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INHIBITION WITH NCO-700, A PROTEASE INHIBITOR, OF DEGRADATION OF CARDIAC MYOFIBRILLAR PROTEINS DURING ISCHEMIA IN DOGS

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Abstract—NCO-700 is a newly synthesized inhibitor of both cathepsin B and calcium-activated neutral protease. We examined whether NCO-700 inhibits degradation of myofibrillar proteins induced by cardiac ischemia in dogs anesthetized with pentobarbital. Cardiac ischemia was produced by complete occlusion of the left anterior descending coronary artery (LAD) for 3 or 6 hr. Myofibrils were prepared from the ischemic myocardium, in which LAD was occluded, and from the nonischemic myocardium, in which LAD was not occluded. Electrophoresis of myofibrils prepared from the ischemic myocardium revealed that there were many degradation bands of myofibrillar proteins as well as the bands corresponding to α-actinin (AN), the 55 kDa protein (55 K), actin (A), tropomyosin (TM), troponin I (TN I), myosin light chain 1 (LC1) and myosin light chain 2 (LC2). In addition, the content of AN, 55 K, A, TM, TN I, LC1 and LC2 in the ischemic myofibrils was lower than that in the nonischemic myofibrils. Treatment with NCO-700 at the total dose of 20 mg/kg, which was injected intravenously before and during ischemia, inhibited both appearance of the degradation bands and the decrease in the content of A, TM, TN I, LC1 and LC2 being produced by cardiac ischemia. NCO-700, however, did not inhibit the decrease in the content of 55K and AN being induced by ischemia.

It is well known that coronary artery occlusion produces ultrastructural changes in intracellular organelles including myofibrils in the myocardium, suggesting that there is degradation of myofibrillar proteins during cardiac ischemia [1–8]. There are many endogeneous proteases in the myocardial cells; for example, cathepsins in lysosomes [9, 10], and calcium-activated neutral proteases (CANP) in cytoplasm [11, 12]. Cathepsins are reported to be released from lysosome into cytoplasm during cardiac ischemia [9, 10], degrading myofibrillar proteins of skeletal muscle [13, 14] and cardiac muscle [15], and therefore cathepsins should be responsible for changes in myofibrils during cardiac ischemia. CANP is another candidate for changes in myofibrils during cardiac ischemia, because it removes Z lines from skeletal and cardiac myofibrils [16, 17] and degrades tropomyosin and troponin in cardiac myofibrils [12, 17], and because calcium concentrations are high in the ischemic cells [1].

NCO-700 (bis[ethyl(2*R*,3*R*)-3-[(*S*)-3-[(*S*)-3-methyl-1-[4-(2,3,4-trimethoxyphenylmethyl)-piperazin-1-ylcarbonyl]butylcarbamoyl]oxiran-2-carboxylate] sulfate) is a newly synthesized inhibitor of both cathepsin B [15, 18] and CANP [19]. The structure of this compound is shown in Fig. 1. Toyooka *et al.* [19] have reported that NCO-700 reduces size of acute myocardial infarction being produced by coronary artery occlusion in rabbits. However, it is unknown whether the beneficial effect of NCO-700 on myocardial infarction is due to inhibition of degradation of myofibrillar proteins. In this paper, therefore, we attempted to examine whether NCO-700 inhibits degradation of myofibrillar proteins induced by coronary artery occlusion in dogs.

MATERIALS AND METHODS

Animals. Thirty-seven mongrel dogs of either sex weighing 9.9 ± 0.4 kg (mean ± S.E.) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). Under artificial respiration, the chest was opened to permit free access to the left ventricular wall. The left anterior descending coronary artery (LAD) was freed from the adjacent tissues, and a silk thread was placed around the LAD for complete occlusion. A wire electrode for epicardial ECG was attached to surface of the left ventricular wall, to which blood was supplied by the LAD. The ST segment was

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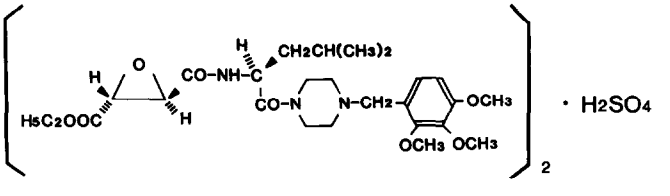

Fig. 1. Chemical structure of NCO-700.

measured from the epicardial ECG, heart rate was counted from the standard ECG (limb lead II). Systolic and diastolic blood pressures were measured in the left carotid artery.

Experimental protocol. After hemodynamic parameters became constant, the experiment was started. Dogs were divided into four groups as follows:

(1) N (nonischemic) group. In this group of dogs (six dogs), neither LAD occlusion nor injection of NCO-700 was performed.

(2) I (ischemic) group. In this group of dogs, LAD was completely occluded but drugs were not injected. According to the duration of complete occlusion, the animals were subdivided into I_3 (seven dogs) and I_6 (six dogs) groups, in which LAD was occluded for 3 and 6 hr, respectively.

(3) NCO-I (NCO-treated ischemic) group. In all the dogs in this group, NCO-700 was injected, and in addition, LAD was completely occluded. The dogs were subdivided into NCO-I_3 (six dogs) and NCO-I_6 (seven dogs) groups, in which LAD was occluded for 3 and 6 hr, respectively. The protocol of administration of NCO-700 was slightly different between NCO-I_3 and NCO-I_6 groups as shown in Fig. 2, but the total dose of NCO-700 was the same (20 mg/kg, i.v.) in both groups.

(4) NCO-N (NCO-treated nonischemic) group. In this group of animals (five dogs), NCO-700 was injected according to the same schedule as in the NCO-I_3 group. The only difference between the NCO-I_3 and NCO-N groups was that LAD was not occluded in the NCO-N group.

At the end of experiment, each of the nonischemic or ischemic myocardium was taken from the left ventricular wall by drill biopsy (a drill having a diameter of about 1 cm was used). The cardiac muscles taken by the drill biopsy were immediately transferred into liquid nitrogen and were kept at −80°, being employed for preparation of myofibrils.

Preparation of myofibrils. Cardiac myofibrils were prepared according to the method of Zak *et al.* [20] with some modifications. The method of preparation of cardiac myofibrils has been described in the previous paper [4]. Briefly, the myofibrils were treated with 1% Triton X-100 for 30 min at 4° for elimination of membrane materials [21]. In order to inhibit degradation of myofibrillar proteins during the course of preparation, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) was added to a buffer solution prepared for extraction of cardiac myofibrils. Protein concentration was determined by the buiret method and the method of Bradford for microassay [22].

Electrophoretic study. Slab gel electrophoresis (14 × 9.5 × 0.1 cm) was performed in the buffer system described by Laemmli [23]. About 25 μg myofibrillar protein was applied on each well in 12% acrylamide gels. Bromphenol Blue was used as a tracking dye. Electrophoresis was carried out at a constant current of 20 mA for about 4 hr. The gels were stained in 0.25% Coomassie Brilliant Blue R 250 and destained with gentle shaking at 37° in 5% methanol and 7% acetic acid. Densitometric tracings were obtained at 570 nm by the use of Shimadzu Dual-Wavelength TLC Scanner (CS-910). The areas under peaks of myofibrillar proteins (α-actinin, the 55 kDa protein, actin, tropomysin, troponin-I, myosin light chain 1 and myosin light chain 2) were determined by planimetry. The content of the myofibrillar proteins was expressed as a ratio (arbitary unit of percentage) of the area under the peaks of corresponding proteins to the amount of total proteins that had been applied on the well.

Drug. NCO-700, donated from Nippon Chemiphar Co. Ltd., was dissolved in saline solution.

Chemicals. PMSF and Coomassie Brilliant Blue were obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals were either reagent or biochemical grade. The 55 kDa protein purified from the dog heart was given by Dr. Umeji Murakami (Department of Chemistry, Asahikawa Medical College). For determination of the mol. wt, a kit of marker proteins containing phosphorylase *b* of the rabbit muscle (94 kDa), albumin of bovine serum (67 kDa), ovalbumin of egg white (43 kDa), carbonic anhydrase of bovine erythrocyte (30 kDa) trypsin inhibitor of soybean (20.1 kDa) and α-lactalbumin of bovine milk (14.4 kDa) was used. The kit was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

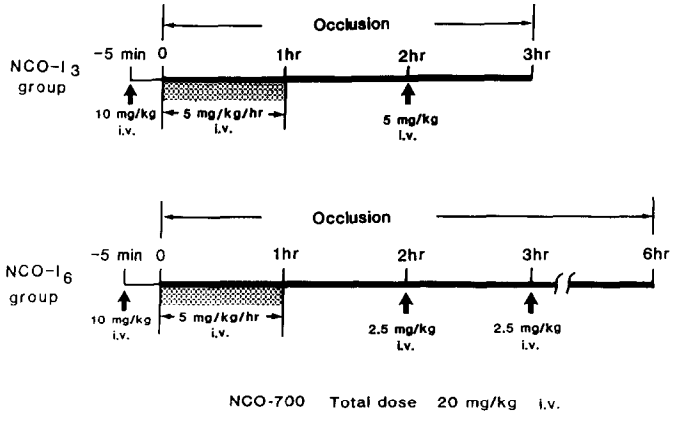

Fig. 2. Protocol of administration of NCO-700 in NCO-I_3 and NCO-I_6 groups. The total dose of NCO-700 is 20 mg/kg in each of the groups.

Statistical analysis. The data were evaluated by analysis of variance (Table 1) or Student's *t*-test (Fig. 3). A P value of 0.05 or less was considered significant. All values were expressed as mean ± S.E.

RESULT

Hemodynamic study

The effect of NCO-700 on heart rate and blood pressure in NCO-N group is shown in Fig. 3. Heart rate significantly increased after a bolus injection of 10 mg/kg of NCO-700, but it returned to the original level rapidly, while it did not change heart rate significantly during and after a constant infusion of 5 mg/kg/hr followed by a bolus injection of 5 mg/kg of NCO-700. Systolic and diastolic blood pressures did not change significantly after the bolus and constant administration of NCO-700, except that there is a decrease in systolic blood pressure at 75 min after the start of constant infusion.

When the LAD was occluded for 3 and 6 hr, ventricular fibrillation (Vf) occurred in some dogs (two out of seven dogs in I_3 group, one out of six dogs in I_6 group, one out of six dogs in NCO-I_3 group, and two out of seven dogs in NCO-I_6 group). The hemodynamic data were obtained from the dogs which survived to the end of the experiment (five dogs in each group). In the I_3 and I_6 groups, ST-segment of epicardial ECG in the ischemic area was elevated by LAD occlusion. Heart rate and blood pressure were not significantly changed by LAD occlusion. In both NCO-I_3 and NCO-I_6 groups, NCO-700 did not attenuate the elevation of ST-segment during LAD occlusion (the data are not shown). There was no significant difference (unpaired analysis) in both blood pressure and heart rate between I_3 and NCO-I_3 groups, and between I_6 and NCO-I_6 groups, except that there was a significant decrease in diastolic pressure only at 121 min after LAD occlusion in NCO-I_3 group (the data not shown). Thus, NCO-700 did not have a striking effect on the elevation of ST-segment induced by LAD occlusion, and did not change both blood pressure and heart rate.

Electrophoretic study. Figure 4 shows a representative slab gel electrophoretogram of myofibrils prepared from the myocardium in each of the groups; myofibrils of N, I_3, NCO-I_3, I_6 and NCO-I_6 groups, and also shows electrophoretic patterns of the 55 kDa protein purified from dog hearts and marker proteins (94, 67, 43, 30, 20.1, and 14.4 kDa). The fixed amount of myofibrils (25 μg) was applied on each well. In the N group, there were the bands corresponding to the myosin heavy chain (HC), α-actinin (AN), the 55 kDa protein (55K), actin (A), tropomyosin (TM), troponin (TN-I), myosin light chain 1 (LC1) and myosin light chain 2 (LC2). In both I_3 and NCO-I_3 groups, a lot of degradation bands were observed between the bands corresponding to AN and 55K, when compared with the electrophoretic pattern in the N group. There was no significant difference in the electrophoretic pattern between I_3 and NCO-I_3 groups. However, a marked difference was found between I_6 and NCO-I_6 groups; there were many degradation bands between the bands corresponding to AN and 55K, whereas there were less degradation bands between the bands of AN and 55K. These results suggest that treatment of the dog with NCO-700 inhibited the degradation of myofibrillar proteins induced by LAD occlusion for 6 hr.

The area under each of the peaks of myofibrillar proteins, having been recorded by a densitometer, was determined by planimetry, and the content of myofibrillar proteins, AN, 55K, A, TM, TN I, LC1 and LC2, was calculated in terms of arbitrary units as described in Methods. In order to know slight changes of small amount of proteins such as TN I and 55K, the densitometric tracing was performed in the high sensitivity range, in which the amount of HC could not be determined. This is because the amount of HC was much greater than that of other myofibrillar proteins. Table 1 shows the relative content of myofibrillar proteins in N, NCO-N, I_3, NCO-I_3, I_6 and NCO-I_6 groups. First, we examined whether or not the treatment with NCO-700 affects the content of myofibrillar proteins even in the non-

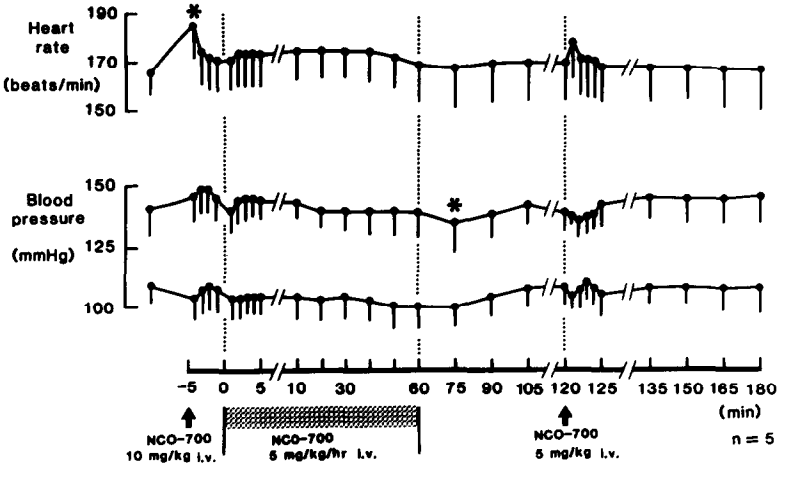

Fig. 3. Effect of NCO-700 on heart rate and blood pressure in the dog with nonischemic heart (NCO-N group). n = number of dogs. Values are expressed as mean ± S.E. (vertical bars). * $P < 0.05$ (Student's *t*-test).

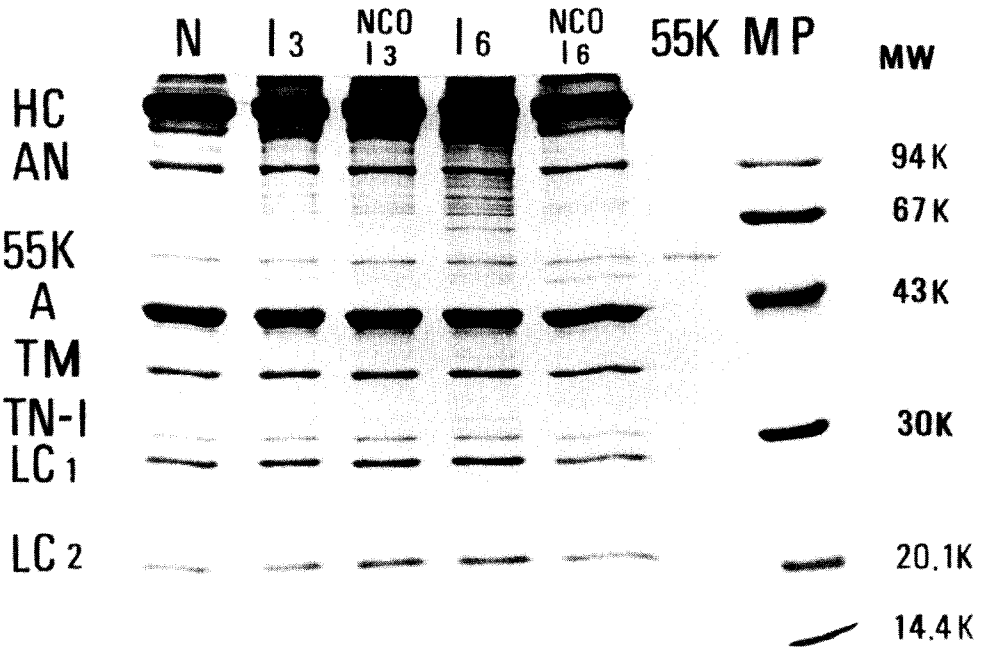

Fig. 4. Slab gel polyacrylamide gel electrophoretic patterns of the isolated cardiac myofibrils. Electrophoresis was carried out in the Laemmli's system. The myofibrillar proteins (25 μg) was applied on each well in the 12% slab gel. N = nonischemic group; I_3 = 3 hr-ischemic group; NCO-I_3 = 3 hr-ischemic group treated with NCO-700; I_6 = 6 hr-ischemic group; NCO-I_6 = 6 hr-ischemic group treated with NCO-700; 55K = the 55 kDa protein; MP = marker proteins containing phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa); HC = myosin heavy chain; AN = α-actinin; A = actin; TM = tropomyosin; TN I = troponin I: LC1 = myosin light chain 1; LC2 = myosin light chain 2; MW = mol. wt.

ischemic normal dogs. There was no significant difference between N and NCO-N groups in the content of all the myofibrillar proteins (Table 1). Second, we examined the effect of ischemia on the content of myofibrillar proteins. The content of all the myofibrillar proteins in both I_3 and I_6 groups was lower than that in N group (Table 1), indicating that the content of myofibrillar proteins decreased in response to ischemia. In NCO-I_3 and NCO-I_6 groups, we examined the effect of NCO-700 on the decrease in myofibrillar proteins induced by ischemia. There were no significant differences between I_3 and NCO-$_3$ groups and between I_6 and NCO-I_6 groups in the content of both AN and 55K, being the structural proteins of Z lines. However, the content of A, TM, TN I, LC1 and LC2 in NCO-I_3 and NCO-I_6 groups was significantly higher than that in I_3 and I_6 groups, respectively (Table 1). These results suggest that the treatment with NCO-700 inhibits the degradation of all the myofibrillar proteins, except AN and 55K, being induced by LAD occlusion for 3 and 6 hr. Another interesting finding was that the content of LC2 in NCO-I_3 group was significantly higher than that in N group (Table 1).

DISCUSSION

In order to determine the content of myofibrillar proteins quantitatively, we performed slab gel electrophoresis of myofibrils and measured the protein content from densitometric tracings of the electrophoretogram. The protein content was expressed as a ratio (arbitary unit) of the area under each of the peaks of corresponding protein to the amount of total proteins that had been applied on the well. Usually the content is expressed by the following two estimations; (I) a ratio of the area under each of the peak to the total area under all of the peaks, or (II) a ratio of the area under each of the peaks to the area under peak of actin. However, these two estimations have disadvantages. In the estimation (I), the ratio of the small amount of proteins, such as troponin I and the 55 kDa protein, is very low, because the area under peaks of both myosin and actin occupies about 80% of the total area under all the peaks [24]; for example, the area under the peak of the 55 kDa protein is only about 1% of the total area [24]. Therefore, an ischemia-induced slight change in the content of myofibrillar proteins, such as troponin I and the 55 kDa protein, cannot be detected by the estimation (I) accurately. In the estimation (II), there is a problem regarding the content of actin; we can use the area under peak of actin as an internal standard or a reference protein, only when the content of actin is constant. However, we have reported that the content of actin itself decreases as the isch-

Table 1. Content of myofibrillar proteins in cardiac myofibrils isolated from nonischemic and ischemic myocardium treated with or without NCO-700

Group	N	NCO-N	I_3	NCO-I_3	I_6	NCO-I_6
n	6	5	5	5	5	5
AN	13.77 ± 0.66 (100%)	11.90 ± 0.29 (86.42%)	9.97 ± 1.52* (72.40%)	12.18 ± 0.52 (88.46%)	8.62 ± 0.86* (62.60%)	11.06 ± 0.76* (80.32%)
55K	3.97 ± 0.33 (100%)	3.48 ± 0.21 (87.66%)	3.24 ± 0.38 (81.61%)	3.85 ± 0.16 (96.98%)	2.28 ± 0.20* (57.43%)	3.00 ± 0.29* (75.57%)
A	82.44 ± 3.14 (100%)	88.44 ± 0.67 (107.28%)	66.15 ± 5.05* (80.24%)	82.34 ± 4.65† (99.88%)	61.12 ± 4.79* (74.14%)	78.45 ± 2.57‡ (95.16%)
TM	18.61 ± 1.05 (100%)	17.15 ± 0.40 (92.15%)	15.82 ± 1.12 (85.00%)	20.20 ± 1.52† (108.54%)	13.41 ± 0.97* (72.06%)	17.87 ± 0.71‡ (96.02%)
TN1	5.83 ± 0.31 (100%)	5.24 ± 0.12 (89.88%)	4.56 ± 0.53* (78.22%)	5.90 ± 0.42† (101.21%)	3.82 ± 0.51* (65.52%)	4.99 ± 0.32‡ (85.59%)
LC1	17.89 ± 0.53 (100%)	18.72 ± 0.96 (104.64%)	13.48 ± 1.54* (75.35%)	19.35 ± 1.29† (119.16%)	11.18 ± 1.52* (62.49%)	15.45 ± 0.97‡ (86.36%)
LC2	11.72 ± 0.39 (100%)	13.45 ± 0.32 (114.76%)	9.34 ± 0.72* (79.69%)	15.00 ± 0.90*† (127.99%)	8.06 ± 1.09* (68.77%)	13.43 ± 0.96‡ (114.59%)

Content of each of the myofibrillar proteins is expressed in terms of arbitrary unit and percentage (see text). N = nonischemic group; NCO-N = nonischemic group treated with NCO-700; I_3 = 3-hr ischemic group; NCO-I_3 = 3-hr ischemic group treated with NCO-700; I_6 = 6-hr ischemic group; NCO-I_6 = 6-hr ischemic group treated with NCO-700; AN = α-actinin; 55K = the 55 kDa protein; A = actin; TM = tropomyosin; TN I = troponin I: LC1 = myosin light chain 1, LC2 = myosin light chain 2; n = number of dogs.

* $P < 0.05$ against the value of respective myofibrillar proteins in N group.

† $P < 0.05$ (between I_3 and NCO-I_3 groups).

‡ $P < 0.05$ (between I_6 and NCO-I_6 groups).

emic period lengthens [4]. Accordingly, actin cannot be used as a standard protein. This is the reason why we determined the content of a myofibrillar protein in terms of the area under the peak of that protein to the total amount of proteins applied. Before starting the examination, we confirmed that the area under the peak of a myofibrillar protein was proportional to the amount of total myofibrillar proteins that had been applied on the well (the data are not shown). Slab gel electrophoresis has an advantage in that many samples can be compared on the same gel at the same time. Therefore, changes in myofibrillar proteins can be detected more accurately by the use of our estimation method than by the use of the estimations (I) and (II).

The purpose of this experiment was to examine whether NCO-700 inhibits degradation of myofibrillar proteins induced by cardiac ischemia. In I_3 and I_6 groups, we examined the effect of myocardial ischemia on composition of myofibrillar proteins. Myocardial ischemia produced by coronary artery occlusion decreased the content of α-actinin, the 55 kDa protein, actin, tropomyosin, troponin I, myosin light chain 1 and myosin light chain 2 (Table 1, I_3 and I_6 groups). In addition, electrophoresis of myofibrils prepared from the ischemic myocardium in I_3 and I_6 groups revealed that there were many degradation bands (Fig. 4, I_3 and I_6 groups). These results are consistent with the results of previous papers which have shown that myofibrillar proteins are degraded during a period of ischemia [4, 5, 6]. In NCO-I_3 and NCO-I_6 groups, it became apparent that treatment with NCO-700 at the total dose of 20 mg/kg inhibited the decrease in the content of actin, tropomyosin, troponin I, myosin light chain I and myosin light chain 2 being produced by ischemia (Table 1). The electrophoretic pattern in NCO-I_6 group was different from that in I_6 group; there were less degradation bands in NCO-I_6 group. These results suggest that treatment with NCO-700 inhibited the degradation of myofibrillar proteins induced by ischemia.

It is possible that many proteases are activated by an increase in intracellular concentrations of hydrogen and calcium ions during cardiac ischemia, and that these proteases degrade myofibrillar proteins [9–12]. Both cathepsin B and CANP are candidates for the proteases being responsible for degradation of myofibrillar proteins [9, 10, 17]. Cathepsin B purified from skeletal muscle degrades myosin and actin [15, 25], and CANP purified from the cardiac muscle degrades tropomyosin, troponin T and troponin I [11, 26]. Hirao *et al.* [15] have reported that NCO-700 inhibits the proteolytic activity of cathepsin B on alkali-denatured casein, and Hara *et al.* [11] have shown that NCO-700 inhibits the activity of CANP purified from monkey cardiac muscles, *in vitro*. In the present study, NCO-700 did not have a striking effect on the heart rate and blood pressures, indicating that NCO-700 does not decrease the oxygen consumption of the heart. Therefore, it is considered that the effect of NCO-700 on the myofibrillar proteins is not due to a decrease in oxygen consumption but probably due to an increase in coronary flow [18] and/or the inhibiting effect of NCO-700 on cathepsin B and CANP. Nevertheless, the treatment with NCO-700 did not inhibit decrease in the content of both α-actinin and the 55 kDa protein, being structural proteins of Z lines. It has been demonstrated that NCO-700 suppresses activities of both CANP and cathepsin B isolated from cardiac muscle, producing 50% inhibition at 46 μM on CANP and at 0.8 μM on cathepsin B [27]; NCO-700 can inhibit the activity of cathepsin B more than

that of CANP at the same concentration. The 55 kDa protein is degraded by CANP but not by cathepsin B [28]. This gives a partial explanation for the fact that NCO-700 did not inhibit the decrease in the content of the 55 kDa protein. Unlike the 55 kDa protein, α-actinin is considerably resistant to digestion by proteases, and the decrease in the content of α-actinin may be due to the release of α-actinin as a result of digestion of another unknown structural protein of Z lines, which is highly sensitive to the protease and involved in anchoring α-actinin to Z lines [24]. It is unknown, however, why NCO-700 did not inhibit the decrease in the content of α-actinin. The content of myosin light chain 2 in NCO-I_3 group was significantly higher than that in N group (Table 1). Because the treatment with NCO-700 of nonischemic dogs did not affect the content of myofibrillar proteins (Table 1, NCO-N group), the increase in the content of myosin light chain 2 in NCO-I_3 group is probably due to appearance of the band of degraded proteins having the mol. wt similar to that of myosin light chain 2.

In conclusion, NCO-700 at the total dose of 20 mg/kg inhibited the ischemia-induced degradation of myofibrillar proteins, such as actin, tropomyosin, troponin I, myosin light chain 1 and myosin light chain 2, but it did not inhibit the ischemia-induced degradation of the 55 kDa protein and α-actinin.

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