MCP-1 Induces Cardioprotection against Ischaemia/Reperfusion Injury: Role of Reactive Oxygen Species

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Abstract

Aim: Monocyte chemoattractant protein-1 (MCP-1: CCL2) has been demonstrated to be involved in the pathophysiology of ischaemic heart disease; however, the precise role of MCP-1 in ischaemia/reperfusion (I/R) injury is controversial. Here, we investigated the role of cardiac MCP-1 expression on left ventricular (LV) dysfunction after global I/R in Langendorff-perfused hearts isolated from transgenic mice expressing the mouse *JE-MCP-1 gene* under the control of the α-cardiac myosin heavy chain promoter (MHC/MCP-1 mice). **Methods and Results:** *In vitro* experiments showed that MCP-1 prevented the apoptosis of murine neonatal cardiomyocytes after hypoxia/reoxygenation. I/R significantly increased the mRNA expression of MCP-1 in the Langendorff-perfused hearts of wild-type mice. Cardiac MCP-1 overexpression in the MHC/MCP-1 mice improved LV dysfunction after I/R without affecting coronary flow; in particular, it ameliorated LV diastolic pressure after reperfusion. This improvement was independent of both sarcolemmal and mitochondrial K_{ATP} channels. Cardiac MCP-1 overexpression prevented superoxide generation in the I/R hearts, and these hearts showed decreased expression of the NADPH oxidase family proteins, Nox1, gp91phox and Nox3 compared with the hearts of wild-type mice. Further, superoxide dismutase (SOD) activity in the hearts of MHC/MCP-1 mice was significantly increased compared with that in the heart of wild-type mice. **Conclusions:** These findings suggested that cardiac MCP-1 prevented LV dysfunction after global I/R through a reactive oxygen species (ROS)-dependent but K_{ATP} channel-independent pathway; this provides new insight into the beneficial role of MCP-1 in the pathophysiology of ischaemic heart diseases.

Key words: apoptosis; cytokines; infection/inflammation; myocytes; oxygen radicals

Introduction

Myocardial infarction (MI) is a common occurrence, and it is predicted that this will be the leading cause of death worldwide in the near future. Over the last 2 decades, coronary reperfusion therapy has become an established treatment for the management of acute MI; however, the restoration of coronary blood flow to a previously ischaemic myocardium results in the 'ischaemia/reperfusion (I/R) injury'. The different clinical manifestations of this injury include myocardial apoptosis, arrhythmias, myocardial stunning, microvascular dysfunction and irreversible cell damage.[1-3] It is widely accepted that the generation of reactive oxygen species (ROS) in the myocardium is the principal mechanism contributing to the pathogenesis of I/R injury.[4]

A number of experimental and clinical studies have demonstrated that chemokines are involved in the pathophysiology of ischaemic heart diseases such as MI and acute coronary syndrome.[2] Among them, monocyte chemoattractant protein-1 (MCP-1/CC chemokine ligand 2, CCL2) is believed to play a crucial role in such processes.[5-7] However, the precise role of MCP-1 in ischaemic heart disease remains controversial. Anti-*MCP-1 gene* therapy improves survival and attenuates left ventricular (LV) dilatation and dysfunction in a murine model of MI.[5] Further, targeted deletion of its receptor CCR2 in mice also improved LV dilatation and dysfunction after MI,[6] suggesting a deleterious role for MCP-1 in post-infarct LV dysfunction and remodeling. In contrast, angiogenic and cardioprotective effects of MCP-1 have been reported.[8, 9] Supporting these reports, we recently demonstrated that cardiac MCP-1 overexpression induced macrophage infiltration, neovascularization and the accumulation of cardiac myofibroblasts, thereby resulting in the prevention of cardiac dysfunction and remodeling after permanent MI.[10] This indicates the beneficial effect of cardiac MCP-1 on MI. The reason for the discrepancy in MCP-1's role in MI remains unclear. However, since the effect of MCP-1 is regulated by its topical concentration,[11,

[12] the beneficial or deleterious effect of MCP-1 might depend on the situation, i.e. local concentration, duration, and time period after MI. In the present study, to clarify the topical role of cardiac MCP-1 after I/R injury, we evaluated LV function in Langendorff-perfused hearts isolated from the transgenic mice expressing the mouse *JE-MCP-1 gene* under the control of the α-cardiac myosin heavy chain promoter (MHC/MCP-1 mice).[13] We demonstrated that the cardiac overexpression of MCP-1 prevented LV dysfunction after I/R through ROS-dependent and K_{ATP} channel-independent pathways. These findings provide new insight into the beneficial role of MCP-1 in the pathophysiology of ischaemic heart diseases.

Materials and Methods

Experimental animals

MHC/MCP-1 mice (background: FVB) were generated as previously described.[13] FVB/N mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and used as age-matched wild-type controls for the MHC/MCP-1 mice. Mice aged 8–12 weeks were used in this study. Our previous report demonstrated no differences in the cardiac function and hypertrophy between wild-type and MHC/MCP-1 mice aged 12 weeks.[10] They were fed a standard diet and water and were maintained on a 12-h light and dark cycle. All experiments in this study were performed in accordance with the Shinshu University Guide for Laboratory Animals which conforms to the NIH Guidelines (NIH Publication No. 85-23, revised 1996).

Cell culture and reagents

Murine neonatal cardiomyocytes were prepared from the ventricles of 1 or 2-day-old mice using the protocol used for rat neonatal cardiomyocyte isolation with minor modifications.[10, 14] Briefly, after dissociation with 0.25% trypsin (Invitrogen, Carlsbad, CA) followed by treatment with 0.8 mg/mL collagenase (Wako Pure

Chemical Industries Ltd., Osaka, Japan), the cells were washed and resuspended in Dulbecco's modified Eagle's medium (DMEM: Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS: Hyclone, Logan, UT). For the isolation of cardiac fibroblasts, the cells were plated onto culture dishes for 1 h during which time cardiac fibroblasts readily attached to the bottom of the culture dishes. The non-attached cells (cardiomyocytes) were removed and plated onto other culture dishes. The isolated cardiomyocytes were grown in 10% FBS-containing DMEM. Primary cardiomyocytes were used in the experiments. To stimulate hypoxia/reoxygenation (H/R), the cells were placed in Aneropac (Mitsubishi Gas Chemical, Tokyo, Japan) for 6 h followed by 24 h of reoxygenation. The levels of PO_2 under the normoxic and hypoxic conditions were 140.7 ± 1.2 mmHg and 67.5 ± 1.5 mmHg, respectively (n=3, $p<0.0001$).

Murine recombinant MCP-1 was purchased from Pepro-Tech Inc. (Rocky Hill, NJ). 5-Hydroxydecanoate (5-HD, a mitochondrial selective K_{ATP} channel blocker); 2, 2'-azobis (2-aminodipropane), menadione sodium bisulphfite (menadione, a superoxide generator), and diphenylene iodonium (DPI), were obtained from Sigma-Aldrich (St. Louis, MO). 1-[5-[2-(5-Chloro-*o*-anisamide) ethyl]-2-methoxyphenyl] sulphonyl]-3-methylthiourea (HMR1098, a sarcolemmal K_{ATP} channel blocker) was kindly provided by Aventis Pharma (Tokyo, Japan). Unless specified otherwise, all other chemicals were obtained from Wako Pure Chemical Industries Ltd. Menadione was dissolved in a perfusate at the time of use. Other chemicals were prepared as stock solutions in dimethyl sulphoxide (DMSO) and stored at –30ºC. The final DMSO concentration in the perfusate was less than 0.1%.

Langendorff-perfused hearts

Mice were anaesthetized with sodium pentobarbital (50 mg/kg) administered intraperitoneally and then treated with 0.2 mL of 1,000 U/mL heparin intravenously. The heart was rapidly excised, cannulated and retrogradely perfused with oxygenated

(95% O_2 and 5% CO_2) modified Krebs-Henseleit solution containing (in mM) 118 NaCl, 25 $NaHCO_3$, 1.2 $MgSO_4 \cdot 7H_2O$, 4.7 KCl, 1.2 KH_2PO_4, 10 glucose and 2.5 $CaCl_2$; pH 7.4; at 37°C and placed in a semiclosed circulating water-warmed (37°C) chamber. The coronary flow rate was adjusted to maintain constant perfusion pressures of 55–70 mmHg. The temperature of the perfusate was maintained at 37°C. All the hearts were subjected to a 15-min stabilization period, followed by 20 min of global ischaemia and then 30 min of reperfusion. Hearts that demonstrated the abnormalities during the stabilization period were discarded.

Coronary perfusion pressure (CPP) was measured using a pressure transducer (Nihon Kohden Co., Tokyo, Japan). The hearts were electrically stimulated at twice the diastolic threshold current with a duration of 1 ms using a polyterafluoroethylene-coated silver bipolar electrode. Pacing was performed from the epicardial surface of the LV wall at a basic cycle length of 150 ms. A polyethylene balloon was inserted into the cavity of the left ventricle through the left atrium to measure the LV pressure and its dp/dt. The balloon was filled with water to adjust the LV diastolic pressure (LVdP) to approximately 10 mmHg. The LV developed pressure (LVDP) was calculated using the following formula: LVDP=LVsP−LVdP. All agents were added to the perfusate. K_{ATP} channel inhibitors started to perfuse at 15 min before ischaemia. Menadione was perfused for 30 min. Only 1 agent was tested in each experiment.

TUNEL assay

Apoptotic myocardial cells in the isolated neonatal murine cardiomyocytes (1×10^6 cells/35 mm tissue culture dish) were identified by terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end-labeling (TUNEL) staining. An *in situ* apoptosis detection kit (Takara Bio Inc., Shiga, Japan) was used according to the manufacturer's instructions. Briefly, cultured cardiomyocytes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), and DNA fragments were

labeled with fluorescein-conjugated dUTP with terminal deoxynucleotidyl transferase. The total cell population and condensed nuclei were estimated by counting the 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich)-stained nuclei in 10 fields of each culture dish. Further, the ratio of TUNEL-positive cells to total DAPI-stained nuclei was calculated (n=3). Additionally, the apoptotic nuclei were calculated as a percentage of the total nuclei based on DAPI staining. Measurements were performed using Scion Image software (Beta 4.03; Scion Corporation, MD). All the measurements were performed in a double-blind manner by 2 independent researchers.

DHE fluorescence assay

The oxidative fluorescent dye dihydroethidium (DHE: Molecular Probes Inc., Eugene, OR) was used to ascertain that murine cardiomyocytes produced the superoxide after I/R (n=4). Briefly, isolated Langendorff-perfused hearts were frozen in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetechnical Co. Ltd., Tokyo, Japan) and cut into 10-μm sections. These enzymatically intact sections were then incubated with DHE (10 μM) in PBS for 30 min at 37°C in a humidified chamber protected from light. The excitation wavelength was 520–585 nm, and emission fluorescence was detected with a 580 nm long-pass filter. All the measurements were performed in a double-blind manner by 2 independent researchers.

Luminol-based chemiluminescence assay

ROS formation was measured using Diogenes Cellular Luminescence Enhancement System (National Diagnostics, Atlanta, GA), according to the manufacturer's instructions. Briefly, neonatal murine cardiomyocytes were trypsinized, washed twice, and resuspended in DMEM (1 x 10^6/mL). The cells were pretreated with MCP-1 (10 ng/mL) or DPI (5 μM) for 30 min, and then stimulated with LY83583 (1

μM). ROS formation was measured immediately after the stimulation (10 second readings) in a Turner Biosystems luminometer (Promega, Madison, WI).

Western blot analysis

Expression of gp91phox and Nox4 was analyzed by Western blotting.[15] The antibodies against of gp91phox and Nox4 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The expression level of TFIIF served as an internal control for protein loading.

Assay of SOD activity

Superoxide dismutase (SOD) activities in the heart and plasma were measured using a SOD assay kit-WST (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. Briefly, heart samples were washed with saline to remove as much blood as possible; the tissue was blotted with paper towels and weighed. Five hundred microlitre of sucrose buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4) was added to the samples and homogenized using a Teflon homogenizer. After the centrifugation of the homogenized sample at 10,000 *g* for 15 min at 4ºC, the supernatant was transferred to a new tube and diluted with distilled water to prepare a sample solution. Since Mn-SOD mainly locates in mitochondria, the data in our experiment show CuZn-SOD activity. Plasma samples were prepared by centrifuging 0.5–1 mL of 10 U/mL heparin-treated peripheral blood at 600 *g* for 10 min at 4ºC.

Real-time RT-PCR analysis

Total cellular RNA was extracted using ISOGEN (Nippon Gene Co. Ltd., Toyama, Japan) or RNA-Bee (Tel-Test, Inc., Friendswood, TX), according to the manufacturer's instructions. Real-time RT-PCR analysis was performed by using the ABI Prism 7000 system (Applied Biosystems, Inc., CA) to detect the mRNA expression

of NADPH oxidase 1 (Nox1), gp91phox (Nox2), Nox3, Nox4, inducible nitric oxide synthase (iNOS), MCP-1, and β-actin. The following primers (oligonucleotide sequences are provided in parentheses in the order of antisense and sense primers) were used: Nox1 (NM_172203.1, 5′-AATGCCCAGGATCGAGGT-3′ and 5′-GATGGAAGCAAAGGGAGTGA-3′), gp91phox (NM_007807.2, 5′-CCACATACAGGCCCCCTTCAG-3′ and 5′-GTTGGGGCTGAATGTCTTCCTCTTT-3′), Nox3 (NM_198958.1, 5′-GCTGGCTGCACTTTCCAAA-3′ and 5′-AAGGTGCGGACTGGATTGAG-3′), Nox4 (NM_015760.2, 5′-TGTTGGGCCTAGGATTGTGTT-3′ and 5′-AGGGACCTTCTGTGATCCTCG-3′), iNOS (NM_010927.2, 5′-GGCAGCCTGTGAGACCTTTG-3′ and 5′-GAAGCGTTTCGGGATCTGAA-3′), MCP-1 (NM_011333.3, 5'-GGCTCAGCCAGATGCAGTTAAC-3' and 5'-GCCTACTCATTGGGATCATCTTG-3'), and β-actin (NM_007393.2, 5′-CCTGAGCGCAAGTACTCTGTGT-3′ and 5′-GCTGATCCACATCTGCTGGAA-3′). The expression levels of each target gene were normalized by subtracting the corresponding β-actin threshold cycle (C_T) values; this was done by using the $\Delta\Delta C_T$ comparative method.

Statistical analysis

Data are represented as mean ± SEM. Multiple group comparison was performed by one-way analysis of variance (ANOVA), followed by Scheffe's F-test for comparison of the means. The comparison between 2 groups was analyzed by an F-test, followed by a two-tailed t-test. Values of $p<0.05$ were considered statistically significant.

Results

Effect of MCP-1 on H/R in cardiomyocytes

To investigate whether MCP-1 exerts a cardioprotective effect on cardiomyocytes, we performed TUNEL staining to assess the anti-apoptotic effect of MCP-1 against 6 h hypoxia followed by 24 h reoxygenation (H/R) using murine neonatal cardiomyocytes *in vitro*. Exposure to the cells with H/R markedly increased the number of TUNEL-positive cells, and this number was significantly decreased when the cells were treated with MCP-1 (Fig. 1A and B). Under the normoxic culture condition, MCP-1 showed no significant effect on the cardiomyocytes. Consistent with this result, DAPI staining showed that MCP-1 treatment significantly decreased the number of the apoptotic cells; these cells were characterized by condensed nuclei (Fig. 1C and D).

Cardioprotective effect of MCP-1 on I/R injury

To investigate whether MCP-1 is upregulated in the myocardium after global I/R, real-time RT-PCR analysis was performed to assess the mRNA expression of MCP-1 in the Langendorff-perfused hearts. Global I/R significantly increased the mRNA expression of MCP-1 in the isolated hearts of wild-type mice (Fig. 2A), suggesting a potential role of cardiac MCP-1 in the I/R injury. To investigate the role of cardiac MCP-1 in the I/R injury, we used MHC/MCP-1 mice and confirmed high levels of MCP-1 mRNA expression in the heart of MHC/MCP-1 mice. We next assessed the LV function and CPP in the hearts isolated from the wild-type and MHC/MCP-1 mice. Under baseline conditions, there was no significant difference in the CPP, LVDP, LDdp/dt, LVsP and LVdP between these mice (Fig. 2B–H). In the wild-type mice, I/R resulted in marked cardiac dysfunction, indicating an increase in the CPP (Fig. 2C) and a significant decrease in the LVDP (Fig. 2D) and LVdp/dt (Fig. 2E and F). However, decreases in the LVDP and LVdp/dt after I/R were remarkably inhibited in the MHC/MCP-1 mice as compared with the wild-type mice. Body weight (data not shown), heart weight (data not shown) and CPP showed no significant difference between the

wild-type and MHC/MCP-1 mice. We further assessed the LVDP, LVsP, LVdP, $LVdp/dt_{max}$ and $-LVdp/dt_{max}$ for 30 min after reperfusion and found that the LV diastolic function rather than the LV systolic function was improved in the MHC/MCP-1 mice (Fig. 2G and H).

Effects of K_{ATP} channel inhibitors

Since ischaemic preconditioning (IPC) is one of the most powerful protective mechanisms known for myocardial I/R injury and is mediated through 2 types of K_{ATP} channels namely the mitochondrial and sarcolemmal K_{ATP} channels in the murine hearts,[16, 17] we examined the effect of K_{ATP} channel blockers on the recovery of LV function after reperfusion in the hearts of the MHC/MCP-1 mice. Treatment with the sarcolemmal K_{ATP} channel blocker HMR1098 (30 μM) demonstrated no significant effects on the CPP, LVDP, and LVdp/dt in the MHC/MCP-1 mice (Fig. 3A–E). Similarly, the mitochondrial specific K_{ATP} channel blocker 5-HD (100 μM) also had no significant effect on the LV function in the hearts of the MHC/MCP-1 mice (data not shown). Further, these K_{ATP} channel blocker did not affect cardioprotective effects of MCP-1 (Fig. 3F and G). These findings suggest that the cardioprotective effect of MCP-1 is mediated through a K_{ATP} channel-independent pathway.

Role of ROS generation

Since ROS have been implicated as the major cause of myocardial I/R injury, cardiac ROS production was evaluated by DHE fluorescence. The heart sections of the wild-type mice demonstrated strikingly enhanced ROS generation after I/R, and this ROS generation was inhibited by the treatment with DPI, an NADPH oxidase inhibitor (Fig. 4A). In addition, ROS generation was markedly inhibited in the hearts of the MHC/MCP-1 mice (Fig. 4B), suggesting that ROS generation played a substantial role in the MCP-1-induced cardioprotective effect. We next investigated the effect of the

pharmacological superoxide generator menadione. Treatment with menadione (100 μM) for 30 min induced superoxide generation and a slight decrease in the LVDP and LVdp/dt in the hearts of the wild-type mice (Fig. 4B–F). Although the levels of superoxide generation induced by menadione was slightly decreased in the MHC/MCP-1 mice (Fig. 4B), no significant differences were observed in the maximum decreases of LVDP and LVdp/dt between the hearts of the wild-type and MHC/MCP-1 mice (Fig. 4C–F). Further, a higher dose of menadione (1,000 μM) did not show the LV contraction but increase the LVdP in the hearts of both mice (Fig. 4C–F).

Nox expression and SOD activity

The NADPH oxidase family of enzymes is reported to be the major sources of ROS in the cardiovascular system.[18, 19] Because Nox1, gp91phox (Nox2), Nox3, and Nox4 in the NADPH oxidase family are present in the murine heart,[18] the mRNA expression of these molecules was assessed by real-time RT-PCR analysis. Under the baseline conditions, the mRNA expression of Nox1, gp91phox, and Nox3 in the hearts of the MHC/MCP-1 mice was lower than that in the hearts of the wild-type mice (Fig. 5A–D). Although the mRNA expression of Nox1, gp91phox, Nox3, and Nox4 was decreased after reperfusion in the wild-type hearts, the expression tended to be unchanged or increased after I/R in the MHC/MCP-1 hearts. We also showed that iNOS mRNA expression tended to be lower than that in the hearts of the wild-type mice under the baseline conditions (Fig. 5E). Western blot analysis showed that protein expression of gp91phox, but not Nox4, tended to be lower in MHC/MCP-1 mice than that in the wild-type mice (Fig. 5F and G).

Cardiac superoxide scavenging systems could participate in the prevention of myocardial I/R injury. To examine whether this system is upregulated in the hearts of MHC/MCP-1 mice, the CuZn-SOD activity in the heart and plasma was evaluated. The CuZn-SOD activity in the hearts showed a significant increase in the MHC/MCP-1

mice as compared with the wild-type mice (Fig. 6A). However, there was no significant difference in the plasma SOD activity between the wild-type and MHC/MCP-1 mice under the baseline conditions (Fig. 6B).

To investigate the role of ROS in MCP-1-induced cardioprotection, we used naphthoquinolinedione LY83583 to generate superoxide in cardiomyocytes. This compound freely crosses cell membranes and generates superoxide via metabolism by cytosolic and membrane-bound NADPH oxidases.[20, 21] LY83583 markedly stimulated superoxide production, and this superoxide production was inhibited by the pretreatment with MCP-1 (Supplemental figure I). As expected, DPI almost completely inhibited LY83583-stimulated superoxide production.

Discussion

The major findings of this study are as follows: (1) Exogenous MCP-1 treatments prevented the apoptosis of cardiomyocytes after H/R *in vitro*; (2) Global I/R significantly increased the mRNA expression of MCP-1 in the hearts of wild-type mice; (3) Cardiac overexpression of MCP-1 improved LV dysfunction after I/R; in particular, it ameliorated LV diastolic pressure after reperfusion; (4) Cardiac overexpression of MCP-1 had no significant effect on coronary flow; (5) The improvement in LV dysfunction in the MHC/MCP-1 mice was independent of both the sarcolemmal and mitochondrial K_{ATP} channels; (6) Cardiac overexpression of MCP-1 inhibited superoxide generation in the hearts with global I/R injury, and these demonstrated decreased expression of the NADPH oxidase family, namely, Nox1, gp91phox and Nox3 as compared with wild-type mice; (7) SOD activity in the hearts of the MHC/MCP-1 mice was significantly increased as compared with that of the wild-type mice. These findings suggested that cardiac MCP-1 prevented LV dysfunction after global I/R through a ROS-dependent but K_{ATP} channel-independent pathway.

Recently, we demonstrated the beneficial role of cardiac MCP-1 in a murine

model of permanent MI;[10] however, there are a number of differences between permanent MI and I/R injury.[2, 3, 22] Reperfusion releases an excess of oxygen-derived ROS and produces I/R injury. In a reperfused heart, the apoptosis of cardiomyocytes and inflammatory responses such as the infiltration of neutrophils and macrophages are much stronger than in an infarcted heart. Recently, Martire et al.[23] reported that the cardiac overexpression of MCP-1 caused a reduction in apoptosis and the infarcted area after I/R through the stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) pathway. Consistent with this, we clearly demonstrated that cardiac MCP-1 attenuated LV dysfunction without any effects on coronary flow after I/R. In particular, the contribution of inflammatory cells to the myocardial damage was eliminated because Langendorff-perfused hearts were used in this study. In addition, MCP-1 exerted anti-apoptotic effects in cultured cardiomyocytes. Taken together, MCP-1 directly induces cardioprotection in cardiomyocytes.

Generally, IPC is known as the protective mechanism for ischaemic myocardial injury whereby brief ischaemia protects against subsequent ischaemic insults.[24] Several triggers have been proposed for IPC, including adenosine, bradykinin, prostaglandins, opioid receptors, nitric oxide, and Ca^{2+}.[25] These triggers lead to the activation of several intracellular pathways that protect cardiomyocytes against myocardial I/R injury.[26] Specifically, the opening of the mitochondrial K_{ATP} channel is believed to be critical for the induction of IPC.[17, 27] However, Suzuki et al.[16] demonstrated that IPC is abolished in sarcolemmal K_{ATP} channel-deficient mice despite intact mitochondrial K_{ATP} channel function; this indicates the important role of both types of K_{ATP} channels in IPC. Since the effect of MCP-1 observed in our study is similar to the effect of IPC, we examined the effect of HMR1098 and 5-HD on cardioprotection by MCP-1. However, neither HMR1098 nor 5-HD prevented the improvement of LV dysfunction in MHC/MCP-1 mice. These results indicate that MCP-1 induced the cardioprotective effect via mechanisms other than those involving

K_{ATP} channels.

During the I/R process, cardiomyocytes are subjected to acute oxygen depletion followed by sudden reoxygenation. The deficient oxygen supply generates ROS, including free radicals (i.e. superoxide) and non-radicals (i.e. hydrogen peroxide).[28] The production of ROS during I/R further promotes cardiomyocyte damage and results in myocardial dysfunction. In our study, superoxide generation was markedly reduced in the hearts of the MHC/MCP-1 mice; this suggests that ROS contributes to the mechanisms by which MCP-1 protects against I/R injury. Interestingly, however, the cardiac overexpression of MCP-1 failed to protect against menadione-induced LV dysfunction although the levels of superoxide generation induced by menadione was slightly decreased in the MHC/MCP-1 mice. There are several possibilities for the reason of this discrepancy: First, the amount of ROS produced by menadione is quite high compared to I/R based on the DHE data. Therefore, MCP-1 may be unable to compensate effectively. Second, menadione-induced signaling for ROS production may not be affected by MCP-1. In this regard, Criddle et al.[29] reported that menadione-induced ROS production is not mediated through NADPH oxidase in murine pancreatic cells. Third, the etiology of LV dysfunction induced by menadione vs. I/R may be different.

The potential sources of cardiac ROS include mitochondria, xanthine oxidases, uncoupled NO synthases (NOS), and NADPH oxidases.[30] NADPH oxidase has been recently addressed as a major source of superoxide in the myocardium.[31] We demonstrated that a NADPH oxidase inhibitor DPI inhibited I/R-induced superoxide generation in the Langendorff-perfused hearts. Consistent with our results, Angelos et al.[32] recently reported that DPI treatment showed a significant reduction in oxygen free radical formation induced by hypoxic reperfusion. These findings indicate that I/R-induced superoxide generation is mediated through, at least in part, activation of NADPH oxidases in the heart. NADPH oxidases appear to be the enzymes whose

primary function is ROS generation; they are also particularly important for redox signaling. Further, ROS derived from NADPH oxidases can induce NOS uncoupling through tetrahyrdrobiopterin oxidation as well as xanthine oxidase activation; thus, they play the role of priming sources for the amplification of ROS production.[30] In mice, the family of NADPH oxidase includes Nox1, gp91phox, Nox3, and Nox4.[18] We observed decreased expression of Nox1, gp91phox, and Nox3 in the hearts of MHC/MCP-1 mice. Regarding the relationship between NADPH oxidase and I/R injury, gp91phox was indicated as essential for the preconditioning of isolated hearts in response to transient global ischaemia but not to a pharmacological agonist, suggesting the beneficial role of gp91phox in the acute response to ischaemia in particular.[19] Although there are some reports in the literature suggesting that iNOS expression may be increased during myocardial I/R,[33] we observed decreased expression of iNOS after I/R. Supporting our finding, Rakhit et al.[34] showed the decrease of iNOS protein due to preconditioning in a simulated ischemia model of cultured cardiomyocytes. Although the reason for this discrepancy is unclear, we speculate that it is due to the experimental conditions such as the protocols and timing for the sample collection. We also observed that the SOD activity was higher in the hearts of the MHC/MCP-1 mice than the wild-type mice. However, since the increase of SOD activity in the myocardium of MHC/MCP-1 mice was small, this might be not sufficient to provide cardioprotection. Therefore, we postulate that the MCP-1-induced cardioprotective effect against I/R was mediated through not only the inhibition of superoxide generation but also the free radical scavenging pathway.

Limitations

Several limitations of this study should be noted. First, to investigate the role of MCP-1 in cardiomyocytes *in vitro*, neonatal cardiomyocytes obtained from the wild-type mice were used in this study. Since the α-cardiac MHC gene is expressed

mainly in the adult cardiac muscle, the expression levels of MCP-1 in the hearts of neonatal MHC/MCP-1 mice are known to be low.[13] Second, even though the present study suggests a role of ROS in the MCP-1-induced cardioprotective effect, we have not identified the species of oxidants and its sources. Since not only NADPH oxidase but also xanthine oxidases and others have been demonstrated as the potential sources of cardiac ROS, further investigations required to elucidate the precise mechanisms underlying MCP-1-induced cardioprotection after I/R.

Conclusion

We demonstrated that cardiac MCP-1 inhibited the generation of ROS, thereby resulting in the prevention of LV dysfunction after global I/R. Although the effect of MCP-1 on I/R injury is currently controversial, several previous studies suggest MCP-1 inhibition as a potential target for therapeutic intervention in patients with ischaemic heart diseases.[5, 6] The findings of our study indicate that further investigations are necessary to elucidate the precise role of MCP-1 in ischaemic heart diseases prior to its clinical application. Further, this study provides new insight into the potential benefit of MCP-1 in ischaemic heart diseases.

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Conflict of Interest

None.

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Figure Legends

Figure 1. Effect of MCP-1 on H/R in cardiomyocytes

Neonatal murine cardiomyocytes were treated with 6 h hypoxia followed by 24 h reoxygenation (H/R) in the presence or absence of MCP-1 (10 ng/mL). (A) TUNEL and DAPI staining was performed. (B) Bar graph shows the quantitative analysis of TUNEL-positive cardiomyocytes/DAPI-stained cells (n=6). $^{###}p<0.001$ vs. H/R; $^{*}p<0.05$, $^{***}p<0.001$ vs. control. (C) Nuclei condensation was evaluated by DAPI staining in the cardiomyocytes under high power fields (n=6). (D) Bar graph shows quantitative analysis of the nuclear condensation in cardiomyocytes/DAPI-stained cells (n = 6). $^{##}p<0.01$ vs. H/R; $^{**}p<0.01$, $^{***}p<0.001$ vs. control.

Figure 2. MCP-1 mRNA expression and the cardioprotective effect of MCP-1

(A) Bar graph shows the mRNA expression of MCP-1 before and at 30 min after reperfusion (I/R) in the isolated hearts of wild-type and MHC/MCP-1 mice (n=5). $^{**}p < 0.01$ vs. Pre. (B–H) Isolated hearts were obtained from wild-type and MHC/MCP-1 mice and analyzed after Langendorff-perfusion. (B) Representative changes in the LVDP and LVdp/dt during I/R are shown (n=7 per group). (C) The time course of CPP in the hearts of the wild-type and MHC/MCP-1 mice during I/R is shown. The time courses of the LVDP (D), LVdp/dt (E), –LVdp/dt (F), LVsP (G) and LVdP (H) of the hearts of the wild-type and MHC/MCP-1 mice during ischaemia and reperfusion are shown (n = 7). $^{*}p<0.05$, $^{**}p<0.01$ vs. wild-type.

Figure 3. Effects of K_{ATP} channel inhibitors

Isolated hearts were obtained from wild-type and MHC/MCP-1 mice. The hearts were analyzed after Langendorff-perfusion in the presence or absence of HMR1098 (30 μM). (A) Representative changes in LVDP and LVdp/dt during 20 min hypoxia followed by 30 min reperfusion (I/R) are shown (n=5 per group). (B) The time course of CPP in the

hearts of the wild-type and MHC/MCP-1 mice during I/R is shown. (C–E) The time courses of LVDP (C), LVdp/dt (D) and –LVdp/dt (E) of the hearts of the wild-type and MHC/MCP-1 mice during ischaemia and reperfusion are shown (n=5). (F–G) Neonatal murine cardiomyocytes were treated with 6 h hypoxia followed by 24 h reoxygenation (H/R) in the presence or absence of MCP-1 and/or HMR1098 (n=3). (F) TUNEL and DAPI staining was performed. (G) Bar graph shows the quantitative analysis of TUNEL-positive cardiomyocytes/DAPI-stained cells (n=6). $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. H/R; $^{*}p<0.05$, $^{***}p<0.001$ vs. control.

Figure 4. Role of ROS generation

(A–B) Heart sections were obtained from wild-type and MHC/MCP-1 mice before (Pre) and at 30 min after reperfusion (I/R) or menadione treatment, and stained with DHE. The results are representative of 3–5 independent experiments. (C) Representative changes in the LVDP and LVdp/dt for 30 min after menadione treatment (n=5). (D−F) Bar graphs show the maximum changes in the LVDP and +LVdp/dt (n=3).

Figure 5. Nox and iNOS expression

Total RNA or cell lysates were extracted from the hearts of wild-type and MHC/MCP-1 mice before (Pre) and at 30 min after reperfusion (I/R). (A–E) Real-time RT-PCR was performed to evaluate the mRNA expressions of Nox1 (A), gp91phox (B), Nox3 (C), Nox4 (D), and iNOS (E) (n=4). (F−G) Western blot analysis was performed to evaluate the protein expression of gp91phox and Nox4 (F). TFIIF served as the loading control. Bar graph shows the relative expression levels quantified by the densitometry (G) (n=3).

Figure 6. SOD activity

Bar graphs show the SOD activities of the hearts (A) and plasma (B) in the wild-type

and MHC/MCP-1 mice under the baseline conditions (n=5). $^{***}p<0.001$ vs. wild-type.

Figure 1

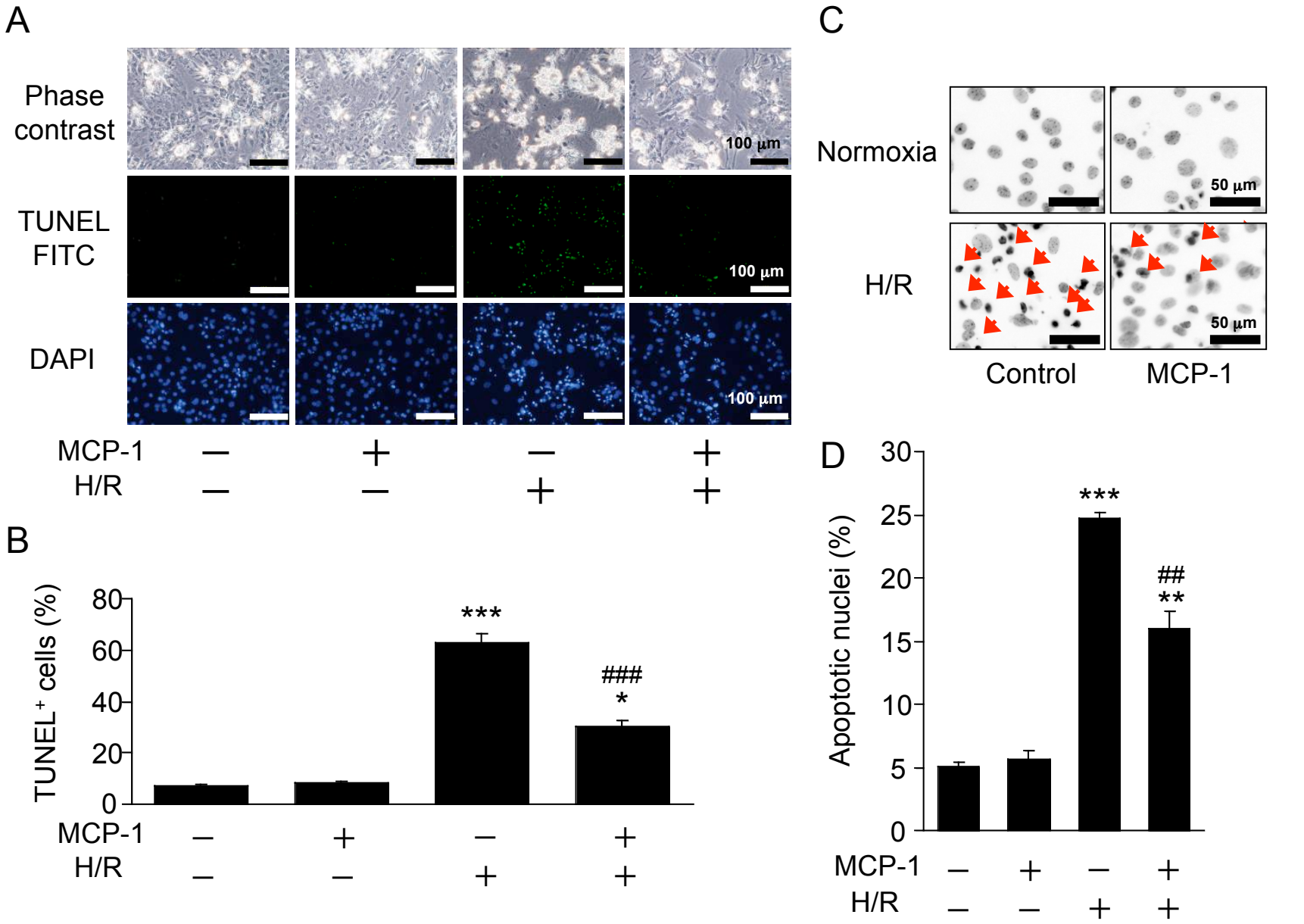

A
Phase contrast
TUNEL FITC
DAPI
100 μm
MCP-1 – + – +
H/R – – + +
B
TUNEL+ cells (%)
80
60
40
20
0
***
###
*
MCP-1 – + – +
H/R – – + +
C
Normoxia
H/R
50 μm
Control
MCP-1
D
Apoptotic nuclei (%)
30
25
20
15
10
5
0
***
##
**
MCP-1 – + – +
H/R – – + +

Figure 2

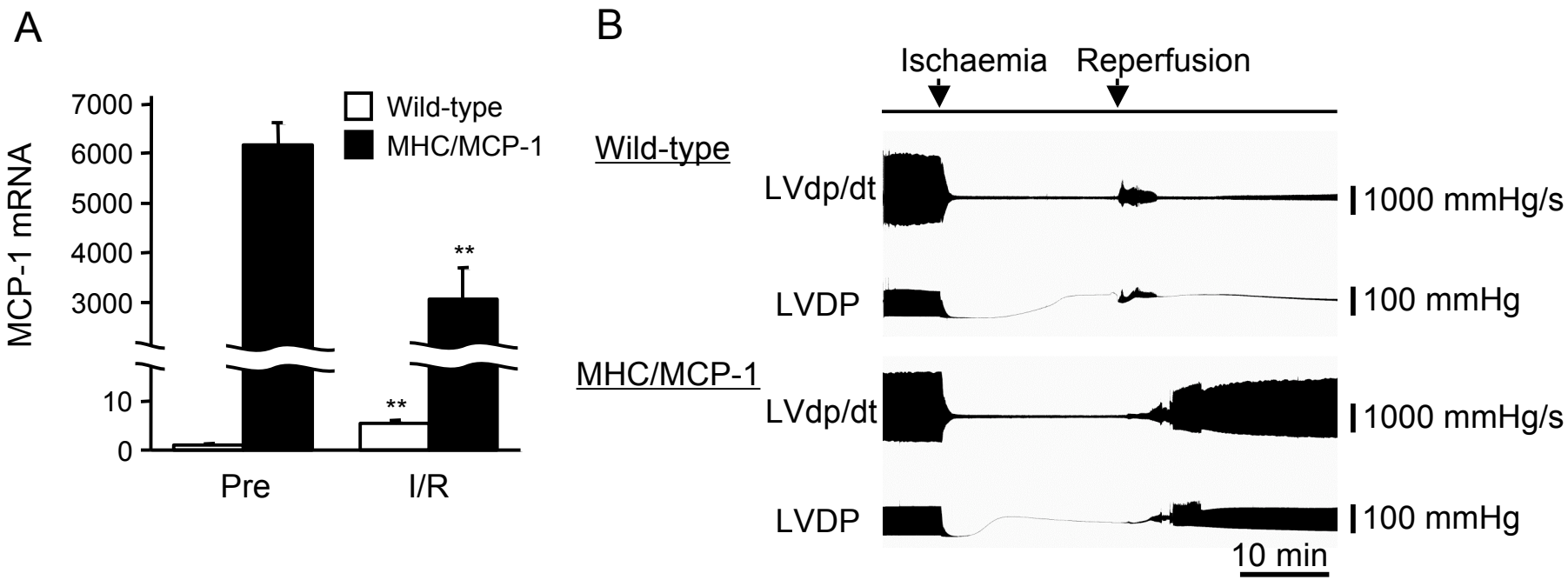

A
MCP-1 mRNA
7000
6000
5000
4000
3000
10
0
Wild-type
MHC/MCP-1
**
**
Pre
I/R
B
Ischaemia
Reperfusion
Wild-type
LVdp/dt
1000 mmHg/s
LVDP
100 mmHg
MHC/MCP-1
LVdp/dt
1000 mmHg/s
LVDP
100 mmHg
10 min

Figure 2

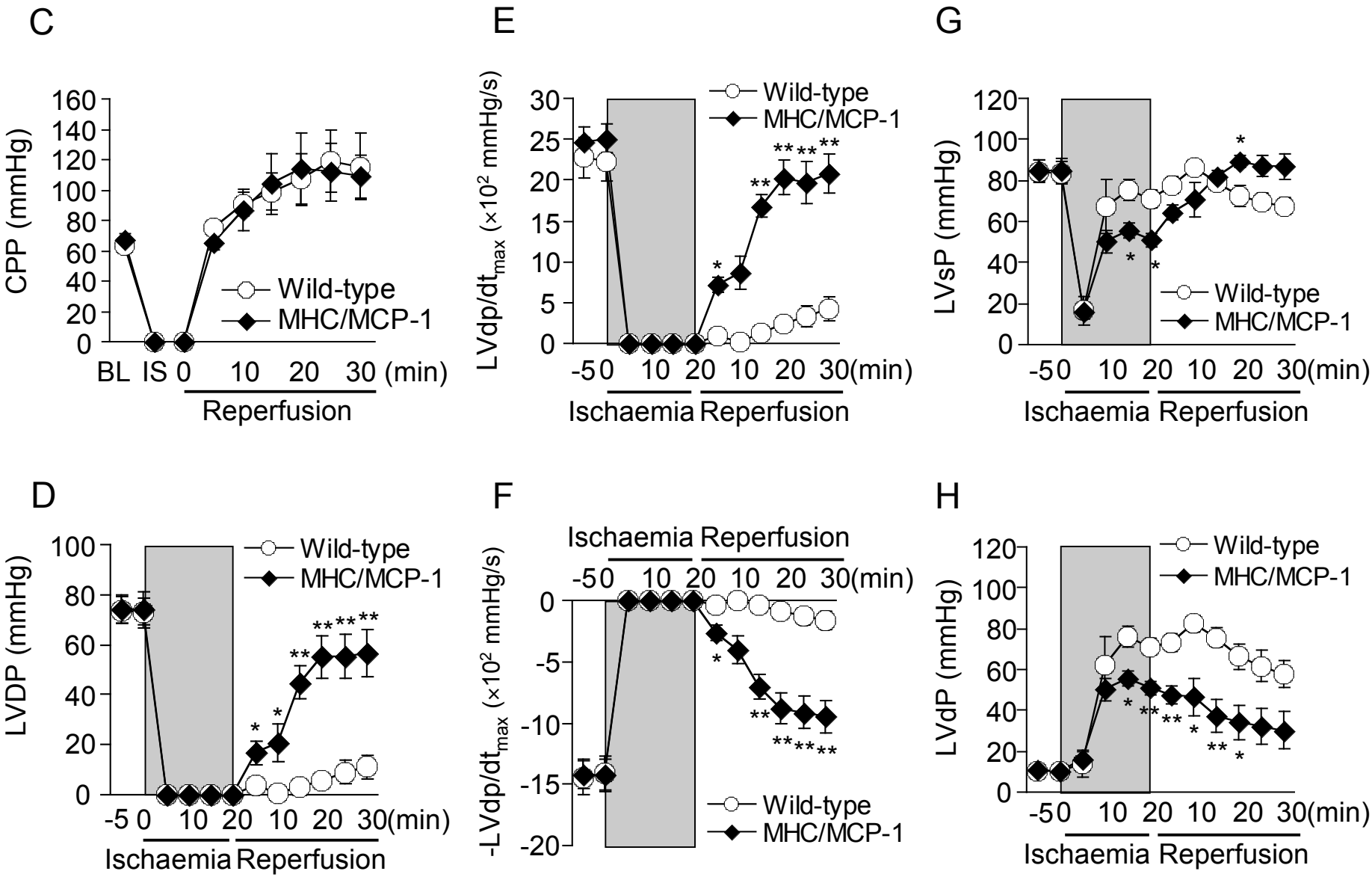

C
CPP (mmHg)
160
140
120
100
80
60
40
20
0
BL IS 0 10 20 30 (min)
Reperfusion
Wild-type
MHC/MCP-1
E
LVdp/dtmax (×10² mmHg/s)
30
25
20
15
10
5
0
-5 0 10 20 10 20 30(min)
Ischaemia Reperfusion
Wild-type
MHC/MCP-1
G
LVsP (mmHg)
120
100
80
60
40
20
0
-5 0 10 20 10 20 30(min)
Ischaemia Reperfusion
Wild-type
MHC/MCP-1
D
LVDP (mmHg)
100
80
60
40
20
0
-5 0 10 20 10 20 30(min)
Ischaemia Reperfusion
Wild-type
MHC/MCP-1
F
-LVdp/dtmax (×10² mmHg/s)
Ischaemia Reperfusion
-5 0 10 20 10 20 30(min)
0
-5
-10
-15
-20
Wild-type
MHC/MCP-1
H
LVdP (mmHg)
120
100
80
60
40
20
0
-5 0 10 20 10 20 30(min)
Ischaemia Reperfusion
Wild-type
MHC/MCP-1

Figure 3

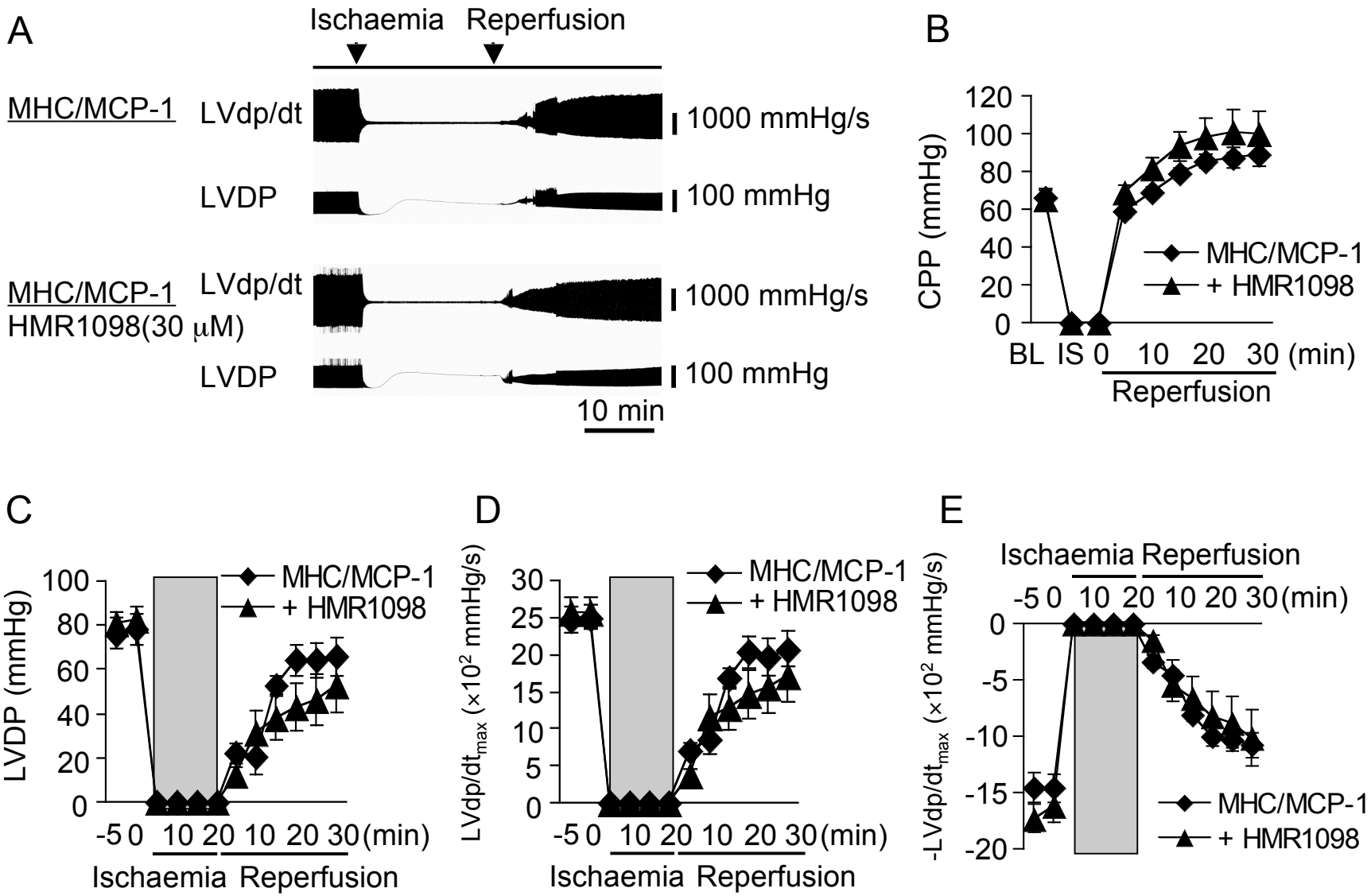

A
Ischaemia
Reperfusion
MHC/MCP-1
LVdp/dt
1000 mmHg/s
LVDP
100 mmHg
MHC/MCP-1
HMR1098(30 μM)
LVdp/dt
1000 mmHg/s
LVDP
100 mmHg
10 min
B
CPP (mmHg)
120
100
80
60
40
20
0
BL IS 0 10 20 30 (min)
Reperfusion
MHC/MCP-1
+ HMR1098
C
LVDP (mmHg)
100
80
60
40
20
0
-5 0 10 20 10 20 30 (min)
Ischaemia Reperfusion
MHC/MCP-1
+ HMR1098
D
LVdp/dtmax (×10² mmHg/s)
30
25
20
15
10
5
0
-5 0 10 20 10 20 30 (min)
Ischaemia Reperfusion
MHC/MCP-1
+ HMR1098
E
Ischaemia Reperfusion
-5 0 10 20 10 20 30 (min)
-LVdp/dtmax (×10² mmHg/s)
0
-5
-10
-15
-20
MHC/MCP-1
+ HMR1098

Figure 3

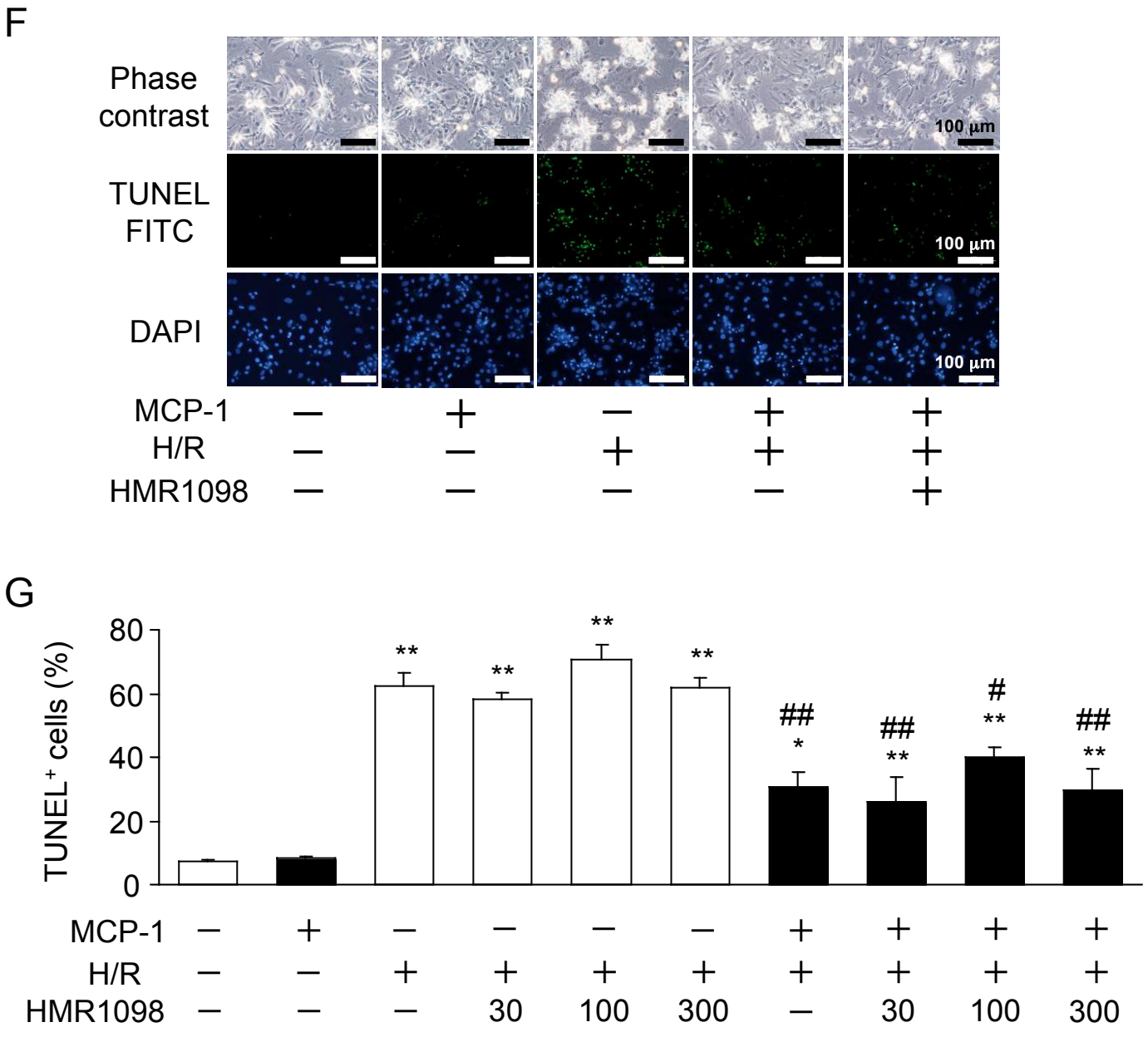

F
Phase contrast
TUNEL FITC
DAPI
100 μm
MCP-1 − + − + +
H/R − − + + +
HMR1098 − − − − +
G
TUNEL+ cells (%)
80
60
40
20
0
**
*
##
#
MCP-1 − + − − − − + + + +
H/R − − + + + + + + + +
HMR1098 − − − 30 100 300 − 30 100 300

Figure 4

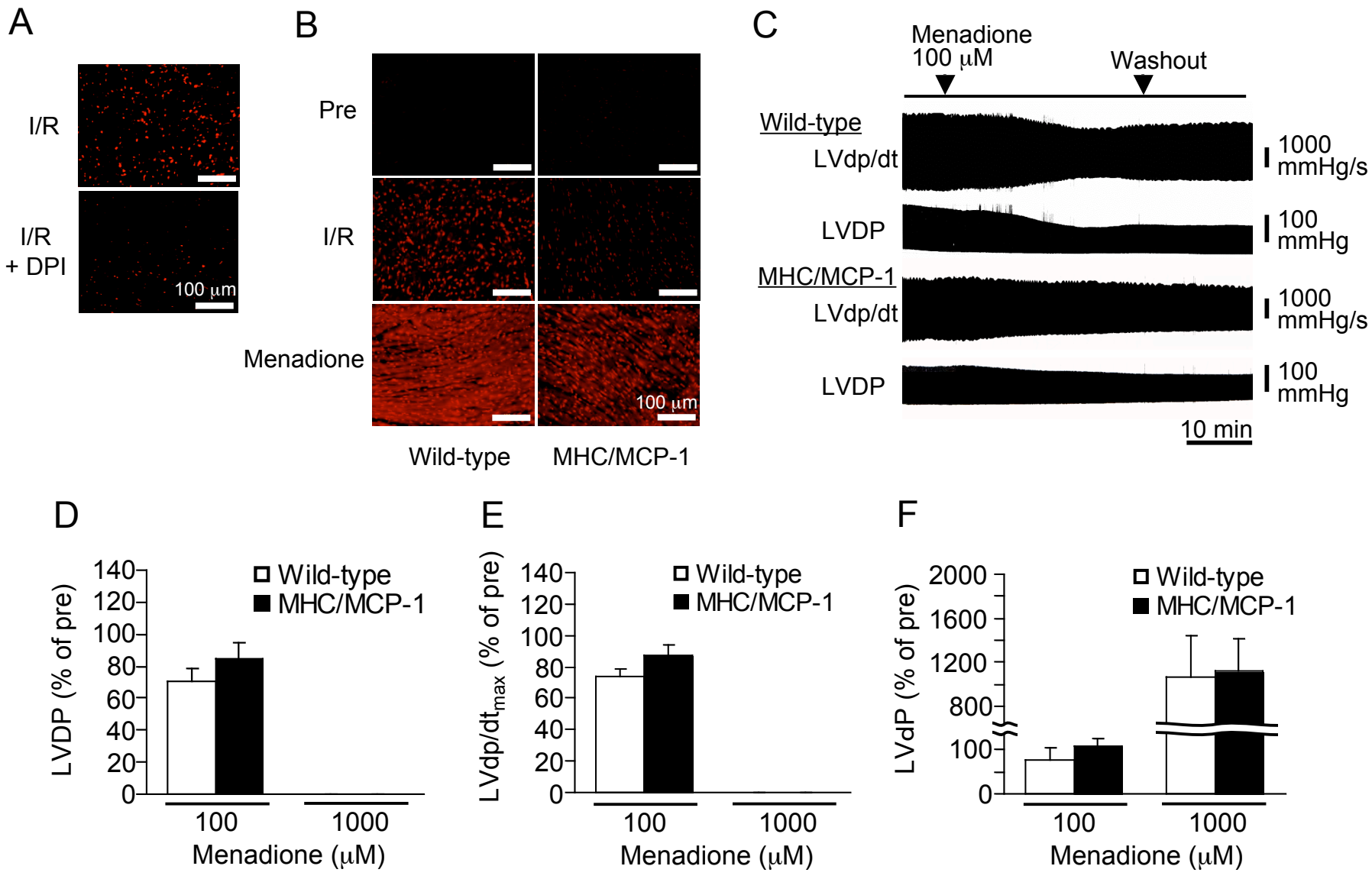
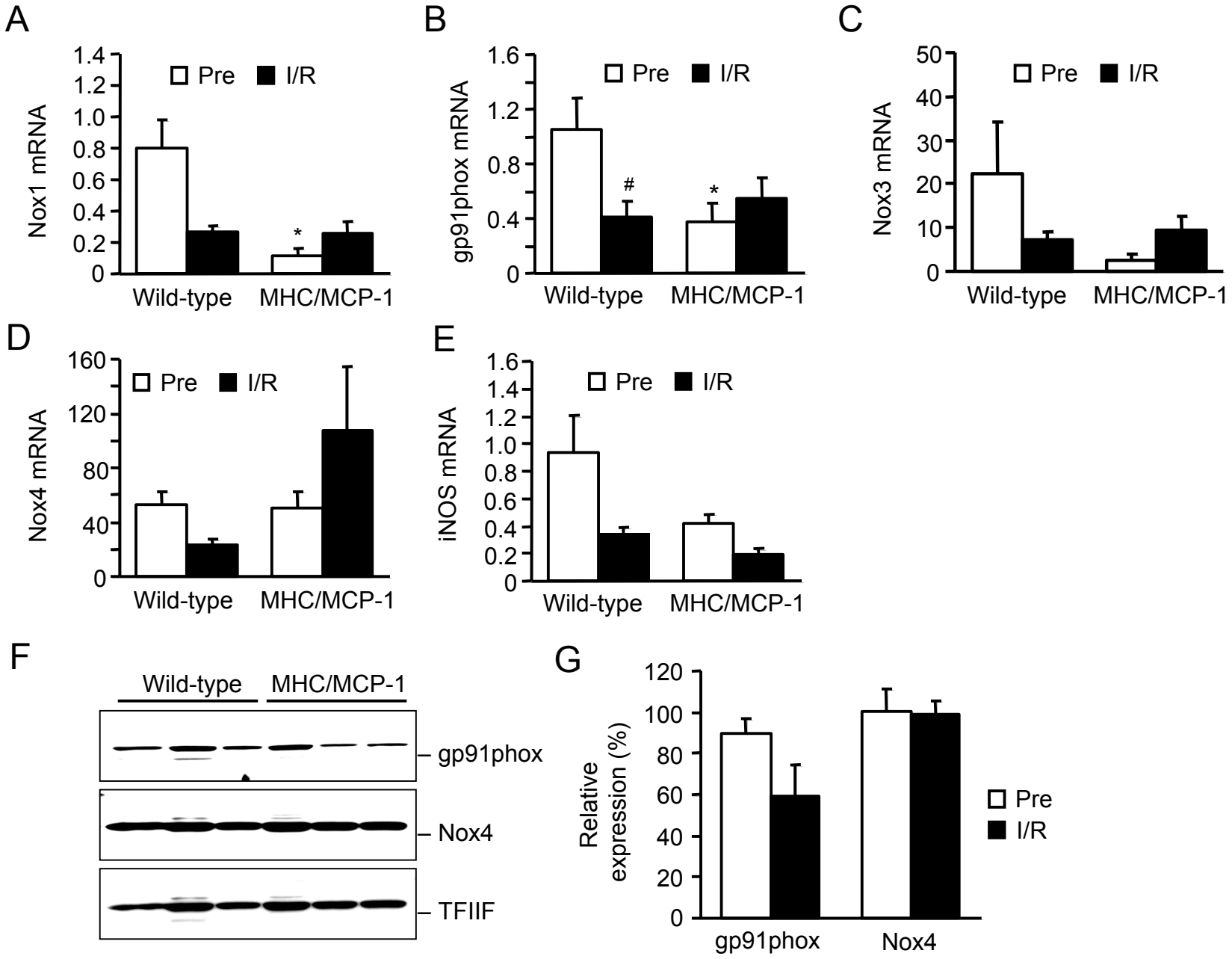
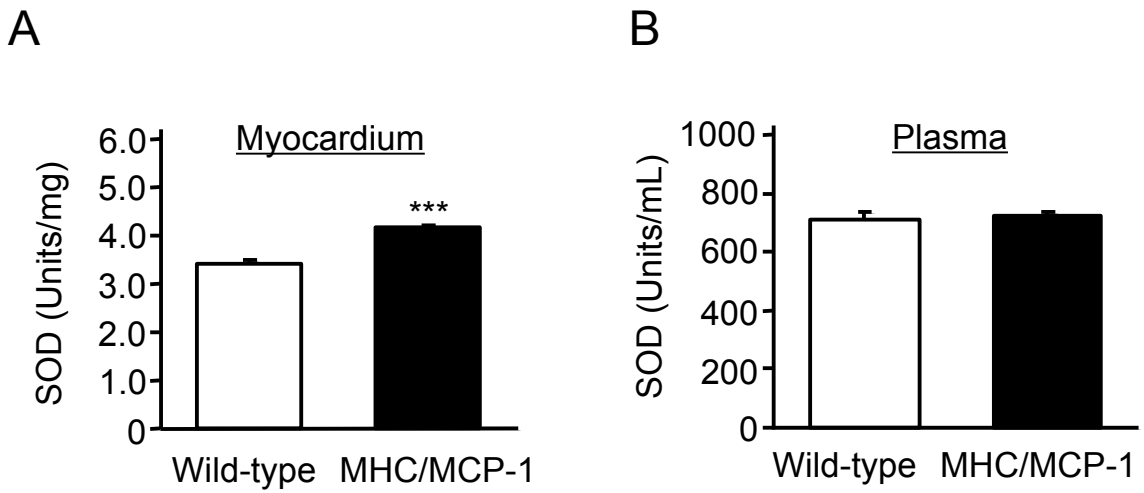

A
I/R
I/R
+ DPI
100 μm
B
Pre
I/R
Menadione
100 μm
Wild-type
MHC/MCP-1
C
Menadione
100 μM
Washout
Wild-type
LVdp/dt
1000
mmHg/s
LVDP
100
mmHg
MHC/MCP-1
LVdp/dt
1000
mmHg/s
LVDP
100
mmHg
10 min
D
LVDP (% of pre)
140
120
100
80
60
40
20
0
Wild-type
MHC/MCP-1
100
1000
Menadione (μM)
E
LVdp/dtmax (% of pre)
140
120
100
80
60
40
20
0
Wild-type
MHC/MCP-1
100
1000
Menadione (μM)
F
LVdP (% of pre)
2000
1600
1200
800
100
0
Wild-type
MHC/MCP-1
100
1000
Menadione (μM)

Figure 5


A
Nox1 mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Pre
I/R
*
Wild-type
MHC/MCP-1
B
gp91phox mRNA
1.6
1.2
0.8
0.4
0
Pre
I/R
#
*
Wild-type
MHC/MCP-1
C
Nox3 mRNA
50
40
30
20
10
0
Pre
I/R
Wild-type
MHC/MCP-1
D
Nox4 mRNA
160
120
80
40
0
Pre
I/R
Wild-type
MHC/MCP-1
E
iNOS mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Pre
I/R
Wild-type
MHC/MCP-1
F
Wild-type
MHC/MCP-1
gp91phox
Nox4
TFIIF
G
Relative expression (%)
120
100
80
60
40
20
0
Pre
I/R
gp91phox
Nox4


A
Myocardium
SOD (Units/mg)
6.0
5.0
4.0
3.0
2.0
1.0
0
***
Wild-type
MHC/MCP-1
B
Plasma
SOD (Units/mL)
1000
800
600
400
200
0
Wild-type
MHC/MCP-1