Revised, September 01, 2013

Title

Milrinone-induced postconditioning reduces hepatic ischemia-reperfusion injury in rats: the roles of phosphatidylinositol 3-kinase and nitric oxide

Short title

Milrinone-induced postconditioning

Category

Transplantation/Immunology

Tomomi Toyoda, M.D.,[a] Shinya Tosaka, M.D.,[a] Reiko Tosaka, M.D.,[a] Takuji Maekawa, M.D.,[a] Sungsam Cho, M.D.,[a] Susumu Eguchi, Ph.D., M.D.,[b] Masahiro Nakashima, Ph.D., M.D.,[c] Koji Sumikawa, Ph.D., M.D.[d]

[a] Department of Anesthesiology, Nagasaki University, Nagasaki, Japan

[b] Department of Surgery, Nagasaki University, Nagasaki, Japan

[c] Department of Tumor and Diagnostic Pathology, Nagasaki University, Nagasaki, Japan

[d] Department of Anesthesiology, Saiseikai Nagasaki Hospital, Nagasaki, Japan

Abstract (237 words)

Background: Ischemic postconditioning (PostC) protects the liver against ischemia-reperfusion (IR) injury. Milrinone, a phosphodiesterase 3 inhibitor, has been reported to exhibit preconditioning properties against hepatic IR injury; however, its PostC properties remain unknown. This study investigated whether milrinone has PostC properties against hepatic IR injury, and the roles of phosphatidylinositol 3-kinase (PI3K) and nitric oxide synthase (NOS).

Materials and methods: Male Wistar rats were separated into six groups: (1) group S: animals that underwent sham operation without ischemia, (2) group C: ischemia followed by reperfusion with no other intervention, (3) group M: milrinone administered immediately after reperfusion, (4) group MW: wortmannin, a PI3K inhibitor, injected prior to milrinone administration, (5) group MN: L-NAME, a NOS inhibitor, injected prior to milrinone administration, and (6) group MD, milrinone administered 30 min after reperfusion. Except for group S, all groups underwent 1 h of warm ischemia of median and left lateral lobes, followed by 5 h of reperfusion. Biochemical liver function analysis and histological examination were performed.

Results: Serum aspartate aminotransferase, alanine aminotransferase, and lactic dehydrogenase levels, histological damage scores, and apoptotic rate in group M were significantly lower than those in group C. The inhibition of PI3K or NOS prevented this protective effect. Milrinone administered 30 min after reperfusion did not show obvious protective effects.

Conclusions: Milrinone-induced PostC protects against hepatic IR injury when it is administered immediately after reperfusion, and PI3K and NOS may play an important role in this protective effect.

Key Words

milrinone; ischemia-reperfusion injury; liver; phosphatidylinositol 3-kinase; nitric oxide synthase

1. Introduction

Major liver surgery, including partial hepatectomy with temporary portal inflow occlusion or liver donor preservation and transplantation, is inevitably associated with ischemia-reperfusion (IR) injury. This hepatic IR injury still remains a factor that contributes to the morbidity and mortality after operation [1]. Various mechanisms have been implicated in hepatic IR injury, including reactive oxygen species (ROS), leukocyte migration and activation, microcirculatory abnormalities, sinusoidal endothelial cell damage, activation of the coagulation cascade, Kupffer cell activation due to the release of inflammatory cytokines, and mitochondrial dysfunction [2,3]. Ischemic preconditioning (PreC), defined as brief periods of ischemia and reperfusion before sustained ischemia, is a promising approach to minimize hepatic IR injury in animals and humans [2-4]. Furthermore, recent studies have proved that several brief cycles of ischemia and reperfusion at the onset of sustained reperfusion after ischemia, termed ischemic postconditioning (PostC), provided protective effects against hepatic IR injury [5-7]. PostC is more likely than PreC to be feasible for clinical application because the onset of reperfusion is more predictable.

Although the mechanisms responsible for ischemic PostC of the liver are not as well characterized as those responsible for PreC, recent studies have shown that the two processes have many similarities, including adenosine- [8], phosphoinositide 3-kinase (PI3K)/Akt- [6], and endothelial and inducible nitric oxide synthase (eNOS and iNOS)-mediated NO production [5], as well as inhibition of the opening of the mitochondrial permeability transition pore (mPTP) [7]. Similarly, studies have demonstrated that some agents, such as adenosine A2A receptor agonist [8],

recombinant erythropoietin (rhEPO) [9], and the volatile anesthetic sevoflurane [10], can be used as pharmacological inducers of PostC as well as PreC.

Milrinone, a phosphodiesterase 3 inhibitor (PDE-I), leads to sequential elevation of intracellular cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase (PKA) activation and exerts positive inotropic and vasodilatory effects [11]. Milrinone has been clinically used for the treatment of acute heart failure or low-output syndrome to facilitate functional recovery from myocardial dysfunction and to preserve the perfusion of major organs [12,13]. It has been reported that milrinone has PreC properties against hepatic IR injury via cAMP/PKA activation [14,15]. Milrinone-induced heart PostC is associated with the activation of PKA/Akt-dependent anti-apoptotic signaling [16]. Furthermore, studies have shown that cardioprotection is associated with NO production following Akt-mediated eNOS activation [17,18]. Given the above background, we designed a study to evaluate the pharmacological PostC properties of milrinone against hepatic warm IR injury, and moreover to clarify the effect of milrinone administration time during reperfusion, that is, administration early and late after reperfusion. We also examined whether milrinone-induced PostC could be mediated by PI3K and NOS.

2. Materials and methods

All experimental procedures and protocols described in this study were approved by the Institutional Animal Care and Use Committee of Nagasaki University School of Medicine, Japan.

2.1. Materials

Milrinone was purchased from Astellas Pharma Co. (Tokyo, Japan). The other chemicals and drugs used were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. Wortmannin was dissolved in dimethyl sulfoxide and diluted tenfold with saline. Milrinone and N-nitro-L-arginine methylester (L-NAME) were diluted and dissolved with saline.

2.2. Surgical procedure and experimental protocol

Male Wistar rats weighing 320 to 430 g were anesthetized with pentobarbital sodium (a 50 mg/kg intraperitoneal bolus). A catheter was inserted into the right jugular vein for administering fluids or drugs. Thereafter, 12 mg/kg/h pentobarbital in Ringer's solution was infused at a rate of 10 ml/kg/h until the experimental procedures had been completed. After tracheotomy, the trachea was intubated with a cannula connected to a small animal ventilator (model SAR-830 CWE, PA, USA), and the lungs were ventilated with pure oxygen. The abdominal cavity was approached through a midline incision and the liver was exposed. At this point, all rats were divided into six groups (n = 10 each). In groups C, M, MW, MN, and MD, total in- and outflows of the median

and left lateral hepatic lobes were occluded by a micro-vascular clip; this yielded approximately 70% hepatic ischemia. The abdomen was covered with saline-humidified gauze during the ischemic period. After 60 min of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion, and the abdominal cavity was closed with a 4-0 silk suture. During surgery, body temperature was monitored using a rectal probe and was maintained with a heating pad and heating lamp at 37°C. Sham-operated animals (group S) went through the same surgical procedure as other animals; however, hepatic vessel clips were not applied. In groups M, MW, and MN, 50 μg/kg milrinone was administered as an i.v. bolus immediately after reperfusion. In groups MW and MN, PI3K inhibitor wortmannin (0.6 mg/kg) and non-selective NOS inhibitor L-NAME (25 mg/kg), respectively, were injected 5 min before the administration of milrinone. In group MD, 50 μg/kg milrinone was administered as an i.v. bolus 30 min after reperfusion. In groups S and C, saline was infused at the same volume as milrinone, wortmannin, or L-NAME. All animals were sacrificed 5 hours after reperfusion or sham surgery, and liver tissues and blood samples were taken for analysis.

2.3. Liver function tests

Blood samples were taken from the inferior vena cava at the end of the experimental protocol. The samples were centrifuged immediately at 3000 rpm for 10 min and then serum samples were stored at -80°C until analysis. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were determined by UV

spectrophotometric enzymatic assay.

2.4. Histological examination

Liver tissue for histological examination was sampled from the left liver lobe at the end of the experimental protocol. The specimens were fixed in 10% buffered formaldehyde solution, embedded in paraffin, and stained using hematoxylin and eosin (HE). Histological analysis, based on the scoring system proposed by Suzuki et al. [19], was applied to all samples in order to determine the degree of sinusoidal congestion, liver cell vacuolization, and necrosis. Histological changes were scored from 0 to 4.

2.5. TUNEL staining

The presence of apoptotic cells in liver tissue was quantified by terminal deoxynucleotidyl transferase-mediated biotin nick end-labeling (TUNEL) staining. The results were scored by counting the number of TUNEL-positive cells as a percentage of the total number of hepatocytes in 6 random high-power fields (magnification ×400) per sample.

2.6. Statistical analysis

The results are expressed as mean ± standard deviation. Differences between experimental groups were analyzed by Student's t test and one-way analysis of variance followed by the Student-Newman-Keuls test. $P < 0.05$ was considered significant. Statistical analysis was

performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

3. Results

Eighty-three rats were used, with 60 successful experiments being achieved. Eight rats were excluded as a result of technical difficulties with the experimental preparation. Circulatory collapse or shock-induced death developed in 15 other rats before completion of the experiment; 4 in the group C, 1 in the group M, 3 in the group MW, 4 in the group MN, 3 in the group MD. These rats were excluded from further analysis.

3.1. Serum AST, ALT, and LDH levels (Fig. 1)

Compared with those in group S, the serum AST, ATL, and LDH levels at 5 hours after reperfusion significantly increased in groups C, M, MW, MN, and MD ($P < 0.01$). In group M, the serum AST, ATL, and LDH levels were significantly lower than in group C ($P < 0.01$). PI3K inhibitor wortmannin and non-selective NOS inhibitor L-NAME abolished the beneficial effects of milrinone-induced PostC. There were no significant differences in the serum AST, ATL, and LDH levels between groups C and MD.

3.2. Histological analysis

Histological analysis showed no pathological findings in group S. According to Suzuki's histological classification with HE staining, group C showed moderate to severe hepatocyte vacuolization with disruption of the lobular architecture and sinusoidal congestion (Fig. 2A); the

Suzuki score was 8.3 ± 1.5 (Fig. 3). In groups MW, MN, and MD, the injury score was severe, as in group C. Compared with group C, group M showed minimal hepatocyte vacuolization and sinusoidal congestion (Fig. 2B); the Suzuki score was 3.0 ± 1.2 ($P < 0.05$) (Fig. 3).

3.3. Evaluation of hepatic apoptosis by TUNEL staining

The occurrence of apoptosis was suppressed in group M compared with that in group C (Fig. 2 and 4). Meanwhile, in groups MW and MN, this protective effect was not shown. In group MD, the occurrence of apoptosis was significantly lower than in group C (Fig. 4).

Discussion

We investigated the pharmacological PostC properties of milrinone against hepatic warm IR injury, and the effect of administration timing or the potential mechanism involved. It was observed that milrinone administered as an i.v. bolus immediately after reperfusion could effectively attenuate liver injury in a model of rat hepatic warm IR. The protective effect of milrinone could be associated with the activation of PI3K and NOS that was abrogated by PI3K inhibition and NOS inhibition, respectively. When milrinone was administered 30 min after reperfusion, the degree of liver function injury as determined by serum examination and histological analysis was not attenuated, while the occurrence of apoptosis was prevented.

Despite the protective effects of ischemic PreC against hepatic IR injury, its utilization as a clinical strategy is limited, mainly because it must be initiated before the ischemic event. In deceased donor liver transplantation, although the onset of ischemia could not be predicted, the onset of reperfusion is more predictable. Both ischemic PreC and PostC of the liver effectively reduced the generation of ROS, the expression of inflammatory cytokines, and apoptosis [20]. Thus, PostC is more likely than PreC to be feasible for clinical application. Although the mechanisms responsible for ischemic PostC of the liver are not fully understood, some studies have shown that the two processes have many similarities [6-8]. Recent studies have demonstrated that some agents, such as adenosine A2A receptor agonist [8], rhEPO [9], and sevoflurane [10], can be used as pharmacological inducers of PostC as well as PreC. Milrinone has been shown to have

PreC properties using an orthotopic liver transplantation model and warm IR injury model in rats [14,15]. The present study is, to our knowledge, the first to demonstrate that milrinone also has PostC properties against hepatic warm IR injury.

The onset of ischemic PreC is triggered by the production of adenosine and by the subsequent stimulation of A2A receptor. A2A receptors have been shown to be coupled to Gs proteins that, through adenylate-cyclase, lead to sequential elevation of intracellular cAMP and PKA/PI3K activation [4]. The PI3K/Akt pathway is an important type of survival signaling that induces various anti-apoptotic molecules in ischemic PreC of the liver [21]. Our results show that pretreatment with a PI3K inhibitor, wortmannin, prevents milrinone-induced PostC of the liver. Ischemia- and A2A receptor agonist- or ginsenoside Rb1-induced PostC markedly enhanced Akt phosphorylation of the liver tissues at reperfusion, and attenuated hepatic warm IR injury [6,8,22]. Dal Ponte et al. [8] also reported that pretreatment with a PI3K inhibitor prevented A2A receptor agonist-induced PostC of the liver. Previous studies showed that milrinone administration stimulated immediate accumulation of cAMP and elevation of PKA in the liver tissue, and pretreatment with a PKA inhibitor prevented milrinone-induced PreC [14, 15]. In heart, milrinone-induced PostC also increased myocardial PKA activity and Akt phosphorylation, and reduced myocardial infarct size and myocyte apoptosis [16]. Thus, it is possible that milrinone-induced hepatic PostC is associated with the activation of PKA and the PI3K/Akt-dependent pathway.

NO appears to be an essential endogenously generated molecule that protects against liver

IR injury [23]. The protective mechanisms of NO have been reported in that NO could reduce neutrophil adhesion to endothelial cells, platelet aggregation, ROS release, and inflammatory cytokine production, as well as ameliorate hepatic microvascular blood flow [23,24]. Our results show that pretreatment with a non-selective NOS inhibitor, L-NAME, prevents milrinone-induced PostC of the liver, which is consistent with previous reports [5,6]. Wang et al. [5] showed that the protective effect of ischemic PostC against hepatic IR injury is closely related to the NO production following the increase in eNOS and iNOS expression and the suppression of tumor necrosis factor-α and macrophage inflammatory protein-2 overproduction. Guo et al. [6] demonstrated that ischemic PostC upregulated the concentrations of NO and attenuated the overproduction of ROS and inflammation, and these protective effects could be abolished by L-NAME. NO may exert cytoprotective effects by preventing mPTP opening after reperfusion through a guanylyl cyclase and cyclic guanosine monophosphate-dependent kinase signaling pathway in hepatocytes [25]. A recent study has shown that the cytoprotective mechanism of ischemic PostC of the liver occurred through inhibition of the opening of mPTP at reperfusion. Our previous study showed that atractyloside, an mPTP opener, prevents milrinone-induced PostC in rat heart [26]. We assume that milrinone can upregulate NOS activity of the liver, leading to inhibition of the opening of mPTP at reperfusion.

The early phase of reperfusion might be important for subsequent reperfusion injury, and manipulation of this early reperfusion phase was critical to the protective effect of ischemic PostC. When the maneuvering of ischemic PostC was delayed for a while, the protective effect was lost

[27]. Since then, PostC maneuvers were initiated within 1 min of reperfusion in the majority of studies. However, a recent study demonstrated that ischemic PostC, when applied within 30 min after the onset of reperfusion, reduced infarct size and DNA fragmentation, and the degree of cardioprotection was inversely proportional to the delay of PostC application [28]. Thus, we studied the effect of milrinone administration time during reperfusion, that is, administration immediately or 30 min after reperfusion. In the present study, milrinone administered immediately after reperfusion but not 30 min after reperfusion exerted obvious PostC properties on the liver. It is possible that milrinone would need to be administered in the early phase of reperfusion to exert PostC properties.

Several limitations of this study should be noted. First, we studied only a single dose of milrinone in rats to examine milrinone-induced hepatic PostC. The dose of milrinone treatment (50 μg/kg) used in this study corresponds to that applied clinically for a loading dose. It is unfortunate that we cannot present the threshold of milrinone for hepatic PostC. Second, we did not measure systemic hemodynamics such as blood pressure and heart rate in this study. It has been shown that milrinone treatment (50 μg/kg) before liver ischemia [15], or after myocardial ischemia [16] did not affect systemic hemodynamics. In another study, wortmannin or L-NAME administered 5 min before reperfusion also did not affect systemic hemodynamics in myocardial infarction models [29]. Taking these facts into consideration, it is not plausible that the present results could be attributed to the systemic hemodynamic changes induced by milrinone. Third, we did not compare the effects of milrinone-induced PostC between orthotopic liver transplantation

model and warm IR injury model in rats. Further studies are thus needed to apply the results of this experimental study to a clinical setting.

In conclusion, PDE-I milrinone-induced PostC alleviated warm IR injury in rat liver. This protective effect was found when milrinone was administered immediately after reperfusion, but not 30 min after reperfusion. Pretreatment of PI3K inhibitor or NOS inhibitor canceled this beneficial effect of milrinone. We conclude that PI3K and NOS could play an important role in the protective effect by milrinone against hepatic IR injury.

Acknowledgements

This work was supported in part by a Grant-in-Aid 23592297 (to Dr. Cho) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest statement

The authors disclose no financial or personal relationships with other people or organizations that could inappropriately influence this work.

References

[1] Serracino-Inglott F, Habib NA, Mathie RT. Hepatic ischemia-reperfusion injury. Am J Surg 2001;181:160.

[2] Carini R, Albano E. Recent insights on the mechanisms of liver preconditioning. Gastroenterology 2003;125:148.

[3] Yan S, Jin LM, Liu YX, Zhou L, Xie HY, Zheng SS. Outcomes and mechanisms of ischemic preconditioning in liver transplantation. Hepatobiliary Pancreat Dis Int 2010;9:346.

[4] Alchera E, Dal Ponte C, Imarisio C, Albano E, Carini R. Molecular mechanisms of liver preconditioning. World J Gastroenterol 2010;16:6058.

[5] Wang N, Lu JG, He XL, et al. Effects of ischemic postconditioning on reperfusion injury in rat liver grafts after orthotopic liver transplantation. Hepatol Res 2009;39:382.

[6] Guo JY, Yang T, Sun Xg, Zhou NY, Li FS, Long D, Lin T, Li PY, Feng L. Ischemic postconditioning attenuates liver warm ischemia-reperfusion injury through Akt-eNOS-NO-HIF pathway. J Biomed Sci 2011;18:79.

[7] Lin HC, Lee TK, Tsai CC, Lai IR, Lu KS. Ischemic postconditioning protects liver from ischemia-reperfusion injury by modulating mitochondrial permeability transition. Transplantation 2012;93:265.

[8] Dal Ponte C, Alchera E, Follenzi A, Imarisio C, Prat M, Albano E, Carini R. Pharmacological postconditioning protects against hepatic ischemia/reperfusion injury. Liver Transpl 2011;17:474.

[9] Shawky HM, Younan SM, Rashed LA, Shoukry H. Effect of recombinant erythropoietin on ischemia-reperfusion-induced apoptosis in rat liver. J Physiol Biochem 2012;68:19.

[10] Beck-Schimmer B, Breitenstein S, Bonvini JM, Lesurtel M, Ganter M, Weber A, Puhan MA, Clavien PA. Protection of pharmacological postconditioning in liver surgery: results of prospective randomized control trial. Ann Surg 2012;256:837.

[11] Yano M, Kohno M, Ohtuka T, Mochizuki M, Yamada J, Kohno M, Hisaoka T, Ono K, Tanigawa T, Kobayashi S, Matsuzaki M. Effect of milrinone on left ventricular relaxation and Ca(2+) uptake function of cardiac sarcoplasmic reticulum. Am J Physiol 2000;279:H1898.

[12] Niemann JY, Garner D, Khaleeli E, Lewis RJ. Milrinone facilitates resuscitation from cardiac arrest and attenuate postresuscitation myocardial dysfunction. Circulation 2003;108:3031.

[13] Jebeli M, Ghazinoor M, Mandegar MH, Rasouli MR, Eghtesadi-Araghi P, Goodarzynejad H, Mohammadzadeh R, Darehzereshki A, Dianat S. Effect of milrinone on short-term outcome of patients with myocardial dysfunction undergoing coronary artery bypass graft: A randomized controlled trial. Cardiol J 2010;17:73

[14] Ikegami T, Nishizaki S, Hiroshige R, Ohta R, Yanaga K, Sugimachi K. Experimental study of a type 3 phosphodiesterase inhibitor on liver graft function. Br J Surg 2001;88:59.

[15] Satoh K, Kume M, Abe Y, Uchinami H, Yakubouski SV, Takahashi T, Sato T, Yamamoto Y. Implication of protein kinase A for a hepato-protective mechanism of milrinone pretreatment. J Surg Res 2009;155:32.

[16] Huang MH, Wu Y, Nguyen V, Rastogi S, McConnell BK, Wijaya C, Uretsky BF, Poh KK, Tan HC, Fujise K. Heart protection by combination therapy with esmolol and milrinone at late-ischemia and early reperfusion. Cardiovasc Drugs Ther 2011;25:223.

[17] Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL: Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: The roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Circulation 2002;105:1497.

[18] Li J, Zhang H, Wu F, Nan Y, Ma H, Guo W, Wang H, Ren J, Das UN, Gao F. Insulin inhibits tumor necrosis factor-alpha induction in myocardial ischemia/reperfusion: Role of Akt and endothelial nitric oxide synthase phosphorylation. Crit Care Med 2008;36:1551.

[19] Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Transplantation 1993;55:1265.

[20] Zhang WX, Yin W, Zhang L, Wang LH, Bao L, Tuo HF, Zhou LF, Wang CC. Preconditioning and postconditioning reduce hepatic ischemia-reperfusion injury in rats. Hepatobiliary Pancreat Dis Int 2009;8:586.

[21] Izushi K, Tsung A, Hossain MA, Fujiwara M, Wakabayashi H, Masaki T, Billiar TR, Maeta H. Ischemic preconditioning of the murine liver protects through the Akt kinase pathway. Hepatology 2006;44:573.

[22] Guo Y, Yang T, Lu J, Li S, Wang L, Long D, Li Q, Feng L, Li Y. Rb1 postconditioning attenuates liver warm ischemia-reperfusion injury through ROS-NO-HIF pathway. Life Sci 2011;88:598.

[23] Abu-Amara M, Yang SY, Seifalian A, Davidson B, Fuller B. The nitric oxide pathway-evidence and mechanisms for protection against ischaemia reperfusion injury. Liver Int 2012;32:531.

[24] Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, Pina E, Geller DA. Factors in the pathophysiology of the liver ischemia-reperfusion injury. J Surg Res 2008;147:153.

[25] Kim JS, Ohshima S, Pediaditakis P, Lemasters JJ. Nitric oxide protects rat hepatocytes against reperfusion injury mediated by the mitochondrial permeability transition. Hepatology 2004;39:1533

[26] Matsumoto S, Cho S, Tosaka S, Higashijima U, Maekawa T, Hara T, Sumikawa K. Hyperglycemia raises the threshold of levosimendan- but not milrinone-induced postconditioning in rat heart. Cardiovasc Diabetol 2012;11:4.

[27] Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME, Kerendi F, Guyton RA, Vinten-Johansen J. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res 2004;62:74.

[28] Roubille F, Franck-Miclo A, Covinhes A, Lafont C, Cransac F, Combes S, Vincent A, Fontanaud P, Sportouch-Dukhan C, Redt-Clouet C, Nargeot J, Piot C, Barrere-Lemaire S. Delayed postconditioning in the mouse heart in vivo. Circulation 2011;124:1330.

[29] Maekawa T, Cho S, Ichinomiya T, Tosaka S, Matsumoto S, Shibata I, Hara T, Sumikawa K. Fasudil administered during early reperfusion protects against myocardial infarction through activation of PI3K/Akt/NOS pathway in rats. Circ Cont 2012:33;96-103.

Figure legends

Fig. 1

Serum AST (A), ALT (B), and LDH (C) levels 5 hours after reperfusion. *Significantly ($P < 0.01$) different from group S. #Significantly ($P < 0.01$) different from group C. Data are mean ± standard deviation (n = 10 in each group). S = sham group, C = control group, M = milrinone group, MW = milrinone + wortmannin group, MN = milrinone + L-NAME group, MD = milrinone delayed by 30 min group

Fig. 2

Comparisons of histopathology for rat liver between control and M-treated groups. A and B (Original magnification×400): Hematoxylin and eosin stains apparently reveals an ischemic change, such as hepatocytic atrophy with occasional cytoplasmic vacuolation and sinusoidal congestion at centrilobular area (zone 3) in control group (A) but not in M-treated group (B). C and D (Original magnification×400): TUNEL-positive cells are found in the centrilobular area showing an ischemic change in the control group (C) but not in M-treated group (D). Asterisks indicate the central vein.

Fig. 3

Analysis of Suzuki's score. *Significantly ($P < 0.05$) different from group S. #Significantly ($P < 0.05$) different from group C. Data are mean ± standard deviation (n = 10 in each group). S =

sham group, C = control group, M = milrinone group, MW = milrinone + wortmannin group, MN = milrinone + L-NAME group, MD = milrinone delayed by 30 min group

Fig. 4

Analysis of apoptosis index. *Significantly ($P < 0.05$) different from group S. #Significantly ($P < 0.05$) different from group C. Data are mean ± standard deviation (n = 10 in each group). S = sham group, C = control group, M = milrinone group, MW = milrinone + wortmannin group, MN = milrinone + L-NAME group, MD = milrinone delayed by 30 min group

Fig 1

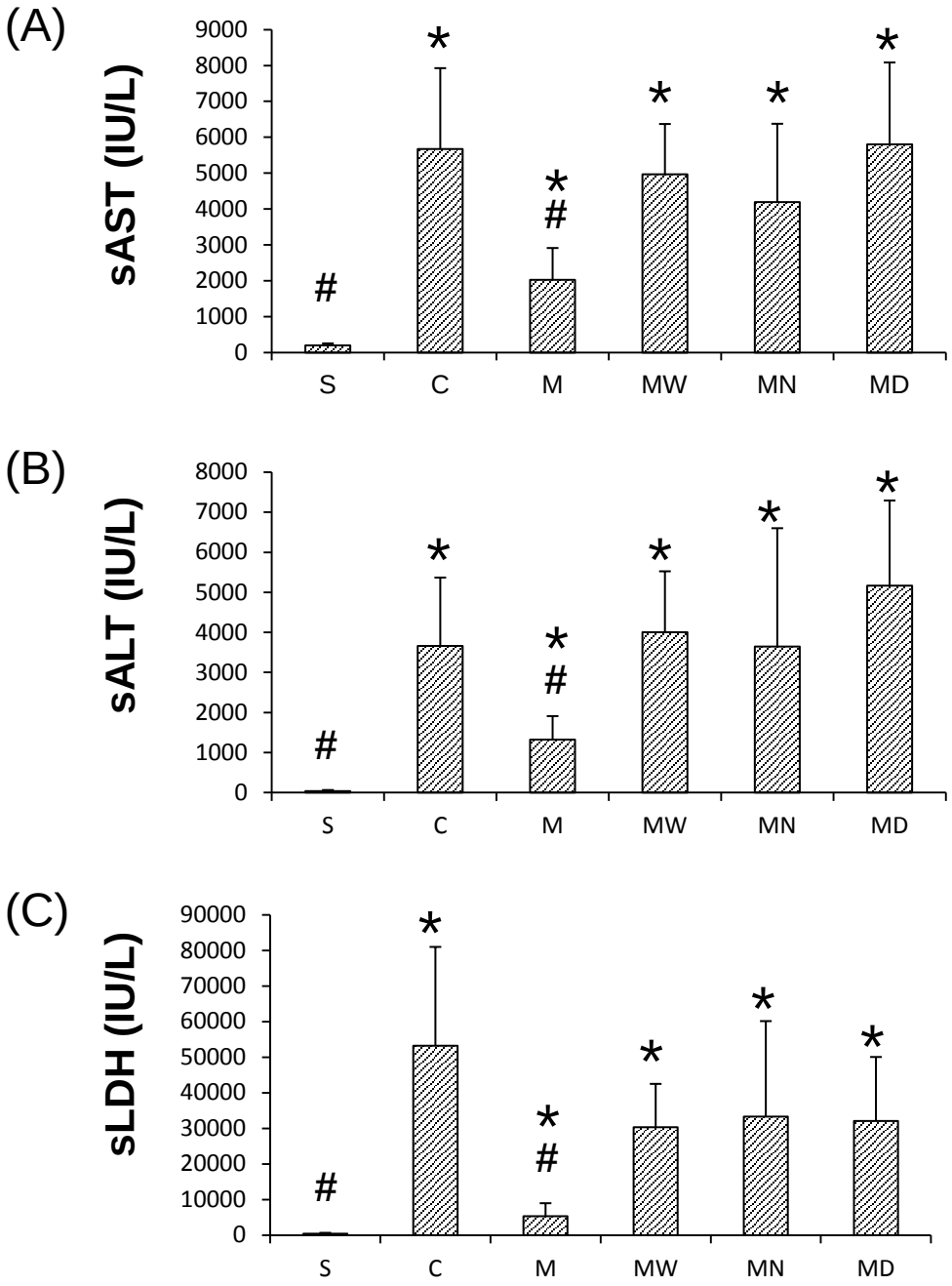
(A)
sAST (IU/L)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
S
C
M
MW
MN
MD
(B)
sALT (IU/L)
8000
7000
6000
5000
4000
3000
2000
1000
0
S
C
M
MW
MN
MD
(C)
sLDH (IU/L)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
S
C
M
MW
MN
MD

Fig 2

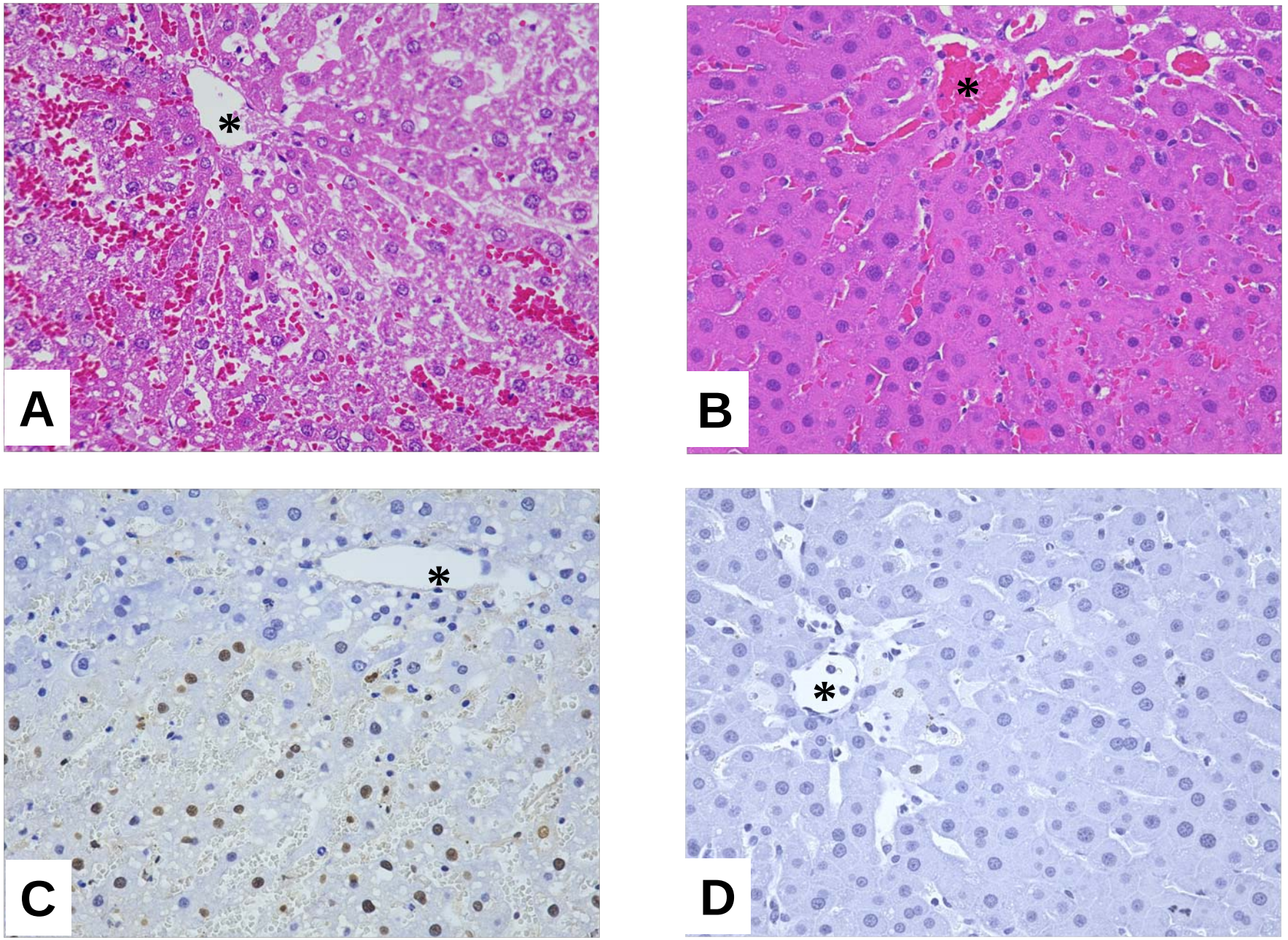

Fig 3

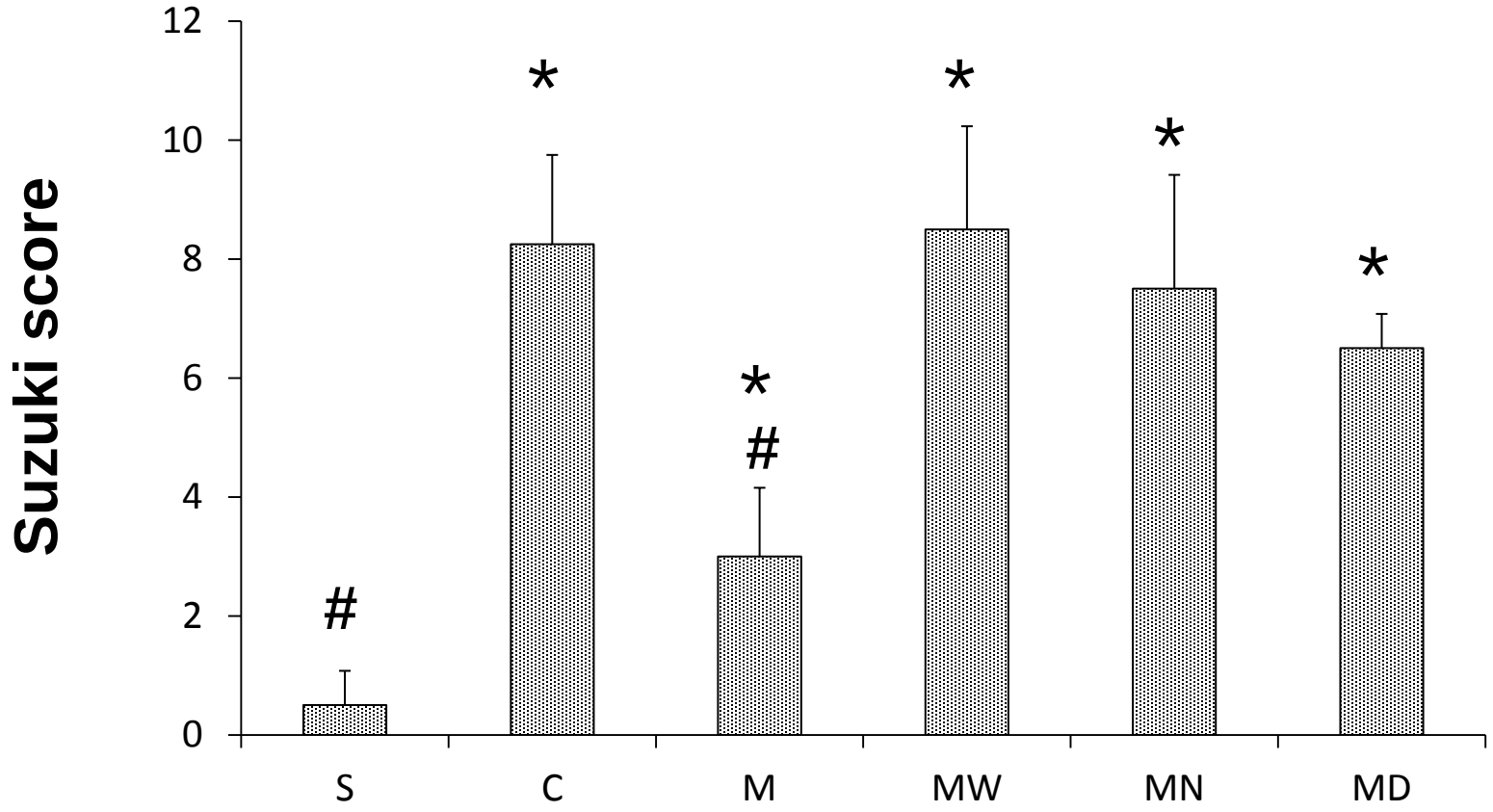
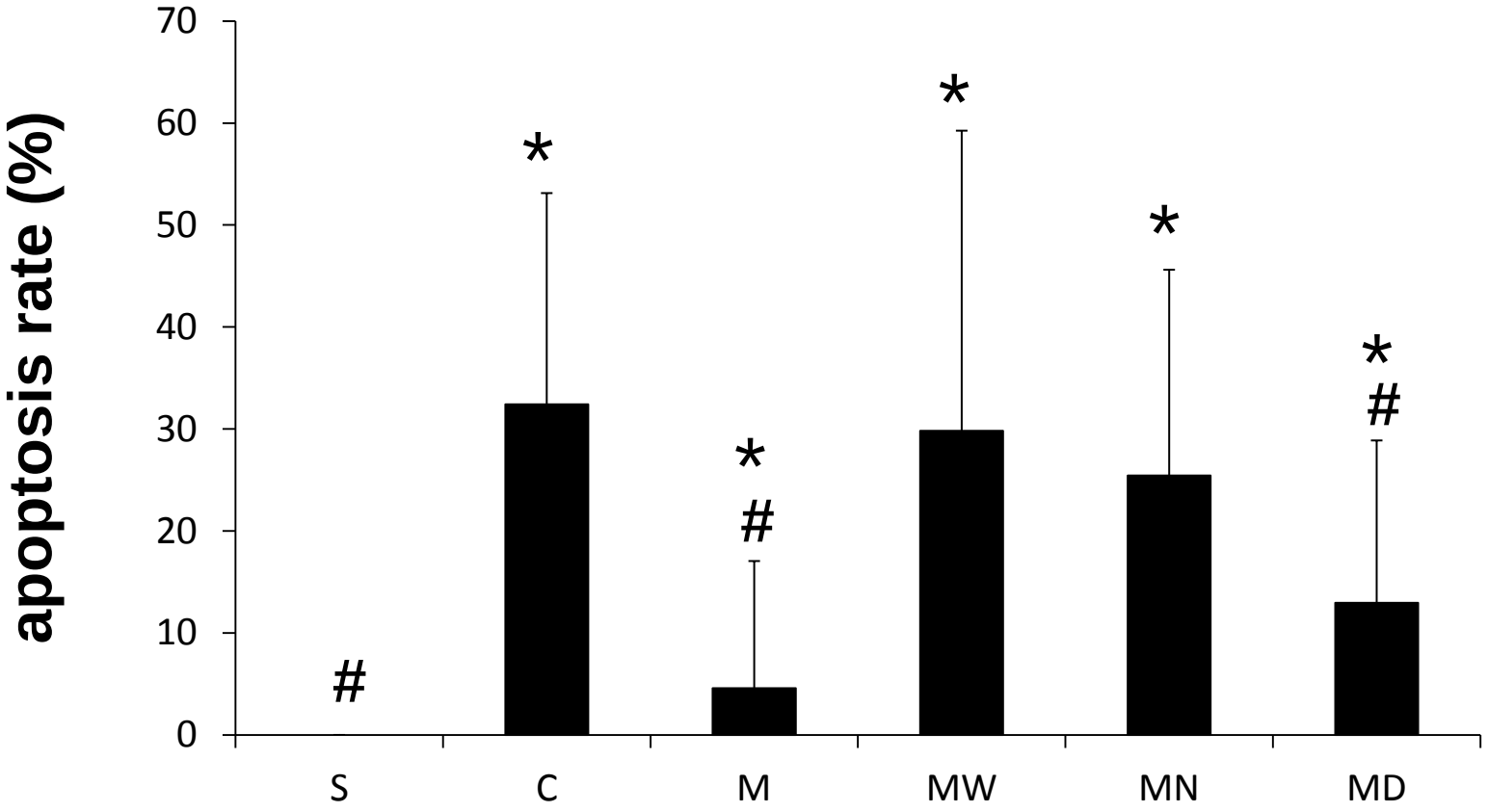
Fig 4