial ROS generation, doxorubicin-induced cell death in different cell lines, and CCl4-induced hepatotoxicity in a rat model.18 NecroX-7, another derivative that is functionally and chemically very similar to NecroX-5, reduced hepatic necrosis to IR injury compared with that in the control group.19 Although the cellular protective effect of NecroX-5 on various in vitro and in vivo models was demonstrated, the mode of action of this compound on the cellular or mitochondrial protective mechanism is still unclear. Therefore, the aim of this study was to validate the effect of NecroX-5 in preventing myocardial infarction (MI) in rats with ischaemic heart disease and to provide support for the use of NecroX-5 as a medication against MI. Thus, we examined the protective effects and the mechanism of action of NecroX-5 on rat heart mitochondria in a hypoxia/reoxygenation (HR) model. We demonstrated that NecroX-5 post-hypoxic treatment improved mitochondrial function in the HR model and suppressed mitochondrial calcium overload. Additionally, we further investigated the possible mechanism of how NecroX-5 inhibits mitochondrial Ca2+ overload in HR. NecroX-5 showed a strong inhibitory effect on the mitochondrial Ca2+ uniporter (MCU) and this novel effect of NecroX-5 may result in preserving mitochondrial function and limiting infarct size in HR.

2. Methods

The details of each experiment are available in the Supplementary material online.

2.1 Ethics statement

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental procedures were reviewed and approved by the Institutional Review Board of Animals, Inje University College of Medicine. Procedures were performed according to the guidelines of the Institutional Review Board on the ethical use of animals.

2.2 Isolation of hearts and cardiac myocytes

Eight-week-old male Sprague–Dawley rats (weight, 200–250 g each) were deeply anaesthetized with sodium pentobarbital (100 mg/kg intraperitoneally).20 The adequacy of anaesthesia was monitored by heart rate, muscle relaxation, and the loss of sensory reflex response, i.e. non-response to tail pinching. Each heart was mounted and perfused with normal Tyrode’s (NT) solution equilibrated with 95% O2 and 5% CO2 at 37°C for 10 min in the Langendorf system to remove all blood. Individual hearts were used for testing infarction size, performing experimental protocols, and isolating myocytes and mitochondria. Single cardiac myocytes were isolated using an enzymatic dissociation method as described previously21 (see Supplementary material online, Methods).

2.3 NecroX-5 concentration and fluorescence

The optimal dose (10 μM) of NecroX-5 was determined in our previous study (see Supplementary material online, Figure S1B and Table S1).18 Before applying fluorescence-based analysis, we tested the fluorescence of NecroX-5 at the various wavelengths (510/580, 488/525, 488/535, and 550/590 nm) using fluorescence reader, SpectraMax M2 (Molecular Device, USA). The fluorescence intensities of NecroX-5 (10 μM) at all wavelengths were not significantly different from NT solution (see Supplementary material online, Figure S2A). In addition, NecroX-5 treatment itself did not altered cells’ autofluorescence in the absence of the fluorescent dye (see Supplementary material online, Figure S2B and C).

2.4 Cardiac and myocyte perfusion

In the HR experimental model, hearts or myocytes were sequentially perfused with NT solution for 5 min and then ischaemic solution containing 140 mM NaCl, 8 mM KCl, 0.5 mM MgCl2, 1.8 mM CaCl2, and 10.0 mM deoxyglucose, HEPES 5.0, pH 6.0 adjusted by NaOH and equilibrated with 100% N2 at 135–140 mmHg for 15 min, followed by perfusion for 40 min with NT solution, or with 10 μM NecroX-5 or 5 μM Ruthenium Red (RuR) in NT solution. Samples were also perfused with 10 μM NecroX-5, 1 mM ouabain, 5 μM RuR, or 100 μM histamine or were co-treated with RuR and NecroX-5.

2.5 Triphenyltetrazolium chloride staining

Infarct size was examined by using tissue triphenyltetrazolium chloride (TTC) staining as described in our previous study (see Supplementary material online, Methods).

2.6 Isolation of mitochondria

Cardiac mitochondria isolation was performed using the differential centrifugation method as described previously5,22 (see Supplementary material online, Methods).

2.7 Measurement of ROS production

Cells were incubated at 37°C with MitoSOX Red (5 μM; excitation/emission: 510/580 nm) to detect changes in mitochondrial O2 levels. After washing twice with the KBr-Brue solution, myocytes were placed in a perfusion chamber at room temperature. The fluorescence intensity was measured every 20 s by using a confocal microscope with LSM-510 META software. Regions of interest in the myocytes were selected to represent changes in fluorescence intensity over time, and the background was identified as an area without cells. MitoSOX Red intensity 2 min after exposure to NT solution expressed as a percentage of the baseline value. The fluorescence intensity was analysed as a percentage of baselines after subtraction of autofluorescence in the absence of the fluorescent dye.

ROS production in isolated mitochondria was also evaluated by flow cytometry as described previously23 (see Supplementary material online, Methods).

2.8 Measurement of mitochondrial membrane potential

To measure ΔΨm, myocytes were stained with 0.1 μM tetracyethylrhodamine ethyl ester (TMRE; excitation/emission: 564/580 nm) for 30 min at room temperature. Next, they were washed and placed in a perfusion chamber. Fluorescence was detected every 30 s by laser scanning confocal microscopy. Images were analysed using LSM-510 META software as described in the section for the measurement of ROS production (Carl Zeiss, Jena, Germany).

The ΔΨm in isolated mitochondria was also evaluated by flow cytometry, as changes in TMRE fluorescence at 580 nm (FL2). The mean values were calculated using CellQuest (version 5.2).

2.9 Oxygen consumption

Mitochondrial oxygen consumption was measured in a 600 μL air-saturated chamber at room temperature, using a fibre-optic oxygen electrode (Instech). The respiration medium consisted of 145 mM KCl, 30 mM HEPES, 5 mM KH2PO4, 3 mM MgCl2, 0.1 mM EGTA, and 0.1% bovine serum albumin at pH 7.4. Mitochondrial state 4 was initiated with the addition of 5 mM glutamate/malate as a substrate. After steady state, state 3 respiration was stimulated by adding 0.5 mM adenosine diphosphate (ADP) to the medium. Oxygen consumption is expressed as nmol O2/min/mg mitochondrial protein. The respiratory control index (RCI) is the ratio of ADP-stimulated state 3 respiration to state 4 respiration.
2.10 Measurement of mitochondrial calcium
Changes in mitochondrial calcium levels were detected with Rhod-2 AM (5 μM; excitation/emission: 533/576 nm). Cells were incubated with Rhod-2 AM using a cool–warm method and washed twice with the Kraft-Brühe solution. The fluorescence intensity was measured using a confocal microscope as described above. We used an MCU inhibitor RuR to validate the effect of NecroX-5 on MCU. Ouabain and histamine were used to overload mitochondrial Ca²⁺ as experimental controls.

2.11 Whole-cell patch-clamp recording of L-type Ca²⁺ current
For recording L-type Ca²⁺ current, myocytes were placed in a chamber mounted on an inverted microscope and continuously perfused with extracellular (bath) solution (143 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 5.0 mM HEPES, and 16.6 mM glucose, adjusted to pH 7.4 with NaOH). When filled with a standard internal solution consisting of 32 mM CsCl, 100 mM Cs-Asp, 10 mM EGTA, 10 mM HEPES, and 5 mM MgATP, adjusted to pH 7.25 with CsOH, and placed into the bath solution, the patch-pipette tip resistances were 2–3 MΩ. Whole-cell patch-clamp recordings were made using an Axon interface and Axonpatch 1C amplifier (Axon Instruments, Union City, CA, USA). Experimental parameters were controlled using PatchPro software. To allow equilibration of the pipette solution with the cytosol, the current recordings were started 4 min after the rupture of the membrane patch. Data were analysed using Origin 8.0 software (Microcal Software, Inc., Northampton, MA, USA).

2.12 Measurement of cardiac function
The hearts were perfused with the Krebs–Henseleit buffer containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.8 CaCl₂, 24.8 NaHCO₃, 1.2 KH₂PO₄, and 10 glucose, which was heated to 37°C and gassed with 95% O₂/5% CO₂. A latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium. The left ventricular pressure and heart rate were continuously recorded with a PowerLab system (AD Instruments, Northampton, MA, USA). For recording L-type Ca²⁺ current, myocytes were placed in a chamber mounted on an inverted microscope and continuously perfused with extracellular (bath) solution (143 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 5.0 mM HEPES, and 16.6 mM glucose, adjusted to pH 7.4 with NaOH). When filled with a standard internal solution consisting of 32 mM CsCl, 100 mM Cs-Asp, 10 mM EGTA, 10 mM HEPES, and 5 mM MgATP, adjusted to pH 7.25 with CsOH, and placed into the bath solution, the patch-pipette tip resistances were 2–3 MΩ. Whole-cell patch-clamp recordings were made using an Axon interface and Axonpatch 1C amplifier (Axon Instruments, Union City, CA, USA). Experimental parameters were controlled using PatchPro software. To allow equilibration of the pipette solution with the cytosol, the current recordings were started 4 min after the rupture of the membrane patch. Data were analysed using Origin 8.0 software (Microcal Software, Inc., Northampton, MA, USA).

2.13 Statistics
Data are presented as mean ± standard error of the mean. The differences between the control and the treatment were evaluated by two-tailed one-way analysis of variance (ANOVA) and the control to treatment comparison over time was tested with a two-tailed two-way ANOVA. We used the Tukey post hoc comparison for the comparison of means using Origin software (version 7.0220, OriginLab, USA). The differences with a P-value of ≤0.05 were considered statistically significant.

3. Results
3.1 NecroX-5 post-hypoxic treatment limits cardiac infarct size in HR and improves cardiac function
The HR treatment induced a severe myocardial infarction (MI) (60.99 ± 1.79% of total area) compared with that in the normal perfusion control (9.27 ± 1.82%), whereas post-NecroX-5 treatment significantly attenuated HR-induced MI (17.38 ± 2.72%; Figure 1A and B; see Supplementary material online, Figure S3). The cell viability of isolated myocytes treated with NecroX-5 was also markedly higher compared with that of the control (95.83 ± 7.22 vs. 62.5 ± 12.5%) (Figure 1C; see Supplementary material online, Figure S4). Treatment with NecroX-5 significantly recovered LVEDP and LVDP during the reoxygenation period (see Supplementary material online, Table S2).

3.2 NecroX-5 post-hypoxic treatment improves mitochondrial oxygen consumption
Mitochondrial oxygen consumption was examined in the HR group and the NecroX-5 post-hypoxic treatment group (Figure 1D). The RCI was significantly higher in the NecroX-5 group (6.77 ± 0.84, n = 6) compared with the HR group (4.54 ± 0.02, n = 6), and oxygen consumption during state 3 mitochondrial respiration was markedly higher in the NecroX-5 group compared with the HR group (12.84 ± 1.31 vs. 9.48 ± 0.31 nmol O₂/min/mg mitochondrial protein, n = 6, respectively), suggesting less collapse of the mitochondrial respiratory chain via NecroX-5. Mitochondrial oxygen consumption during the resting state (state 4) did not differ significantly between the HR and NecroX-5 groups (2.09 ± 0.06 vs. 1.91 ± 0.10 nmol O₂/min/mg mitochondrial protein, n = 6 respectively).

3.3 NecroX-5 decreases mitochondrial oxidative stress induced by reoxygenation
NecroX-5 markedly suppressed the overproduction of mitochondrial ROS during reoxygenation in both isolated cardiomyocytes (Figure 2A and B) and isolated mitochondria (Figure 2C and D). Mitochondrial O₂⁻ production increased in both groups during reoxygenation, but the increase was attenuated in the NecroX-5 group compared with the HR group (Figure 2A and B). In the flow cytometry analysis, the intensities of MitoSOX Red and H₂DCF-DA were significantly diminished by NecroX-5 post-treatment compared with HR as described in Figure 2C and D and Supplementary material online, Figure S5A and B.

3.4 NecroX-5 post-hypoxic treatment preserves ΔΨm
TMRE intensity was markedly attenuated in the NecroX-5-treated group compared with that in the HR group in both isolated cardiomyocytes (Figure 2E and F) and isolated mitochondria (Figure 2G), particularly with prolonged reoxygenation times. At the end of the experimental protocol, the TMRE intensities of the HR and NecroX-5 groups were depolarized to 11.10 ± 7.12 and 64.86 ± 3.47%, respectively, of the basal level (n = 6). In the mitochondria, which were isolated after the reoxygenation period, ΔΨm in the NecroX-5 group was ~1.4-fold higher than that in the HR group (Figure 2G; see Supplementary material online, Figure S5C).
3.5 NecroX-5 attenuates mitochondrial calcium overload

HR-induced mitochondrial Ca\(^{2+}\) overload was strongly attenuated in the NecroX-5 group compared with the HR group (Figure 2H–J). Mitochondrial Ca\(^{2+}\) levels gradually increased during the hypoxic period and peak Ca\(^{2+}\) levels occurred at early reoxygenation times. The increase in Ca\(^{2+}\) was more pronounced in the HR group and reached a maximum at the end of the reoxygenation period. The high mitochondrial Ca\(^{2+}\) level in the HR group finally led to hyper-contracture and cardiac cell death; however, NecroX-5 treatment prevented HR-induced Ca\(^{2+}\) overload, hyper-contracture, and cardiac cell death (Figure 2I).

Mitochondrial Ca\(^{2+}\) uptake during reoxygenation is dependent on the MCU.\(^{25}\) To determine whether NecroX-5 can inhibit MCU and thereby reduce mitochondrial Ca\(^{2+}\) overload, we compared the effects of NecroX-5 with those of the MCU inhibitor RuR. During the reoxygenation period, both the NecroX-5 and RuR groups showed a similar tendency towards reduced mitochondrial Ca\(^{2+}\) (Figure 3A–C). The post-hypoxic treatment of RuR also markedly decreased the mitochondrial Ca\(^{2+}\) level in comparison with HR and a limited infarct size (18.04 ± 1.51 vs. 60.99 ± 1.79%, n = 6; Figure 3D–F), indicating that inhibiting MCU after hypoxia is beneficial against HR injury.

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We further tested whether NecroX-5 has a direct effect on HR-independent mitochondrial calcium overload. Ouabain, an Na\(^{+}/\)K\(^{+}\) ATPase inhibitor in the plasma membrane, was used to induce HR-independent mitochondrial Ca\(^{2+}\) overload (Figure 4A–C).\(^{21}\) Treatment with ouabain alone induced maximal mitochondrial Ca\(^{2+}\) overload; consequently, the myocytes were contracted (data not shown). Both NecroX-5 and RuR reduced the ouabain-induced mitochondrial Ca\(^{2+}\) overload.

We also tested whether pre-treatment with NecroX-5 before the hypoxic or ouabain treatment could suppress mitochondrial Ca\(^{2+}\) overload. Unlike post-treatment, pre-treatment with NecroX-5 did not inhibit the HR or ouabain-induced mitochondrial Ca\(^{2+}\) overload (see Supplementary material online, Figure S6) and did not inhibit the collapse of ΔΨm during reoxygenation (data not shown).

To confirm our hypothesis that MCU is a molecular target of NecroX-5, we tested the MCU inhibitory effect of NecroX-5 in the presence of histamine, an activator of MCU. Histamine treatment induced a mitochondrial Ca\(^{2+}\) peak, and NecroX-5 suppressed this effect of histamine on mitochondrial Ca\(^{2+}\) uptake (Figure 4D and E). These results indicate that NecroX-5 can prevent HR-induced mitochondrial Ca\(^{2+}\) overload by directly inhibiting MCU.

3.6 NecroX-5 has no effect on the L-type Ca\(^{2+}\) channel

We investigated whether Ca\(^{2+}\) regulation by the L-type Ca\(^{2+}\) channel was involved in the effect of NecroX-5 on mitochondrial Ca\(^{2+}\). The patch-clamp recordings of L-type Ca\(^{2+}\) current in the presence and absence of NecroX-5 demonstrated that NecroX-5 had no effect on the L-type Ca\(^{2+}\) channel activity (Figure 5).
4. Discussion

The objective of the present study was to evaluate the cardioprotective effect of NecroX-5 against HR injury, elucidate the possible mechanism for limiting cardiac mitochondrial damage, and validate the use of this compound as a medication against ischaemic heart disease. During the first few minutes of reoxygenation, mitochondria encounter harsh environmental changes, with mitochondrial Ca\(^{2+}\) overload and a burst of mitochondrial ROS production. Replacing these rapid changes with gradual increases in mitochondrial ROS production and Ca\(^{2+}\) accumulation would offer a better chance for the survival of mitochondria during reoxygenation. In the present study, we showed that post-hypoxic treatment with NecroX-5 provides a strong protection of rat heart mitochondria against reoxygenation injury by reducing mitochondrial oxidative stress, preserving \(\Delta \Psi_m\), improving mitochondrial oxygen consumption, and attenuating mitochondrial Ca\(^{2+}\) accumulation. In addition, mitochondrial Ca\(^{2+}\) overload induced by HR, ouabain, or histamine was eliminated with NecroX-5 treatment, suggesting that NecroX-5 may act as an inhibitor of MCU.

4.1 Post-hypoxic treatment with NecroX-5 protects cardiac mitochondria against reoxygenation damage

In the present study, the mitochondrial protective role of NecroX-5 was clearly demonstrated by the improvement in mitochondrial oxygen consumption, the preservation of \(\Delta \Psi_m\), and the reduction in mitochondrial oxidative stress and the Ca\(^{2+}\) overload under the HR condition.

IR significantly damages the electron transport chain (ETC), and therefore impairs the mitochondrial coupling of the electron flow (from NADH to \(O_2\) through the ETC) and the proton ejection from mitochondrial matrix, consequently leading to a lower

Figure 2  Effect of NecroX-5 post-hypoxic treatment on mitochondrial function. (A) The graph shows the changes in the mitochondrial O\(_2\) levels as indicated by the relative intensity of MitoSOX Red in myocytes of the HR and NecroX-5-treated groups. (B) The relative MitoSOX Red intensities recorded before hypoxia (a and d), during hypoxia (b and e), and on reoxygenation (c and f) then expressed as a histogram. (C) NecroX-5 treatment reduced the O\(_2\) level in mitochondria which were isolated at the end of HR treatment. The fluorescence intensity of MitoSOX red was measured by fluorescence-associated cell sorter (FACS) in the FL2 channel. (D) NecroX-5 treatment reduced the H\(_2\)O\(_2\) level in mitochondria which were isolated at the end of HR treatment. The fluorescence intensity of H\(_2\)DCFDA was measured by FACS in the FL1 channel. (E) The graph indicates the changes in \(\Delta \Psi_m\), as determined by the relative intensity of TMRE, in HR, and NecroX-5-treated myocytes. (F) The graph shows the relative TMRE intensity recorded at indicated times. (G) The TMRE intensity in mitochondria isolated at the end of the reoxygenation period was evaluated by FACS. (H–J) The dynamic changes in mitochondrial Ca\(^{2+}\) levels measured by the change in Rhod 2AM intensity in the HR and NecroX-5-treated groups during the time course and at the indicated times are shown. The mean values were calculated using CellQuest. n = 6 for each group. a.u., arbitrary unit; \(\ast P < 0.05\) vs. HR, \(\ast\ast P < 0.05\) vs. before the hypoxic period in the same group, and \(\ast\ast\ast P < 0.05\) vs. hypoxic period in the same group.
electrochemical gradient, which is further used to synthesize ATP. Although no difference in state 4 of oxygen consumption was observed between the HR and NecroX-5 groups, significantly higher ADP-dependent oxygen consumption (state 3 respiration) and markedly improved $\Delta \Psi_m$ after prolonged reoxygenation could account for the tighter coupling between the mitochondrial respiratory chain and oxidative phosphorylation in the NecroX-5 group. In agreement with our results, ischaemic preconditioning protected state 3 respiration against IR damage.

The breakdown of $\Delta \Psi_m$ also contributes to the destabilization of action potential repolarization during IR, leading to arrhythmias. In this study, the higher $\Delta \Psi_m$ in the NecroX-5-treated group compared with that in the HR group (Figure 2E–G) was essential in avoiding reoxygenation-induced hyper-contracture, a marker of irreversible damage, and to improve post-ischaemic recovery. Therefore, NecroX-5 preserved mitochondrial integrity and myocyte viability, consistent with smaller infarct size (Figure 2A and B), which is highly associated with the preservation of cardiac performance in the NecroX-5 group after HR treatment (see Supplementary material online, Results and Table S2).

Reoxygenation-induced mitochondrial oxidative stress was also attenuated in the NecroX-5-treated group (Figure 2A–D). This finding was consistent with our previous study and supports an antioxidant effect of NecroX-5. Mitochondria are thought to be both a source of ROS generation and a target of ROS-induced damage in HR. Ischaemia damages the ETC and increases the net production of ROS from complex I to complex III, which in turn results in further mitochondrial ROS generation. Inhibiting ROS levels via ROS scavengers could suppress mitochondrial depolarization. Previous studies have also shown that the release of mitochondrial ROS may be involved in the disruption of mitochondrial Ca$^{2+}$ homeostasis and that a reduction in mitochondrial ROS production and mitochondrial Ca$^{2+}$ concentration could prevent the opening of mPTPs.

The influx of Ca$^{2+}$ into the mitochondria during reoxygenation was inhibited in the NecroX-5 group, which had a lower mitochondrial Ca$^{2+}$ content compared with the HR group (Figure 2H–J), especially during reoxygenation. Owing to the high mitochondrial Ca$^{2+}$ content in the HR group, the myocytes of the HR group began to hyper-contract. In a previous study, mitochondrial depolarization and elevated mitochondrial Ca$^{2+}$ concentration were associated with reoxygenation-induced hyper-contraction. Mitochondrial Ca$^{2+}$ uptake can either inhibit or stimulate ROS production, depending on the respiratory substrate and the effect of Ca$^{2+}$ on $\Delta \Psi_m$, which has been implicated in triggering mitochondrial DNA mutations and cell death.

In our study, post-hypoxic treatment with NecroX-5 not only limited mitochondrial ROS generation but also inhibited mitochondrial Ca$^{2+}$ accumulation, which may further prevent the opening of mPTPs, as suggested in previous studies. Moreover, maintaining the mitochondrial Ca$^{2+}$ homeostasis function could contribute to the antioxidant effect of NecroX-5.

4.2 NecroX-5 may act as a novel MCU inhibitor

An essential finding of our study was a novel effect of NecroX-5 as an MCU inhibitor under pathological Ca$^{2+}$ overload conditions. A
Figure 4 Effect of NecroX-5 on mitochondrial Ca\(^{2+}\) levels. (A) The graphs shows the dynamic changes in mitochondrial Ca\(^{2+}\) levels, measured as changes in Rhod 2AM intensity, induced by ouabain, RuR, or NecroX-5 treatment during the time course of the experiment. (B and C) \(^{\#}P < 0.05\) vs. none treated control, \(^{\#\#}P < 0.05\) vs. time point 'a' in the same group, and \(^{\dagger}P < 0.05\) vs. time point 'b' in the same group. Cardiac images and Rhod 2AM intensities before ouabain, with ouabain, and without or with drug treatment are shown. (D and E) The graph demonstrates the disappearance of the histamine-induced mitochondrial Ca\(^{2+}\) peak after NecroX-5 administration, as evidenced by relative Rhod 2AM intensity, during the treatment period or at the indicated times. Myocytes in the histamine and histamine + NecroX-5 groups were captured at the indicated times (a and b). \(n = 6\) for each group; \(^{\#}P < 0.05\) vs. His, \(^{\#\#}P < 0.05\) vs. time point 'a' in the same group, and \(^{\dagger}P < 0.05\) vs. time point 'b' in the same group. His, histamine; Oua, ouabain; RuR, Ruthenium red.

Figure 5 Effect of NecroX-5 on the activation of L-type Ca\(^{2+}\) current in rat ventricular myocytes. (A) Superimposed current traces elicited by pulses from \(-40\) to \(+50\) mV in \(10\) mV steps from a holding potential of \(-40\) mV under control conditions (left panel), with \(10\) \(\mu\)M NecroX-5 in the bath solution (right panel). (B) Plot of the amplitude of peak inward current normalized to membrane capacitance (pA/pF) against the test potential under the control condition (black circles) and in the presence of NecroX-5 (blue circles). (C) The current traces recorded at 0 mV membrane potential under control conditions (left panel), in the presence of NecroX-5 (middle panel), and under wash-out conditions (right panel). (D) Peak inward currents measured at 0 mV under control conditions, in the presence of NecroX-5, and under wash-out conditions. \(n = 3\) for each group.
comparison of the NecroX-5 effect with the MCU blocker RuR and MCU activator histamine suggested that NecroX-5 inhibited HR-induced Ca\(^{2+}\) overload by directly inhibiting MCU. During ischaemia or hypoxia, the depletion of Na\(^+\) and a loss of ΔΨm and ΔpH elicited by uncouplers dramatically inhibits mitochondrial Ca\(^{2+}\) uptake via MCU. Thus, Ca\(^{2+}\) uptake into mitochondria occurs largely via the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger, whereas mitochondrial Ca\(^{2+}\) was mainly taken up through the MCU during the reoxygenation period. Thus, the inhibition of MCU presented cardio-protective effects through interactions with mPTP and the mitochondrial ATP-sensitive potassium channel. However, the MCU inhibitor RuR showed no protective effect in a model of HR damage at concentrations that inhibit changes in the Ca\(^{2+}\) level. In contrast, Figureondo et al. showed a protective role of RuR upon reperfusion via a recovery of energy and myocardial contraction and a decrease in necrotic cell death.

NecroX-5 strongly attenuated mitochondrial Ca\(^{2+}\) accumulation during reoxygenation. We hypothesized that this compound could affect the uptake of Ca\(^{2+}\) into mitochondria through MCU. RuR and NecroX-5 similarly attenuated mitochondrial Ca\(^{2+}\) accumulation induced by ouabain or hypoxia. Co-treatment of RuR and NecroX-5 did not have any additional MCU inhibitive effect compared with single treatment (see Supplementary material online, Figure S3), which clearly demonstrated that NecroX-5 has the same target as RuR. The effect was not seen with NecroX-5 pre-treatment (see Supplementary material online, Figure S6). Thus, the effect of NecroX-5 appears to be mediated by high Ca\(^{2+}\) concentration in mitochondria. Experiments using histamine to induce an elevation of mitochondrial Ca\(^{2+}\) confirmed the effect of NecroX-5 on MCU (Figure 4D–F). Histamine is reported to induce the release of Ca\(^{2+}\) from the endoplasmic reticulum by phosphorylation mediated by protein kinase C, which further increases the mitochondrial Ca\(^{2+}\) content via MCU. This stimulation disappeared in the presence of NecroX-5.

Additionally, elevated mitochondrial Ca\(^{2+}\) has been reported to be a consequence of cytosolic Ca\(^{2+}\) overload through L-type Ca\(^{2+}\) channels; this induces ΔΨm disruption subsequent to ATP depletion and leads to apoptotic cell death. Although increased Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels during ischaemia and after MI prevents depressed myocardocyte contractility, it may increase the risk of ischaemic injury and exacerbate depressed cardiac pump function. In the present study, the administration of NecroX-5 did not affect L-type Ca\(^{2+}\) current (Figure 5), suggesting that cardiac contraction initiated by this channel would not be affected by NecroX-5. Therefore, we propose that NecroX-5 directly regulates myocardial Ca\(^{2+}\) homeostasis via MCU and suppresses mitochondrial damage in a model of HR injury.

Dysregulated calcium homeostasis is an important factor involved in generating an arrhythmia, which is a major cause of sudden cardiac death. Lowering mitochondrial Ca\(^{2+}\) influx by inhibiting MCU is a promising approach to reduce arrhythmia followed by reperfusion or other pathological conditions. In this context, the newly demonstrated effect of NecroX-5 on MCU will be a useful experimental tool and provide insight into the regulatory mechanism of MCU and its anti-arrhythmia role. However, the molecular mechanism underlying NecroX-5-induced inhibition of MCU and its effect on preventing arrhythmia should be addressed in future by studies more extensive in terms of the molecular reaction. Although the precise mechanism of action for NecroX-5 is still unclear, our results demonstrated significant cardiac protection by NecroX-5 when administered during reoxygenation. Because heart attacks are usually unpredictable, the actions of this compound that take place during reperfusion could be a potent way to ameliorate post-hypoxic cardiac injury. First, NecroX-5 still worked well and showed a cytoprotective effect, whereas little or no cytoprotective effect was observed in cells treated with other agents. Secondly, the synthesized NecroX-5 is chemically and functionally similar to NecroX-7, which was presented as the most efficacious and safe compound to prevent IR injury during liver surgery. It was also tested and investigated as a new drug in a Phase 1 clinical trial with indications for MI. Finally, the current data further establish the dual protective role of NecroX-5 during reoxygenation by inhibiting both mitochondrial ROS generation and mitochondrial Ca\(^{2+}\) accumulation as a strategy to limit HR damage.

However, limitations of this study must be acknowledged. We were unable to conduct all experiments at temperatures that mimic the body due to inadequate equipment. Cell-based analyses were carried out at room temperature. However, other experiments such as cardiac function, infarct size, isolated myocytes, and Langendorff perfusion protocols were conducted at 37°C. Collectively, NecroX-5 showed protective functions on treated samples under any temperature condition. Because we focused our research interest in the mitochondrial MCU and cardioprotective effect of NecroX-5, the effect of NecroX-5 on the activity of TCA cycle remained unclear. In the future study, investigation of the NecroX-5 effect in the NADH and FADH\(^{2+}\) production and various enzymatic activities in TCA cycle during HR injury will provide more insight of the cellular protective effect of NecroX-5 in the view of mitochondrial energetics.

In conclusion, our data demonstrate that the new agent NecroX-5 exerts a novel cardioprotective effect on rat heart mitochondria against HR damage by inhibiting MCU activity. NecroX-5 is a promising agent for the attenuation of myocardial damage resulting from reoxygenation injury.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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### References


