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Effect of MCI-186 on Postischemic Reperfusion Injury in Isolated Rat Heart

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MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one) is a newly developed antioxidant which has been shown to reduce brain edema in cerebral ischemia through inhibition of the lipoxygenase pathway of arachidonic acid. However, its effect on myocardial reperfusion injury after prolonged ischemia has not yet been demonstrated. We compared the mode of the effect of MCI-186 and recombinant human CuZn superoxide dismutase (rh-SOD) in isolated perfused rat hearts subjected to 60-min ischemia followed by 60-min reperfusion. Left ventricular developed pressure (LVDP), necrotic area and the release of creatine phosphokinase (CPK) and endogenous CuZn superoxide dismutase (endoge-SOD) were measured to evaluate myocardial damage. The decrease in left coronary flow (CBF) was measured as an index of the damage of left coronary circulation. MCI-186 (17.5 mg/L) was perfused for 10 min in the MCI group and rh-SOD (70 mg/L) was perfused during the reperfusion period in the SOD group starting 5 min prior to reperfusion. The release patterns of CPK and endoge-SOD were analyzed to elucidate the difference in the mode of protection of MCI-186 and rh-SOD. The LVDP remained higher in both MCI and SOD groups than that of control (76 ± 1, 77 ± 2 and 69 ± 1% of preischemic value, respectively). The necrotic area was significantly attenuated in both MCI and SOD groups compared with that in the control group (16 ± 1, 14 ± 1 and 32 ± 1%, respectively, $p<0.05$). Total CPK release was lower in both MCI and SOD groups than in the control (78 ± 7, 100 ± 2 and 116 ± 4 × 10^3 units/g myocardium respectively). The decrease in CPK release was more marked in the MCI group than that in the SOD group ($p<0.05$). The reduction in CBF was significantly attenuated by the treatment with rh-SOD or MCI-186, but the effect was much higher in the SOD group than in the MCI group (69 ± 5, 58 ± 2, and 48 ± 2% in SOD, MCI and control groups, respectively). The release pattern of endoge-SOD was identical to that of CPK and thus this did not distinguish the mode of effect of MCI-186 from that of rh-SOD. These results indicate that MCI-186 reduces reperfusion injury in isolated perfused hearts with prolonged ischemia and the effect is more closely related to the reduction of myocyte damage than the preservation of the coronary circulation.

Key words: Isolated rat heart, reperfusion injury, antioxidant, MCI-186, SOD

INTRODUCTION

Free radical-mediated reperfusion injury has been the focus of intense research since its initial

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description in 1981.[1] Although various types of radical scavengers and antioxidants have been shown to effectively reduce reperfusion injury, each have some limitations as a therapeutic agent for reperfusion injury mainly due to their low accessibility to tissue or rapid clearance from the body.[2,3] MCI-186, a newly developed antioxidant which has hydroxyl radical scavenging activity, has been shown to reduce brain edema in cerebral ischemia through the inhibition of the lipoxygenase pathway of arachidonic acid.[4,5] In addition to its excellent antioxidant effect, the promising features of MCI-186 are that 1) it is lipophilic, and 2) readily accessible to tissue, and 3) that an effective tissue level can be maintained with one intravenous bolus injection.[4,6–9] As the effect of MCI-186 on myocardial reperfusion injury after prolonged ischemia has not been shown, we examined its effect on isolated perfused rat hearts subjected to ischemia-reperfusion and compared the characteristics of myocardial protection with that of rh-SOD. rh-SOD was selected as a comparative antioxidant because it does not enter the myocardium and its antioxidant action is solely based on dismutation of superoxide. To clarify the difference between the characteristics of protection of MCI-186 and rh-SOD, the myocardial damage and impairment of coronary flow after reperfusion were examined. In addition, the influence of these agents on the creatine phosphokinase (CPK) release pattern during reperfusion was examined in comparison with that of endoge-SOD release, based on the following hypothesis: the inequality of the release patters of CPK and endoge-SOD can distinguish the site of protection at the subcellular level offered by MCI-186 and rh-SOD since CPK is found in both cytosolic and mitochondrial compartments but endoge-SOD is localized in the cytosolic compartment. Since the time course of endoge-SOD release after ischemia-reperfusion has not been examined to date, the detailed comparison of CPK and endoge-SOD release patterns may offer new insight regarding the mechanisms by which the enzymes are released.

MATERIALS AND METHODS

Isolated Perfused Heart Preparation

Adult male Wistar rats (weighing 350–405 g) were given intraperitoneal injection of heparin and pentobarbital 50 mg kg^{-1}. After isolation all hearts were cooled about 15 seconds in ice cold saline before mounting on a Langendorff apparatus via the aortic root and retrograde perfusion at a constant perfusion pressure of 120 cm of water was started. Modified Krebs-Henseleit buffer was used, pH 7.40, containing the following (in millimolar concentration): NaCl 118, KCl 4.7, $NaHCO_3$25, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $KHPO_4$ 1.2 and glucose 11. The perfusate was filtered through a cellulose nitrate membrane (5.0 μm pore size), saturated with a gaseous mixture containing 95% O_2 and 5% CO_2. The temperature of the whole system was maintained at 36.5°C using a water bath and a water jacket. A latex balloon was inserted into the left ventricular cavity through the left atrium for the measurement of left ventricular pressure. The left ventricular end diastolic pressure was adjusted to 4 cm of water. The heart was paced at a fixed rate of 300 beats min^{-1}. In hearts of all three groups a ligature was placed around the left coronary artery (LCA) for the subsequent LCA occlusion and reperfusion.

Protocol of the Experiment

After an initial 20 min stabilization period, all hearts in the following three groups were subjected to the LCA occlusion for 60 min, followed by reperfusion for another 60 min: Control group (n = 5), no treatment during ischemia and reperfusion periods; MCI group (n = 5), MCI-186 perfused for 10 min, and SOD group (n = 5), rh-SOD perfused for the whole period of reperfusion starting 5 min prior to reperfusion. CBF and LVDP were continuously monitored and CPK and endoge-SOD in the coronary effluent was determined. CPK was measured by an autoanalyzer AU550 (Olympus, Tokyo), and the results were expressed

as units/gram of myocardium with normalization using the wet heart weight. Rat heart endoge-SOD was measured using rat specific monoclonal antibody by an enzyme-linked immunosorbent assay (ELISA)[10] and the results were expressed as units/gram of myocardium with normalization calculated using the wet heart weight.

Measurement of Risk and Necrotic Areas

At the end of the experiment LCA was re-ligated and Evans blue dye (0.5%, 0.4 ml) was injected at the aortic root to delineate the risk area. The necrotic area was determined by triphenyltetrazolium chloride (TTC) staining after the heart was sliced (2 mm thick) perpendicular to the apical-basal axis. Both surfaces of the sections we photographed using color slide film. The risk area was determined using a computer-assisted image analysis system (MCID, Imaging Research, St. Catherine's, Canada) and the necrotic area was expressed as the percentage of the risk area.

Evaluation of Left and Right Coronary Flow

Total coronary flow was continuously monitored by electromagnetic flowmeter (Nihon Koden Co., Tokyo) which was connected to the perfusion apparatus. The individual left and right coronary flows were obtained as shown in Figure 1; the CBF immediately after occlusion represents the right coronary flow and the reduced part represents the LCA flow prior to the occlusion state. Each individual flow was also measured at the end of 60 min reperfusion by re-occluding the LCA and changes in the flow was expressed as the percentage of the flow prior to the occlusion state.

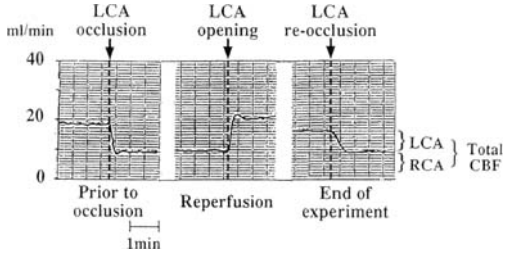

FIGURE 1 Records of coronary flow prior to the occlusion state, at LCA occlusion, at reperfusion and at re-occlusion.

Drugs

MCI-186 was obtained from the research Center of Mitsubishi Kasei Corporation (Yokohama, Japan), and rh-SOD was donated by Nippon Kayaku Co. Ltd. (Tokyo, Japan). All other chemicals and materials were of the highest analytical grade available and were purchased from local commercial sources.

Statistics

All data are expressed as mean ± SEM. Differences were considered significant with $p<0.05$. Differences among the groups were analyzed by one-way analysis of variance (ANOVA). Statistical comparisons were made using Fisher PLSD.

RESULTS

The changes in the CBF and LVDP during ischemia and reperfusion period in the three groups are shown in Figures 2 and 3. During ischemia these values did not differ among the three groups. CBF was 67 ± 2, 72 ± 2 and 77 ± 1% of the

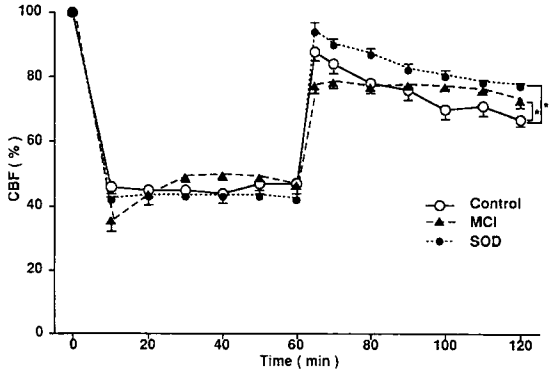

FIGURE 2 The time course of coronary flow among the three groups. The results are expressed as the percentage of pre-ischemic control (mean + or − SEM). *denotes significant difference ($p<0.05$).

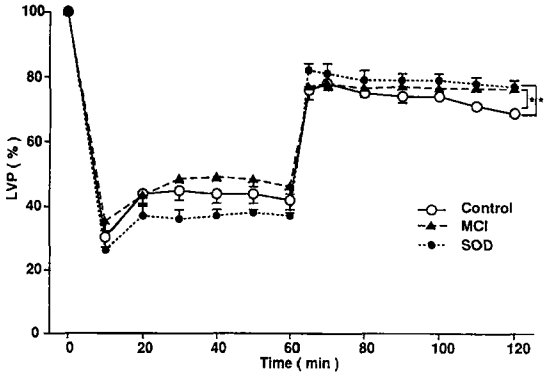

FIGURE 3 The time course of left ventricular developed pressure among the three groups. The results are expressed as the percentage of preischemic control (mean + or – SEM).

preischemic value in the control, MCI and SOD groups, respectively. CBF in MCI and SOD groups were significantly higher than that of the control group ($p<0.01$) but there was no difference in CBF between the MCI and SOD groups. The LVDP was 69 ± 1, 76 ± 1 and $77 \pm 2\%$ of the preischemic value in the control, MCI and SOD groups, respectively. LVDP in the SOD and MCI groups were higher than that in the control group ($p<0.01$) but LVDP did not differ among the MCI and SOD groups. The total CPK release during the whole period of reperfusion was 116 ± 4, 78 ± 7 and $100 \pm 2 \times 10^3$ units/gram of myocardium in the control, MCI and SOD groups, respectively (Figure 4). There was a significant difference among the three groups with the lowest amount of CPK in the MCI group and the highest amount in the control. The total endoge-SOD release during the whole period of reperfusion was 14 ± 1, 8 ± 1 and $12 \pm 1 \times 10^3$ units/gram of myocardium in the control, MCI and SOD groups, respectively. The least amount of endoge-SOD was released from the hearts of MCI group and there was no significant difference in the control and SOD groups. The risk area of the left ventricle showed no significant difference among the three groups (48 ± 1, 45 ± 1 and $47 \pm 2\%$ in the control, MCI and SOD groups, respectively) indicating that a similar amount of tissue was jeopardized by the occlusion of LCA in each group. However the necrotic area expressed as a percentage of the area at risk (Figure 5) was 33 ± 1, 16 ± 1 and $15 \pm 1\%$ in the control, MCI and SOD groups, respectively. The MCI group demonstrated a smaller necrotic area than the control group; necrotic area did not differ between the MCI and SOD groups. These findings of a high CBF and LVDP, low CPK release, small endoge-SOD release and small necrotic area in the MCI group indicate that MCI-186 treatment at the initiation of reperfusion attenuates ischemia-

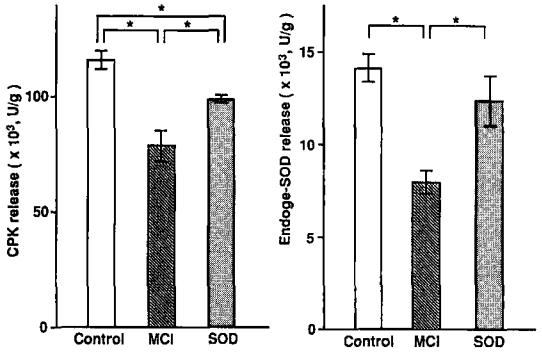

FIGURE 4 The total amount of CPK release and endogenous-SOD release among the three groups. Bar height represents mean ± SEM.

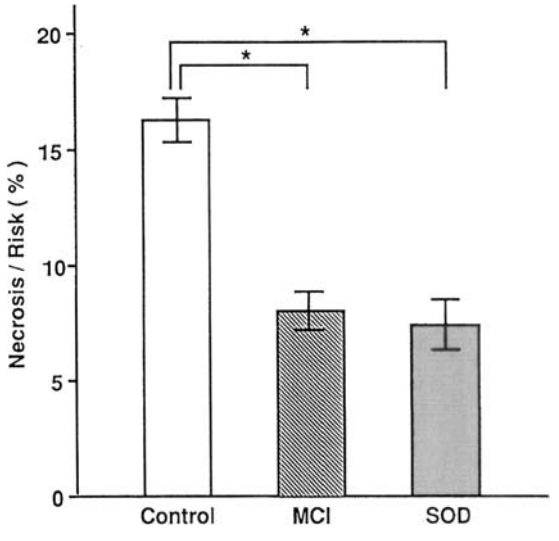

FIGURE 5 Percentage of necrosis/risk among the three groups. Bar height represents mean ± SEM.

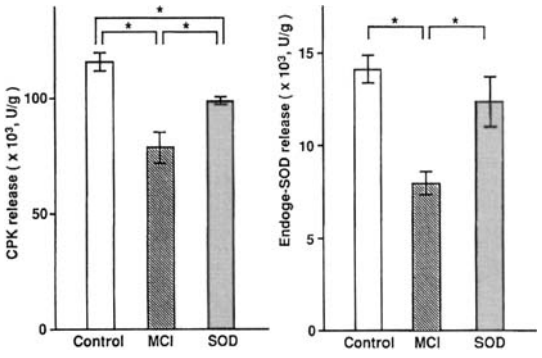

FIGURE 6 The left coronary artery flow (LCA) and right coronary artery flow (RCA) at the end of the experiment in the three groups. Bar height represents mean ± SEM.

reperfusion injury. Figure 6 shows the LCA flow and RCA flow at the end of the ischemia-reperfusion (60 min/60 min). The LCA flow was 48 ± 2, 58 ± 2 and 69 ± 5%; and the RCA flow was 94 ± 3, 96 ± 2 and 93 ± 3% of the preischemic value in the control, MCI and SOD groups, respectively. The LCA flow in the SOD group was the highest among the three groups ($p<0.01$) and the RCA flow did not differ among the three groups. These data indicate that both MCI-186 and rh-SOD attenuated the reduction of coronary flow in the vasculature subjected to ischemia-reperfusion injury and the degree of attenuation was greater in the SOD group than in the MCI group.

The peak release of CPK and endoge-SOD occurred at 2 min after the reperfusion and then the release decreased rapidly irrespective of the total amount of CPK and endoge-SOD release (Figure 7 A, B). Although the endoge-SOD release pattern did not differ overall from the CPK release pattern, a more sustained release of endoge-SOD was observed in the SOD group.

DISCUSSION

The present study showed that both MCI-186 and rh-SOD treatment protected hearts against reperfusion injury in isolated perfused heart which were rendered to regional ischemia. The LV function was significantly better preserved and the necrotic area was significantly smaller in the MCI and SOD groups than in the control. Coronary flow of the LAD was also better maintained in the MCI and SOD groups than in the control group, indicating that both MCI-186 and rh-SOD attenuated myocytes and vascular damage. These are some what unexpected results as there are distinct differences between the structure of MCI-186 and rh-SOD; the former has a low molecular weight (MW 174.2) and is lipophilic, whereas the latter is an enzyme of 31863 MW which has been shown not to enter myocytes.[11,12] The antioxidant actions of these agents are also shown to be different; MCI-186 does not react with superoxide, scavenges hydroxyl radicals, inhibits iron-dependent

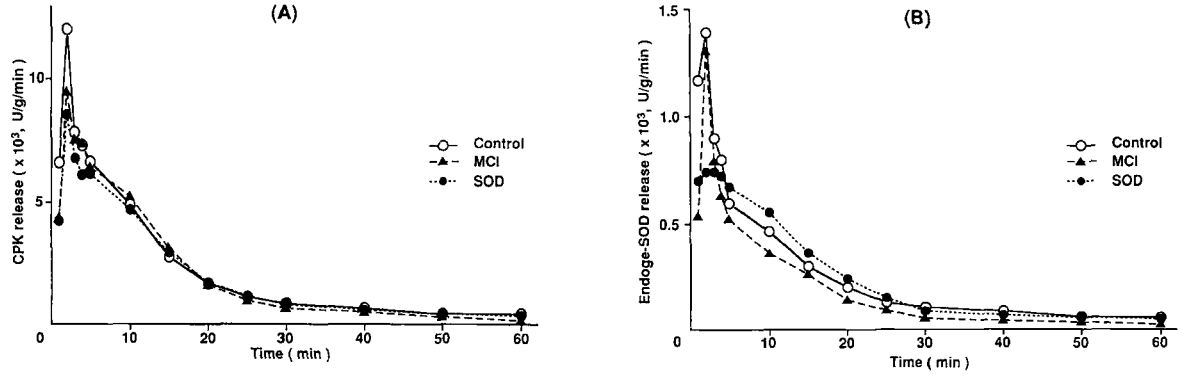

FIGURE 7 A, B The time course of CPK (A) and endoge-SOD (B) release during reperfusion in the three groups. The results are expressed as units/ml of coronary effluent/min, with normalization using wet heart weight.

lipid peroxidation and depresses the lipooxygenase pathway.[4–8] rh-SOD scavenges superoxide but does not directly inhibit either the iron-dependent lipid peroxidation or lipooxygenase pathway.[13,14] Nevertheless, both agents demonstrated similar protective effects against reperfusion injury, which indicates that the major free radical initially generated in this experiment was superoxide. rh-SOD attenuated the superoxide-induced chain reaction by eliminating the initial free radical of superoxide and MCI-186 blocked the process of subsequent chain reactions including the hydroxyl radical and arachidonic pathways. In the detailed comparison of the effects of MCI-186 and rh-SOD, a significant difference was found in the LAD flow between the two groups; the SOD group showed higher flow than the MCI group. A plausible reason is that the elimination of superoxide which reacts with nitric oxide (NO) at a diffusion limited rate, increased the effective NO concentration and resulted in vasodilatation.[15] The higher LAD flow appeared to influence release of CPK and endoge-SOD since the total amounts of released CPK and endoge-SOD were higher in the SOD group than in the MCI group. The underlying mechanism for the higher enzyme releases in the SOD group may be due to the accelerated washout of enzymes by high LAD flow, which falsely increase the amount of released enzymes which were collected for a limited period of 60 min and may not be due to the larger size of necrotic area since the LV function and necrotic area did not differ in the MCI and SOD groups. Therefore, if the effluent collection was prolonged for a longer period, such as two hours, the total amount of enzyme would likely become similar to that in the MCI group.

Regarding the release pattern, it was quite an unexpected result that endoge-SOD showed a pattern identical to that of CPK in all groups, considering the different localization of these enzymes and the different site of action of MCI-186 and rh-SOD: 1) endoge-SOD is localized only in the cytosol, 2) in contrast, the CPK activity which we measured represents both cytosolic and mitochondrial components in which mitochondria composes 15% of CPK activity in myocytes, 3) rh-SOD works at the endothelial site and interstitial space but MCI-186 can enter the cells. Since this is the first study, to our knowledge, in which a serial determination of endoge-SOD release was examined, the pattern can not be analyzed in relation to previous results. The one available report which measured Mn SOD release from patients with myocardial infarction with successful thrombolytic therapy demonstrated sustained release; the release pattern showed two peaks, the early peak occurred at 16 h and late peak at 108 h and the late peak was attributed to the induction of Mn SOD from myocytes.[16] This report however can not be compared with the present results since several factors differ. Mn SOD is located in the mitochondria and reperfusion in the clinical situation cannot be as complete as in an experiment. The nearly identical release patterns of CPK and endoge-SOD implicates that the difference in the molecular size or the cellular location of the enzyme does not influence their release patterns. Endoge-SOD release is merely a manifestation of cell membrane damage; detailed examination of the release pattern fails to provide additional insight into the analysis of the pathological process. It might be argued that Mn SOD may have contributed to some part of the measured endoge-SOD in the present study but this can be denied since the antibody against rat CuZn SOD utilized in this study did not crossreact with rat Mn SOD or rh-SOD (data not shown).

Regarding the dose and infusion period in the present study, we selected the most effective doses and infusion periods of MCI-186 (10 μM, 17.5 mg/L and 10 min) and rh-SOD (224000 units/L and 65 min) based on well established dose dependency studies of both agents against reperfusion injury to elucidate the characteristics of their protection.[2,4,6,7] Besides, we performed additional dose dependency study of MCI-186 effect to determine the minimum and maximum doses to produce the effect in this experimental model. MCI-186 of doses 5 μM (8.25 mg/L) and 7.5 μM

(12.4 mg/L) did not improve the LV function (100% and 102% of control value) and not attenuated the CPK release (104% and 110% of the control value). High dose (15 μM, 25.75 mg/L) or same dose (10 μM, 17.5 mg/L) with prolonged infusion (65 min) demonstrated the reduction in CPK release to the same level as that produced by 17.5 mg/L but did not improve the LV function.

The beneficial effect of MCI-186 and likely mechanism underlying its effect agreed with the previous *in vivo* rat heart study of a 10-min ischemic period.[9] As 10-min ischemia produces a condition known as myocardial stunning in which generation of superoxide-related molecules is shown,[17] their report supports the present results showing that an underlying mechanism for the effect of MCI-186 is the blockade of superoxide-related injury.

In conclusion MCI-186 can reduce myocardial reperfusion injury and the impairment of coronary flow in the reperfused vasculature. The characteristics of effectiveness are similar to those of rh-SOD for myocardial damage but are less pronounced for coronary arterial damage. The release pattern of endoge-SOD is identical to that of CPK, indicating that the implication of endoge-SOD release is the same as that of CPK release.

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