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MCP-1 mediates ischemia-reperfusion-induced cardiomyocyte apoptosis via MCPIP1 and CaSR

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Zhang W, Zhu T, Chen L, Luo W, Chao J. MCP-1 mediates ischemia-reperfusion-induced cardiomyocyte apoptosis via MCPIP1 and CaSR. Am J Physiol Heart Circ Physiol 318: H59–H71, 2020. First published November 27, 2019; doi:10.1152/ajpheart.00308.2019.—Monocyte chemotactic protein-1 (MCP-1) plays a crucial role in ischemia-reperfusion (I/R) injury; however, the detailed mechanism of MCP-1 in I/R injury-induced cardiomyocyte apoptosis remains unclear. In this study, we explored the cascade downstream of I/R-induced MCP-1 that modulates cell apoptosis and determined whether Ca2+-sensing receptors (CaSRs) are involved in the process. Protein levels were detected in a cardiac muscle cell line (HL-1) and primary cultured neonatal mouse ventricular cardiomyocytes using Western blotting and immunocytochemistry. Released MCP-1 was detected using ELISA. Both Hoechst staining and flow cytometry methods were used to measure cell apoptosis. Specific pharmacological inhibitors of CC chemokine receptor 2 (RS-102895) and CaSR (NPS-2143) as well as a CaSR activator (evocalcet) were applied to confirm the roles of these factors in I/R-induced cell apoptosis. I/R inhibited cell viability and upregulated cell apoptosis. Moreover, I/R induced the release of MCP-1 from both HL-1 cells and primary cardiomyocytes. Further research confirmed that CaSR acted as an upstream effector of monocyte chemotactic protein-1-induced protein-1 (MCPIP1) and coordinately regulated cell apoptosis, which was verified by addition of an inhibitor or activator of CaSR. Moreover, MCPIP1 induced cell apoptosis through endoplasmic reticulum (ER) stress but not autophagy induced by I/R. Based on these findings, I/R-induced MCP-1 release regulates cardiomyocyte apoptosis via the MCPIP1 and CaSR pathways, suggesting a new therapeutic strategy for I/R injury.

NEW & NOTEWORTHY Ischemia-reperfusion (I/R)-induced monocyte chemotactic protein-1 release regulates cardiomyocyte apoptosis via the monocyte chemotactic protein-1-induced protein-1 (MCPIP1) and Ca2+-sensing receptor pathway. The functional changes mediated by MCPIP1 involve the activation of endoplasmic reticulum stress, but not the autophagy pathway, after I/R injury. MCP-1/CCL2; MCPIP1; apoptosis; CaSR; ischemia-reperfusion; MCP-1/CCL2; MCPIP1

INTRODUCTION

Myocardial ischemia-reperfusion (I/R) involves tissue ischemia with inadequate oxygen followed by successful reperfusion, which initiates a wide and complex array of injuries and induces impairment of remote organ function (1, 32, 46). Increasing evidence indicates that myocardial apoptosis (1), intracellular Ca2+ overload, and irreversible cell damage (1) occur after I/R injury. Accumulating research has indicated that myocardial apoptosis involving chemokines such as monocyte chemotactic protein-1 (MCP-1)/C-C motif chemokine ligand 2 (CCL2) (45), activated Ca2+-sensing receptors (CaSR; see Ref. 44), autophagy (18, 30), and endoplasmic reticulum (ER) stress (42) occurs in the pathophysiological process associated with I/R injury. Lu et al. reported that CaSR induced Ca2+ release from the sarcoplasmic reticulum (SR) into the mitochondria and induced cardiomyocyte apoptosis through the SR and mitochondrial apoptotic pathway in heart failure (22). Qi et al. reported that CaSR activation leads to the apoptosis of cardiomyocytes in diabetic cardiac injury through the induction of Ca2+ overload and the activation of the mitochondrial pathway (29); in short, the underlying mechanism must be investigated further to develop better therapy for ischemic heart disease.

Based on increasing evidence, C-C motif chemokine-MCP-1/CCL2 and its downstream molecule, monocyte chemotactic protein-1-induced protein-1 (MCPIP1), facilitate myocardial inflammation and endothelial dysfunction in response to I/R injury (6, 13, 25, 36, 46). MCP-1/CCL2 is a potent mononuclear cell chemoattractant involved in various diseases characterized by monocyte-rich leukocyte infiltration (3, 5, 8, 41), and it is essential for the development of restenotic changes after coronary intervention because it mediates monocyte infiltration and subsequent activation (16, 34). For instance, MCP-1/CCL2 induces cardiac cell death and ventricular dysfunction through a novel transcription factor (45). MCP-1/MCPIP1 contributes to cardiomyoblast death in patients with heart failure and is associated with autophagy resulting from ER stress (42). On the other hand, knockdown of CC chemokine receptor 2 (CCR2), a specific receptor of MCP-1/CCL2, inhibits neointimal formation, monocyte recruitment, and neointimal macrophage content (31) as well as increases macrophage infiltration and infarct size following arterial injury (9, 14). Moreover, the role of MCP-1 in I/R injury is controversial (23, 26). One paper reported that cardiac MCP-1 prevented left ventricular dysfunction after I/R through a reactive oxygen species-dependent pathway and played a beneficial role in the pathophysiology of ischemic heart diseases (45). However, other reviews considered that innate immunity mediated the...
overexpression of MCP-1 by ischemic preconditioning through mitogen-activated protein kinase activation (23).

The present study explored the mechanism by which MCP-1/CCL2 regulates I/R-induced cardiomyocyte apoptosis. The research results in this study will aid clinicians and researchers in understanding the mechanisms that regulate MCP-1/CCL2 expression and its functional relevance to I/R injury and provide insights into potential therapeutic targets for the treatment of myocardial infarction.

MATERIALS AND METHODS

Reagents. Fetal bovine serum, normal goat serum, Dulbecco’s modified Eagle’s medium (DMEM; No. 1200-046), and 10× MEM (11430-030) were obtained from Life Technologies. Amphotericin B (BP2645) and GlutaMax supplement (35050-061) were obtained from Gibco, and penicillin/streptomycin (15140-122) was obtained from Fisher Scientific. Antibodies specific for CaSR (sc33821, rabbit), MCPIP1 (sc136750, goat), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc32233, mouse) were obtained from Santa Cruz Biotechnology. Antibodies specific for caspase-3 (19677, rabbit), beclin (BECN) 1 (11306, rabbit), LC3B (14600, rabbit), binding immunoglobulin protein (BiP, 11387, rabbit), and C/EBP homologous protein (CHOP, 15204, rabbit) were acquired from ProteinTech, Biotechnology. The transfection reagent used for the siRNAs was purchased from Santa Cruz Biotechnology.

Cell culture. HL-1 cells (cardiac muscle cell line) were purchased from ScienCell and maintained following the manufacturer’s protocol. Primary cultured neonatal mouse ventricular cardiomyocytes were prepared using a previously described method (39).

Establishment of the oxygen-glucose deprivation model. The oxygen-glucose deprivation model, which was a modified version of a previously described method, was applied (46, 47). First, the normal culture medium was replaced with serum-free DMEM before the start.Fig. 1. Ischemia-reperfusion (I/R) induced cell apoptosis in vitro. A: representative blots showing that I/R induced apoptotic marker expression in a time-dependent manner in HL-1 cells. B: densitometric analyses of the ratio of Bax/Bcl-Xl in 5 independent experiments. *P < 0.05 compared with the 0-h group. C: densitometric analyses of the ratio of cleaved caspase-3/caspase-3 in 5 independent experiments. *P < 0.05 compared with the 0-h group. D: representative images of Hoechst 33342 staining depicting HL-1 cell apoptosis at 24 h after I/R. Red arrows indicate apoptotic cells. Scale bar = 10 μm. E: percentages of apoptotic cells from 6 separate experiments. *P < 0.05 compared with the control group. F: I/R decreased HL-1 cell viability in a time-dependent manner, as shown by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. *P < 0.05 compared with the corresponding time point of the control group.
of the experiment. Following pregassing with 95% N₂-5% CO₂ for a minimum of 5 min, the ischemic buffer, consisting of (in mM) 1 NaH₂PO₄, 24 NaHCO₃, 2.5 CaCl₂, 118 NaCl, 16 KCl, 0.5 sodium EDTA, and 20 sodium lactate (pH 6.8, 37°C), was added to the cells, which were then placed in a sealed chamber containing the deoxygenation reagent, resulting in the consumption of oxygen and the production of CO₂. This Anaero-Pack system (Mitsubishi Gas Chemical) created near-anaerobic conditions, with an O₂ concentration of <1% and a CO₂ concentration of ~5%, and the cells were subjected to a 1-h incubation at 37°C (35). The partial pressure of oxygen (PO₂) in the medium was directly measured in several experiments using a phosphorescence decay method to directly determine the efficacy of the equilibration system (5, 21). The actual PO₂ values of the supernatant were 4.5 ± 0.3 mmHg (mean ± SE). To induce I/R injury, cells in a 24-well plate were treated with an ischemic buffer solution (1 mL/well) for 2 h before a 24-h incubation with glucose-containing DMEM at 37°C in a 95% O₂-5% CO₂ atmosphere (reperfusion).

In vivo myocardial I/R model. Male C57BL/6 mice (12–20 wk old, 22–30 g) were purchased from Nanjing Medical University Laboratories (Nanjing, China) and divided randomly into two groups as

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**Fig. 2. Ischemia-reperfusion (I/R) induced the release of monocyte chemotactic protein-1 (MCP-1) in vitro.** A: ELISAs showing the MCP-1 level in the supernatant from cultured HL-1 cells that were exposed to I/R. *P < 0.05 compared with the 0-h time point, n = 5. B: representative blots showing the effect of I/R on MCP-1/C-C motif chemokine ligand 2 (CCL2) receptor-CC chemokine receptor 2 (CCR2) in HL-1 cells. C: densitometric analyses of the expression of CCR2 in 5 independent experiments. *P < 0.05 compared with the 0-h group. D: effect of I/R on monocyte chemotactic protein-1-induced protein-1 (MCPIP1) expression in HL-1 cells as determined by real-time PCR. *P < 0.05 vs. 0 h. E: representative blots showing that I/R induced the expression of Ca²⁺-sensing receptor (CaSR) in a time-dependent manner in HL-1 cells. F: densitometric analyses of the expression of CaSR in 5 independent experiments. *P < 0.05 compared with the 0-h group. G: representative images of immunocytochemical staining showing that MCPIP1 colocalized with CaSR in HL-1 cells in response to I/R injury. Scale bar = 10 μm.

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follows: an ischemia group (ischemia for 30 min) and reperfusion group (ischemia for 30 min followed by reperfusion for 2.5 h). Briefly, the mice were anesthetized with 100 mg/kg ketamine and were then intubated and mechanically ventilated by connecting the endotracheal tube to a scientific ventilator (Harvard model) at a respiratory rate of 138 breaths/min with a tidal volume of 20 mL/kg body wt. A left thoracotomy was performed to expose the heart. The left anterior descending coronary artery was then transiently ligated (or tied with a slipknot) using a 6-0 polypropylene suture for a 30-min ischemic period. After 30 min of ischemia, microsurgical scissors were used to cut the knot in the ligature (or the slipknot was released), and the heart was perfused for 2.5 h. Immediately after finishing the reperfusion time, the mice were euthanized, and the heart was excised for the next experiment (7, 27). All animal procedures were performed in strict accordance with the Animal Research: Reporting of In Vivo Experiments guidelines, and the animal protocols were approved by the

Fig. 3. Expression of monocyte chemotactic protein-1-induced protein-1 (MCPIP1) and Ca\textsuperscript{2+}-sensing receptor (CaSR) in mice. Representative images of immunohistochemical staining showing that MCPIP1 colocalized with CaSR in the hearts of mice exposed to ischemia-reperfusion (I/R) conditions. Scale bar = 10 μm.

Fig. 4. Monocyte chemotactic protein-1 (MCP-1) and Ca\textsuperscript{2+}-sensing receptor (CaSR) mediated cell apoptosis induced by ischemia-reperfusion (I/R). A: MCP-1 (25 μg/mL) decreased cell viability in a time-dependent manner, as shown by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. *P < 0.05 compared with the 0-h group (n = 3). B: flow cytometric results showing that MCP-1 (25 μg/mL) on cell apoptosis. Data are presented as means ± SE (n = 3). *P < 0.05 compared with the control group. C: representative blots showing the effect of MCP-1 (25 μg/mL) on CaSR and monocyte chemotactic protein-1-induced protein-1 (MCPIP1) expression in a time-dependent manner in HL-1 cells induced by I/R. D: densitometric analyses of the expression of CaSR and MCPIP1 in 5 independent experiments. *P < 0.05 compared with the 0-h group. E: representative images of Hoechst 33342 staining showing that I/R-induced HL-1 cell apoptosis was attenuated by pretreatment with RS-102895 or NPS-2143. Red arrows indicate apoptotic cells. Scale bar = 10 μm. F: percentages of apoptotic cells from 6 separate experiments. P < 0.05 compared with control (*) and I/R (#) groups. G and H: flow cytometric results showing that RS-102895 prevented I/R-induced cell apoptosis. Data are presented as means ± SE (n = 3). P < 0.05 compared with the control group (*) and the I/R control group compared with the I/R + RS-102895 group (#). I: pretreatment with RS-102895 or NPS-2143 restored the I/R-induced decrease in HL-1 cell viability, as shown by the MTT assay. Data are presented as means ± SE (n = 3). P < 0.05 compared with the control (*) and I/R (#) groups (n = 3).
Institutional Animal Care and Use Committee of the Medical School of Southeast University.

**Hoechst staining.** Cells were fixed and stained with 5 μM Hoechst 33324 (Invitrogen) for 15 min at room temperature to quantify the number of apoptotic cells (46). The morphological features of apoptotic cells (cell shrinkage and chromatin condensation and fragmentation) were monitored using fluorescence microscopy (TE2000E microscope; Nikon).
Monocyte chemotactic protein-1 (MCP-1) is involved in the regulation of cell apoptosis via monocyte chemotactic protein-1-induced protein-1 (MCPIP1) and CaSR in I/R-induced endothelial cell dysfunction (38). How-ever, whether MCPIP1 and CaSR coordinately regulate I/R-induced endothelial cell apoptosis requires further investigation. As shown in Fig. 2A, the MCP-1 level in the supernatant of HL-1 cells transiently and rapidly increased after I/R. Interestingly, the expression of CCR2, a specific receptor of MCP-1, showed a decreasing trend at 12 h after I/R that was statistically significant only 24 h after I/R, indicating that a negative feedback mechanism may be involved in this process (Fig. 2, B and C). The rapid and sustained increase in the expression of MCPIP1 at the transcript and protein levels after I/R was further explored (Fig. 2, D–F). Previous data from our laboratory suggested that CaSR functions in I/R-induced cardiomyocyte death in vivo (39, 40). Recent research showed a link between MCPIP1 and CaSR in I/R-induced endothelial cell dysfunction (38). However, whether MCPIP1 and CaSR coordinately regulate I/R-induced cardiomyocyte apoptosis remains unknown. We first measured the expression of CaSR in HL-1 cells after I/R. As noted above, the expression of MCPIP1 at the transcript and protein levels after I/R was further explored (Fig. 2, D–F). Previous data from our laboratory suggested that CaSR functions in I/R-induced cardiomyocyte death in vivo (39, 40). Recent research showed a link between MCPIP1 and CaSR in I/R-induced endothelial cell dysfunction (38). However, whether MCPIP1 and CaSR coordinately regulate I/R-induced cardiomyocyte apoptosis remains unknown. We first measured the expression of CaSR in HL-1 cells after I/R. As noted above, the expression of MCPIP1 at the transcript and protein levels after I/R was further explored (Fig. 2, D–F). Previous data from our laboratory suggested that CaSR functions in I/R-induced cardiomyocyte death in vivo (39, 40). Recent research showed a link between MCPIP1 and CaSR in I/R-induced endothelial cell dysfunction (38). However, whether MCPIP1 and CaSR coordinately regulate I/R-induced cardiomyocyte apoptosis remains unknown. We first measured the expression of CaSR in HL-1 cells after I/R.
shown in Fig. 2, E and F, I/R increased the CaSR level in a time-dependent manner. Furthermore, the colocalization of CaSR and MCPIP1 after I/R was confirmed by immunofluorescence staining in vitro (Fig. 2G) and in vivo (Fig. 3).

**MCP-1 and CaSR mediated cell apoptosis after I/R.** To further investigate the involvement of functional changes in MCP-1 and CaSR in I/R-induced cell apoptosis, we applied MCP-1 and specific pharmacological inhibitors of CCR2 and CaSR to further identify the mechanism of I/R-induced apoptosis. As shown in Fig. 4, A–D, treatment with MCP-1 (25 μg/mL) decreased cell viability (Fig. 4A) and induced cell apoptosis (Fig. 4B), as well as upregulation of CaSR and
MCPIP1 expression (Fig. 4, C and D), confirming the role of MCP-1 on cell apoptosis. Interestingly, exogenous application of MCP-1 did not further increase the apoptosis induced by I/R, which may be because the maximum effect of endogenous MCP-1 induced by I/R was reached. Moreover, either the CCR2 inhibitor RS-102895 (3) or the CaSR inhibitor NPS-2143 (28) significantly attenuated I/R-induced apoptosis in HL-1 cells (Fig. 4, E–H). Similarly, pretreatment with RS-102895 or NPS-2143 restored the decreased cell viability after I/R (Fig. 4I). Correspondingly, RS-102895 pretreatment significantly reduced MCPIP1 and CaSR levels in HL-1 cells after I/R (Fig. 5, A and B), and NPS-2143 also exhibited an inhibitory effect on the apoptotic marker cleaved caspase-3 (Fig. 5, C and D). Interestingly, NPS-2143 inhibited MCPIP1 expression but not CaSR expression (Fig. 5, E–G), whereas knockdown of MCPIP1 had no effect on the expression of CaSR induced by I/R (Fig. 5, H–J). This effect was further explored with a CaSR activator (evocalcet), which aggravated MCPIP1 expression induced by I/R (Fig. 5, K and L). These data suggest that CaSR, as an upstream effector of MCPIP1, mediated cell apoptosis induced by I/R.

Knockdown of MCPIP1 inhibited ER stress activation by I/R. A recent study identified a link between MCPIP1 and ER stress (33); therefore, we further investigated the inositol-requiring protein-1α (IRE1α) pathway after I/R. As shown in Fig. 6, A and B, I/R administration increased the expression of IRE1α and CHOP, as well as the binding immunoglobulin protein (BiP), a master regulator of the ER stress response. To further investigate the function of MCPIP1 in ER stress, we used siRNA to specifically silence MCPIP1. As shown in Fig. 6, C and D, knockdown of MCPIP1 inhibited the upregulation of IRE1α and CHOP as well as BiP levels induced by I/R. Correspondingly, the CaSR inhibitor NPS-2143 decreased the expression of IRE1α and CHOP as well as BiP levels induced by I/R (Fig. 6, E and F). To this end, salubrinal, a specific inhibitor of ER stress, was applied. As shown in Fig. 6, G and H, salubrinal obviously reversed cell apoptosis, as indicated by a decrease in condensation and shrinkage of nuclei induced by I/R, and the CaSR activator increased cell apoptosis induced by I/R (Fig. 6, I and J). Taken together, these results demonstrated that MCPIP1 mediated I/R-induced apoptosis via the activation of ER stress.

MCP-1 did not affect I/R-induced functional changes related to cell autophagy. Many studies have been performed to investigate the interaction between MCPIP1 and autophagy (18), and the cross talk between autophagy and apoptosis has also been extensively studied and shown to affect many physiological and pathological processes (28). Based on the above research results, we also explored whether MCPIP1 induced cell apoptosis through the autophagy pathway. As shown in Fig. 7, A and B, the levels of proteins related to autophagy, such as BECN and LC3B (MAP1LC3B), were elevated after I/R, as was P62 (an indicator of inhibition of autophagic clearance). However, knockdown of MCPIP-1 did not affect the upregulation of autophagy markers induced by I/R (Fig. 7, C and D), thus ruling out an interaction between MCPIP1 and autophagy in the I/R setting.

I/R induced the release of MCP-1 in primary cultured cardiomyocytes. To validate our findings on MCP-1 and its downstream events, primary cultured neonatal mouse ventricular cardiomyocytes were used. As shown in Fig. 8A, I/R induced a transient and rapid release of MCP-1 from primary cardiomyocytes at 1 h after reperfusion, and the duration of release was shorter than that in HL-1 cells. On the other hand, I/R also upregulated the protein level of MCPIP1 in primary cardiomyocytes, but the duration of MCPIP1 upregulation was also shorter than that in HL-1 cells (Fig. 8B and C). The above phenomenon of increased MCPIP1 levels was further confirmed in vivo, since MCPIP1 showed a significant increase in the hearts of I/R mice (Fig. 8D). I/R induced a similar release of MCP-1 and upregulation of MCPIP1 in vivo and in vitro, suggesting that a general mechanism is involved in I/R injury (Fig. 8E).

DISCUSSION

Cardiovascular disease with I/R injury is one of the leading causes of death worldwide. Cardiomyocyte apoptosis occurs in the early stages of reperfusion and is associated with the release of cytokines and chemokines as well as oxidative stress (46–48). The chemokine MCP-1/CCL2, one of the main factors that mediates the recruitment of mononuclear cells, is involved in I/R-induced cell apoptosis. As a continuation of our previous investigation on the role of MCP-1/MCPIP1 in I/R-induced alterations in cell function, the aim of the current study was to investigate the downstream events mediated by cardiomyocyte-derived MCP-1 in I/R injury-induced cell apoptosis.

An increasing number of studies have reported the release of MCP-1 from cardiomyocytes following I/R, but there are conflicting reports on the outcome of MCP-1 release. For example, MCP-1 was shown to induce the release of cytokines and growth factors, leading to effective repair and scar formation, which may be beneficial for I/R injury repair (15, 19). On the other hand, sustained release of MCP-1 was shown to cause an inflammatory response that led to irreversible tissue damage.
In the present study, I/R induced a transient and rapid release of MCP-1 from both HL-1 cells and primary ventricular cardiomyocytes, which correlated with a decrease in cell viability. Interestingly, CCR2, the specific receptor of MCP-1, was also decreased at 24 h after I/R, which may be because of negative feedback after continuous upregulation of MCPIP1. This may explain why some inhibitors become less effective after long-term treatment. Thus, the current study suggests that MCP-1 expression contributes to cardiomyocyte apoptosis following I/R.
MCPIP1, a novel zinc finger protein, is the main downstream molecule that mediates the inflammatory effect of MCP-1, and it is a newly discovered protein that was induced by MCP-1 in human peripheral blood monocytes (45). MCPIP1 plays an important role in inflammatory diseases, such as pneumoconiosis (18), neuronal injury (20), and I/R injury (46), by regulating gene transcription (45), mRNA degradation (24), cell apoptosis (42, 43), autophagy (18, 30), and differentiation (37). A recent study also suggested that MCPIP1 regulates autophagy via p53, a tumor suppressor and an important regulator of cell apoptosis (17, 18). Previous data from our laboratory also showed that MCPIP1 mediated I/R-induced endothelial cell dysfunction via autophagy (46), indicating a key role for MCPIP1 in I/R. The previous data suggested that MCPIP mediates the transcriptional activity of genes involved in cell death by I/R (45). Our study results are

Fig. 7. Monocyte chemotactic protein-1-induced protein-1 (MCPIP1) did not affect ischemia-reperfusion (I/R)-induced functional changes related to cell autophagy. A: representative blots showing that I/R increased the expression of P62, beclin (BECN), and LC3B in HL-1 cells. B: densitometric analyses of the enhanced P62, BECN, and LC3B levels after I/R in 5 independent experiments. *P < 0.05 compared with the control group. C: representative blots showing that knockdown of MCPIP-1 did not influence P62, BECN, and LC3B levels in HL-1 cells after I/R. D: densitometric analyses showing that MCPIP-1 knockdown did not affect the expression of P62, BECN, or LC3B after I/R in 5 independent experiments. *P < 0.05 compared with the control group.

Fig. 8. Ischemia-reperfusion (I/R) induced the release of monocyte chemotactic protein-1 (MCP-1) in primary cultured cardiomyocytes. A: ELISAs showing the MCP-1 level in the supernatant from primary cultured cardiomyocytes that were exposed to I/R. *P < 0.05 compared with the 0-h time point, n = 5. B: representative blots showing the effect of I/R on the monocyte chemotactic protein-1-induced protein-1 (MCPIP1) level in primary cultured cardiomyocytes. C: densitometric analyses of the MCPIP1 level in 5 independent experiments. *P < 0.05 compared with the 0-h group. D: representative blots showing the effect of I/R on the MCPIP1 levels in mouse hearts (C57BL/6, male, 12–20 wk old, 22–30 g). E: schematic diagram showing the mechanisms by which MCP-1 mediates I/R-induced cardiomyocyte apoptosis.
in accord with the finding that elevated MCPIP1 expression was induced at the transcriptional level (Fig. 2D) by I/R treatment. In the current study, MCPIP1 expression was rapidly increased in vivo and in vitro in both HL-1 cells and primary ventricular cardiomyocytes after I/R; thus, the downstream events represent an interesting research topic.

CaSR has been shown to be involved in I/R-induced cardiomyocyte death (39). Zheng et al. suggested the involvement of CaSR in cardiac apoptosis through the mitochondrial death pathway during I/R (44). However, the detailed mechanism remains unknown. CaSR not only responds to extracellular Ca\textsuperscript{2+} but is also activated by many ligands, such as divalent and trivalent cations, \( \epsilon \) amino acids, and polyamines (2). Although most reports indicated a role for CaSR in apoptosis, CaSR also promotes osteoblast proliferation during bone remodeling (10). In the current study, NPS-2143, a CaSR-specific inhibitor (28), rescued I/R-treated cells from apoptosis. Furthermore, the specific CCR2 inhibitor RS-102895 inhibited the I/R-induced upregulation of CaSR, clearly indicating an association between MCP-1 and CaSR. To further understand the roles of MCP-1 and CaSR after I/R, we used a CaSR-specific inhibitor or knocked down MCPIP1 and assessed the underlying mechanism between MCP-1 and CaSR. The results suggested that CaSR, as an upstream effector of MCPIP1, was involved in I/R-induced cell apoptosis; however, the underlying mechanism requires further study. Various physiological and pathological conditions may affect ER homeostasis and ultimately cause ER stress (42, 43). Furthermore, an increasing amount of data has reported that autophagy may also participate in irreversible cell injury and cell death under extreme conditions (44, 45). Although MCP-1 is associated with the interplay between ER stress and autophagy in I/R, it has received little attention. Surprisingly, our results demonstrated that I/R increased the expression of IRE1\( \alpha \) and CHOP as well as BiP, the master regulator of the ER stress response (Fig. 6, A and B). Moreover, either the knockdown of MCPIP1 (Fig. 6, C and D) or the inhibition of CaSR (Fig. 6, E and F) inhibited protein expression associated with ER stress and decreased cell apoptosis behavior and cell nuclear morphology changes (Fig. 6, G and H). Interestingly, the results of CaSR activator-induced cell apoptosis further demonstrated that MCPIP1 mediated cell apoptosis involving the activation of ER stress after I/R. To obtain more robust evidence, we extended the study and confirmed the role of autophagy induced by I/R. The results confirmed that I/R-induced autophagy elevated the expression of P62, BECN1, and LC3B (Fig. 7, A and B); however, limited changes were observed in these protein levels via knockdown of MCPIP1 and CaSR (Fig. 7, C and D). These results further indicate that MCPIP1 was not involved in autophagy and did not affect the functional changes associated with autophagy induced by I/R. To further understand the detailed mechanism of cell apoptosis by I/R injury, clinical samples related to I/R injury should be applied in future studies.

In summary, our study identified a mechanism by which MCP-1 regulated I/R-induced cardiomyocyte cell death via MCPIP1 and CaSR. Furthermore, we determined that the I/R-mediated expression of both MCPIP1 and CaSR affected cell apoptosis, resulting in I/R injury (Fig. 8E). Moreover, the functional changes mediated by MCPIP1 did involve the activation of ER stress but not the autophagy pathway after I/R injury. These findings have implications for I/R injury in individuals with heart failure, and a better understanding of the mechanisms regulating MCP-1 may aid in the development of adjunct therapeutic strategies to treat individuals with I/R injuries.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

W.Z., T.Z., and J.C. conceived and designed research; W.Z., L.C., and W.L. performed experiments; W.Z., L.C., and W.L. analyzed data; W.Z., T.Z., W.L., and J.C. interpreted results of experiments; W.Z., T.Z., L.C., and W.L. prepared figures; W.Z. and T.Z. drafted manuscript; T.Z. and J.C. edited and revised manuscript; J.C. approved final version of manuscript.

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MCP-1 IN CARDIOMYOCYTE APOPTOSIS AFTER I/R


