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Comparison of the Protective Effect of *N*-Acetylcysteine by Different Treatments on Rat Myocardial Ischemia-Reperfusion Injury

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Abstract. Reactive oxygen species have been known as important contributors to ischemia /reperfusion (I/R) injury. Studies on the beneficial effect of *N*-acetylcysteine (NAC), a potent antioxidant, on limiting infarct size induced by I/R yielded contrasting results. The present study was undertaken to compare the effect of NAC by different administration methods on infarct size in a rat myocardial I/R model. Rats underwent 30 min of left coronary occlusion followed by 4 h of reperfusion. Treatment with continuous infusion of NAC (150 mg/kg per hour) from 30 min before occlusion for 2 h (until 1 h after the start of reperfusion) produced a significant limitation of the infarct size as a percentage of the ischemic area (8%) compared to the non-treated control (60%). However, bolus injection of 150 mg/kg at 30 min prior to occlusion and 5 min prior to reperfusion failed to reduce it (56%) although the total dose is the same. The decreased total glutathione content and glutathione peroxidase activity in the ischemic region were recovered in the continuous infusion group, but not in the bolus injection group. The increased myeloperoxidase activity and phosphorylation of inhibitor κB after I/R were inhibited by the continuous treatment. These results indicate that the protective effect of NAC on myocardial infarction induced by I/R was different depending on the administration method. It is necessary to maintain blood concentration during the early period of reperfusion to obtain the beneficial effect of NAC.

Keywords: *N*-acetylcysteine, myocardial ischemia/reperfusion injury, oxidative stress, continuous infusion, bolus injection

Introduction

Ischemia/reperfusion (I/R) injury is an important clinical problem associated with procedures such as thrombolysis, angioplasty, and coronary bypass surgery that are commonly used to establish the blood reflow and minimize the damage of the heart due to ischemia. Reactive oxygen species (ROS) generated during I/R are thought to be a critical trigger of I/R injury characterized by myocardial stunning, arrhythmia, and necrosis. With reperfusion following ischemia, a burst and sustained release of ROS leads to peroxidation of membrane lipids, denaturation of proteins, and modification of DNA, all of which ultimately lead to cell death in the necrosis process of myocardium. The cell damage induced by ROS can also initiate local inflammatory responses, which then lead to further oxidant stress-mediated tissue injury (1).

Ischemia causes a depletion of antioxidant systems present in myocardium, and the myocardium is therefore at risk during reperfusion. Among various antioxidant systems, the glutathione/glutathione peroxidase system seems more important in the myocardium (2). Glutathione peroxidase reduces reactive oxygen intermediates, hydrogen peroxide, and other glutathione-dependent enzymes, including glutaredoxin and glutathione *S*-transferase, inhibit lipid peroxidation (3).

N-Acetylcysteine (NAC), a precursor of glutathione, has been used effectively to replenish intracellular glutathione stores in the I/R condition (4). In addition, NAC can directly scavenge radicals (5). Therefore, many studies have been made to explore the preventive effect

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of NAC against myocardial I/R injury. NAC has been used in I/R injury more or less empirically in cardiology. However, there are the contradictory reports on the beneficial effect of NAC on limiting infarct size in clinical (6, 7) and animal studies (8 – 11). To clarify the reason for the different results, the present study was undertaken to compare the effect of NAC by various administration protocols on infarct size in a rat myocardial I/R model.

Materials and Methods

Materials

NAC, 2,3,5-triphenyltetrazolium chloride (TTC), protease inhibitor cocktail, Evans blue, and cumene hydroperoxide were purchased from Sigma (St. Louis, MO, USA). Anti-phosphorylated-IκB-α antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase–conjugated secondary antibody was purchased from Cell Signaling Technology Ltd. (Beverly, MA, USA). Hexadecyltrimethylammonium bromide was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). All other compounds were of reagent grade.

Surgical preparation of animal model

Male Wistar rats weighing 130 – 150 g were obtained from Japan SLC (Shizuoka). Rats were anesthetized by sodium pentobarbital (50 mg/kg, i.p.) and ventilated with a Harvard ventilator (120 strokes/min, tidal volume 6 – 7 mL/kg). The left jugular vein was cannulated for drug administration. The heart was exposed via thoracotomy, and the left anterior descending artery was ligated proximal with a 7-0 silk suture for 30 min. Then the ligature was released and the heart was reperfused for 4 h.

All experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee.

Drug treatment

Four different treatments of NAC are shown in Fig. 1. In NAC1 group, NAC at a dose of 150 mg/kg was bolusly injected i.v. two times, at 30 min before occlusion and 5 min before reperfusion. In the NAC2 group, NAC was continuously administered i.v. at the infusion rate of 150 mg/kg per hour from 30 min before occlusion for 2 h (until 1 h after the start of reperfusion). In the NAC3 group, NAC (150 mg/kg) was bolusly injected i.v. 5 min before occlusion. In the NAC4 group, NAC (150 mg/kg per hour) was continuously administered i.v. from 5 min before reperfusion to 1 h after reperfusion. NAC solution was prepared to 150 mg/mL.

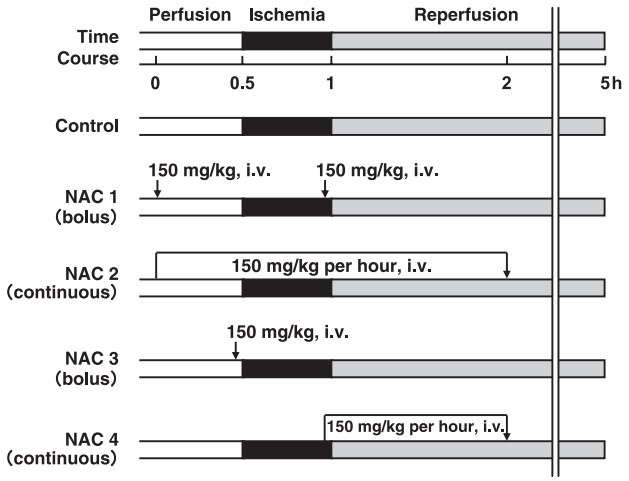

Fig. 1. Experimental protocol. Closed black and gray bars indicate ischemia and reperfusion, respectively. NAC1: *N*-acetylcysteine (NAC: 150 mg/kg) was administered i.v. at 30 min before occlusion and 5 min before reperfusion. NAC2: NAC was administered i.v at the infusion rate of 150 mg/kg per hour from 30 min before occlusion for 2 h. NAC3: NAC (150 mg/kg) was administered at 5 min before occlusion. NAC4: NAC was administered (150 mg/kg per hour) from 5 min before reperfusion to 1 h after reperfusion.

Measurement of myocardial infarct size

The excised heart was quickly perfused with 1.5% TTC in 20 mM sodium phosphate buffer (pH 7.4) from the aorta retrogradely and incubated in saline at 37°C for 2 min to determine infarct area (IN), and then 1% Evans blue dye in saline was injected after the ligation again to determine ischemic area (IS). The right ventricle was removed, and the left ventricle was cut transversally from the apex to the ligation site into eight slices of equal thickness. The left ventricle area (LV), IS, and IN were measured by using a computerized digital image analysis MacScope (Mitani Corporation, Fukui).

Biochemical analysis

The excised heart was washed with saline to remove blood. The tissue sample from the ischemic region was homogenized in 9 vol of 10 mM potassium phosphate buffer (pH 7.4) and centrifuged at 3500 × *g* for 60 min at 4°C to obtain the supernatant.

Lactate dehydrogenase (LDH) activity was measured using a LDH assay kit (Wako). Total glutathione content and glutathione peroxidase activity were measured by the spectrometric methods of Owens and Belcher (12) and Chow and Tappel (13), respectively.

Measurement of tissue myeloperoxidase (MPO) activity

MPO activity was measured as an index of leukocyte accumulation according to the method of Bradley et al. (14). The sample of the ischemic region was homo-

genized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide and centrifuged at 1500 × *g* for 15 min at 4°C. The MPO activity of the supernatant was measured with a standard chromogenic spectrophotometric method. Kinetic change in absorbance at 460 nm was measured and MPO activity was expressed as units/mg protein. The protein concentration was determined by the method of Lowry et al. (15).

Western blot analysis for the phosphorylated inhibitor κB (IκB)

Phosphorylated IκB expression in the ischemic region was measured by western blot analysis. Briefly, tissue samples were homogenized in lysis buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100) with a protease inhibitor cocktail (1/1000 v/v), and insoluble materials were removed by centrifugation at 4°C. The solubilized lysates were resolved by SDS-PAGE electrophoresis under reducing conditions at a concentration of 20 μg protein of each sample per lane. Detection of phosphorylated IκB-α protein was performed with an anti-phosphorylated-IκB-α antibody. The immunocomplex was visualized using an ECL-plus kit (Amersham, London, England).

Simulation of plasma NAC concentration

The plasma concentration curve of NAC in rats was simulated by using the following equations (16):

[Bolus injection]

$$C = \frac{D\,(\alpha - k_{off})}{V_d\,(\alpha - \beta)} \exp(-\alpha t_1) + \frac{D\,(k_{off} - \beta)}{V_d\,(\alpha - \beta)} \exp(-\beta t_1)$$

[Continuous infusion]

during infusion

$$C = \frac{k_0}{V_d k_e}\left[1 + \frac{\beta - k_e}{\alpha - \beta}\exp(-\alpha t_2) + \frac{k_e - \alpha}{\alpha - \beta}\exp(-\beta t_2)\right]$$

after infusion

$$C = \frac{k_0\,(\alpha - k_{off})}{V_d\,(\alpha - \beta)\alpha} \exp(-\alpha t_3) + \frac{k_0(k_{off} - \beta)}{V_d\,(\alpha - \beta)\beta} \exp(-\beta t_3)$$

, where C is plasma NAC concentration (protein-unbound form), D is dose of NAC (μmol), k_0 is infusion rate of NAC (μmol/h), t_1 is time after bolus injection (h), t_2 is time after start of infusion (h), t_3 is time after end of infusion (h), k_{on} is the first-order protein binding rate constant (0.23/h), k_{off} is the dissociation rate constant (0.57/h), k_e is the first-order elimination rate constant (4.3/h), V_d is distribution volume (391 mL/kg), and α and β are hybrid parameters ($\alpha + \beta = k_{on} + k_{off} + k_e$ and $\alpha \times \beta = k_{off} \times k_e$).

Statistical analysis

Data are each expressed as the mean ± S.E.M. Comparisons between multiple groups were performed by ANOVA followed by Scheffé's multiple comparison test. A value of $P<0.05$ was considered statistically significant.

Results

Effect on infarct size

The ischemic area (IS) expressed as a percentage of the left ventricle area (LV) was about 60%, and no difference was found among the five groups (Fig. 2). Infarct size expressed as a percentage of the IS was 60 ± 4% in control rats. The treatment with continuous infusion of NAC (150 mg/kg per hour) from 30 min before occlusion for 2 h (NAC2) produced a significant limitation of the infarct size compared to the control. However, the bolus injection of NAC (150 mg/kg) at 30 min prior to occlusion and 5 min prior to reperfusion (NAC1) failed to reduce the infarct size.

The altered bolus treatment NAC3, in which NAC (150 mg/kg) was injected at 5 min before occlusion, showed no reduction of infarction size. The NAC4 treatment, in which NAC (150 mg/kg per hour) was administered from 5 min before reperfusion to 1 h after reperfusion, produced a significant limitation of the infarct size compared to the control, but it was less effective than the NAC2 treatment.

Effect on tissue LDH leakage

Tissue damage causes the release of LDH from damaged tissue. Therefore, the loss of tissue LDH activity in the ischemic region is a biochemical index of tissue I/R injury. The tissue LDH activity after I/R in

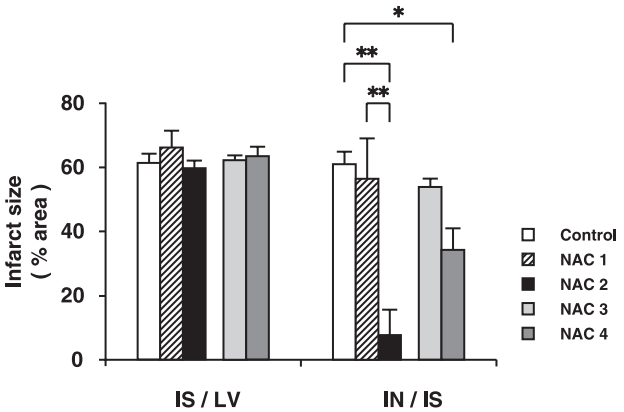

Fig. 2. Effect of *N*-acetylcysteine by different treatments on myocardial infarct size after ischemia/reperfusion in rats. IN: infarct area, IS: ischemic area, LV: left ventricle area. Data are each reported as the mean ± S.E.M. of 6 – 10 animals. **P*<0.05 and ***P*<0.01.

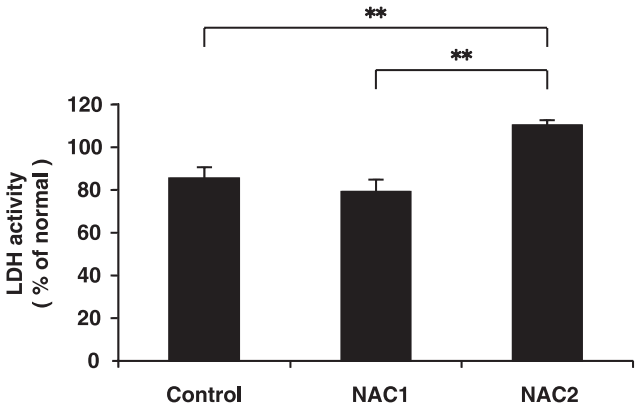

Fig. 3. Effect of *N*-acetylcysteine by different treatments on loss of LDH activity in rat myocardium after ischemia/reperfusion. Results were expressed as the percentage of normal rats. Data are each reported as the mean ± S.E.M. of 6 animals. ***P*<0.01.

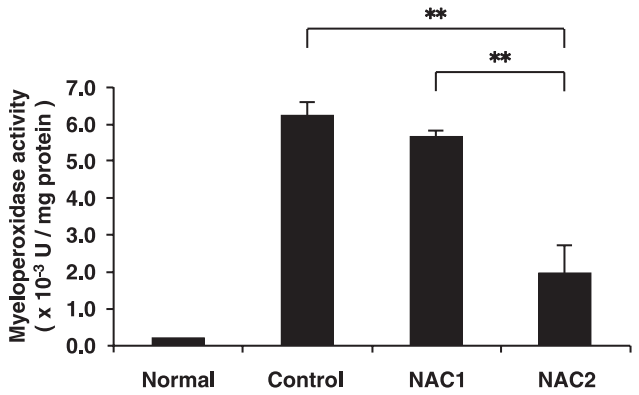

Fig. 4. Effect of *N*-acetylcysteine by different treatments on myeloperoxidase activity in rat myocardium after ischemia/reperfusion. Data are each reported as the mean ± S.E.M. of 5 animals. ***P*<0.01.

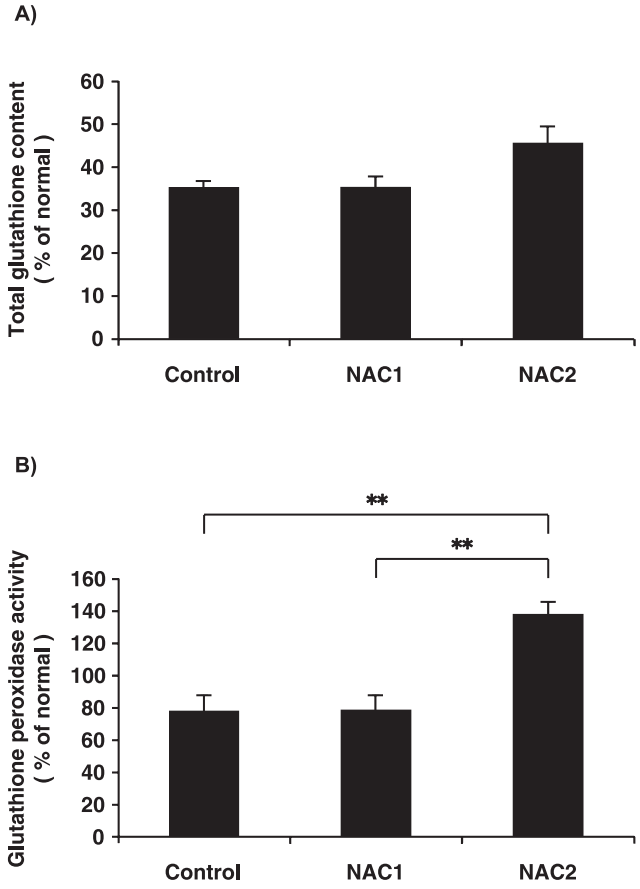

Fig. 5. Effect of *N*-acetylcysteine by different treatments on total glutathione content (A) and glutathione peroxidase activity (B) in rat myocardium after ischemia/reperfusion. Results were expressed as the percentage of normal rats. The value of total glutathione content and glutathione peroxidase activity in normal rats is 0.56 μmol/mg tissue and 0.15 IU/mg protein. Data are each reported as the mean ± S.E.M. of 6 animals. ***P*<0.01.

control rats was significantly lower than that in normal rats. The NAC2 treatment inhibited the loss of LDH activity, while the NAC1 treatment did not (Fig. 3). These results strongly confirmed the difference in effects of NAC on infarct size between the NAC1 and NAC2 treatments.

Effect on tissue MPO activity

Tissue MPO activity in myocardium hardly could be detected in normal rats. But, a significant increase in MPO activity in the ischemic region was found in control rats, which indicates leukocyte accumulation. NAC inhibited the increase of MPO activity by the continuous treatment (NAC2), but not by the bolus treatment (NAC1) (Fig. 4).

Effect on total glutathione content and glutathione peroxidase activity

Total glutathione content and glutathione peroxidase activity in the ischemic region were decreased in control rats compared to normal rats. The decreased total glutathione content and glutathione peroxidase activity by I/R were recovered by the NAC2 treatment, but not by the NAC1 treatment. The recovery of the enzyme activity found in the NAC2 group was greater than that of glutathione content (Fig. 5).

Effect on IκB phosphorylation

Phosphorylation of IκB induces the release of IκB from nuclear factor κB (NF-κB), a stress-sensitive transcription factor, resulting in the activation of NF-κB. The influence of I/R on IκB phosphorylation is shown in Fig. 6A. The rise of IκB phosphorylation was observed

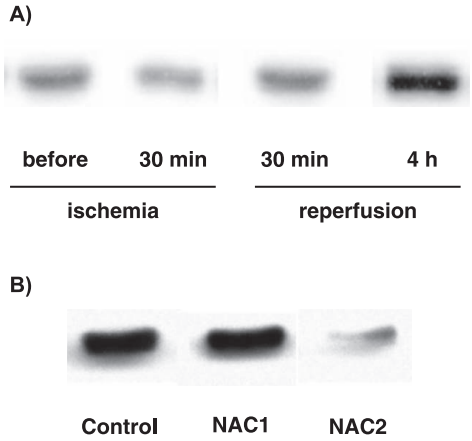

Fig. 6. Western blot analysis of inhibitor κB phosphorylation in rat myocardium after ischemia/reperfusion at the indicated time (before ischemia, 30 min ischemia, 30 min reperfusion, 4 h reperfusion) after ischemia/reperfusion (A), and at 4 h after reperfusion with different treatment of *N*-acetylcysteine (B). The rise of inhibitor κB phosphorylation found at 4 h after reperfusion was inhibited by the NAC2 treatment, but not by the NAC1 treatment.

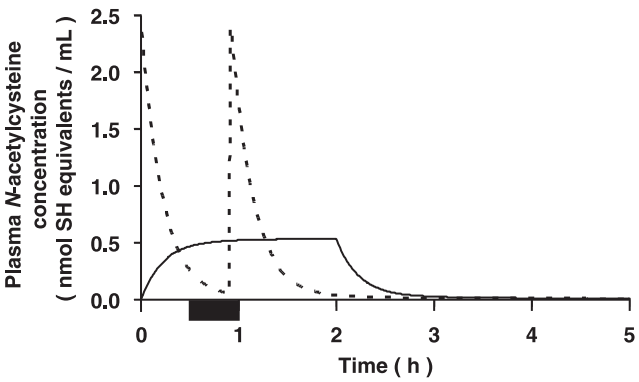

Fig. 7. Simulation curve of plasma *N*-acetylcysteine concentration with NAC1 and NAC2 treatments. Broken line: NAC1, solid line: NAC2. Bar shows occlusion period.

at 4 h after the start of reperfusion, but not at 30 min ischemia and at 30 min after reperfusion. The rise of Iκ B phosphorylation found at 4 h after reperfusion was inhibited by the NAC2 treatment, but not by the NAC1 treatment (Fig. 6B).

Simulation of plasma NAC concentration

The simulated plasma NAC concentration curve in rats is shown in Fig. 7. In the NAC2 treatment group, the plasma concentration reaches to the plateau phase before the ligation, and it is kept during ischemia and the first 1 h of reperfusion. On the other hand, the maximum concentration is five times higher in the NAC1 than that in the NAC2, but the concentration decreases rapidly with a half-life of about 15 min.

Discussion

The present study demonstrated that the preventive effect of NAC on rat myocardial injury after 30 min of ischemia followed by 4 h of reperfusion was different depending on the administration method even though the total dose was the same (300 mg/kg). The continuous infusion of NAC (150 mg/kg per hour) for 2 h from 30 min before occlusion of the coronary artery (NAC2) significantly limited the extent of infarction. However, the bolus injection of NAC (150 mg/kg) at 30 min before occlusion and 5 min before reperfusion (NAC1) failed to reduce the infarct area.

To prevent damage by ROS, various antioxidant defense systems are present in myocardial tissue: superoxide dismutase, catalase, glutathione peroxidase, and other endogenous antioxidants such as a vitamin E. Ischemia depletes these antioxidant systems, and the myocardial tissue is at risk because of a burst and sustained release of ROS produced during I/R. The glutathione/glutathione peroxidase system appears to be more active and important in the myocardium (2). Enhancement of the myocardial glutathione system contributed to cardioprotection against I/R (17). In this study, the recovery of glutathione peroxidase activity with glutathione content in myocardial tissue was shown by the NAC2 treatment, but not by the NAC1 treatment that failed to prevent the I/R injury. Plasma concentration of NAC decreases rapidly with a half-life of about 15 min (16) as shown in Fig. 7. The disposition of NAC to heart is poor (18). Considering from the pharmacokinetic properties, maintaining the plasma concentration for a certain period like the NAC2 treatment may be required to distribute a sufficient amount of NAC quantity to recover the glutathione antioxidant system in the heart. On the contrary, bolus injection of NAC seems to recover the glutathione content in the liver and limit the hepatic I/R injury (4, 19) because NAC is easily distributed in liver (18).

The lower plasma concentration of NAC at occlusion was not considered to be a reason for the failure of the bolus treatment of NAC, because the altered bolus treatment NAC3 in which NAC was injected 5 min before occlusion showed no reduction of the infarct size. On the other hand, the continuous treatment of NAC from 5 min before reperfusion (NAC4) produced the significant limitation of the infarct size, but it was less effective than the NAC2 treatment. These results indicate that it is necessary to maintain plasma NAC concentration for the early period after reperfusion to obtain its beneficial effect on myocardial I/R injury. Maintaining of the plasma NAC concentration from pre-occlusion may be more effective. Kingma and Rouleau

(10) and Andreadou et al. (11) could not confirm the beneficial effect of NAC on limiting infarct size by a continuous infusion of NAC starting before ischemia and ending before or 15 min after reperfusion. Considering the observations in the previous studies with the results of this study, the failures in preventing the I/R injury might be attributed to the short period of the infusion after reperfusion. On the other hand, bolus injection of NAC was reported to have a beneficial effect in canine myocardial I/R injury (8, 9). Therefore, the possibility could not be excluded that the beneficial effect of NAC is obtained at the higher dose in the bolus treatment group. However, in this experimental condition, the beneficial effect could not be obtained even at double the dose (300 mg/kg, twice) in the bolus treatment group.

NF-κB is a well-known transcription factor sensitive to oxidative stress. NF-κB can rapidly activate the expression of genes involved in the inflammatory response, such as proinflammatory cytokines and adhesion molecules (20, 21). The activation of NF-κB was observed during I/R (22, 23) and is considered to contribute to the occurrence and development of myocardial infarction (24, 25). In most cells, NF-κB is present in the cytoplasm binding with an inhibitory protein called IκB. Phosphorylation of IκB induces the release of IκB from NF-κB, which causes translocation of NF-κB to the nucleus and its binding to κB DNA sites (20). ROS induce IκB phosphorylation by the activation of kinases located at the upstream of the NF-κB pathway (26). The increase of IκB phosphorylation was observed after 4 h of reperfusion in this study. The continuous treatment of NAC (NAC2) inhibited the increase of IκB phosphorylation, which was consistent with the result that the increased MPO activity in the ischemic region after reperfusion was inhibited. The results were supported by the study of Kin et al. (27) that showed the reduction by NAC of nuclear NF-κB expression after I/R in the rat myocardium. Staal et al. (28) reported that NAC inhibited the activation of NF-κB via increasing the intracellular glutathione level, which appeared to be an important regulator of the activity of NF-κB. Therefore, it seems that the inhibition of IκB phosphorylation was not found in the NAC1 treatment because of no recovery of the glutathione/glutathione peroxidase system.

In conclusion, the protective effect of NAC on myocardial infarction induced by I/R was different depending on the administration method. To obtain the beneficial effect of NAC, it is necessary to maintain blood concentration during the early period of reperfusion. If the continuous treatment of NAC could be started before ischemia, a more potent effect can be obtained.

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