A PROTEASE INHIBITOR, NCO-700, IMPROVES THE CONTRACTILE FUNCTION IN STUNNED CANINE MYOCARDIUM

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To explore the role of calcium-dependent protease in the stunned myocardium, open-chest dogs underwent 15 min of left anterior descending coronary artery occlusion followed by 2 h of reperfusion. Dogs received a single bolus intravenous injection of either the protease inhibitor NCO-700 (n=6) or saline (n=6) 1 min before reperfusion followed by a 30-min infusion at the same dose. Regional myocardial function was assessed in terms of systolic wall thickening with an epicardial Doppler probe. The two groups exhibited comparable systolic thickening under baseline conditions and similar degrees of dyskinesis during occlusion. After reperfusion, recovery of contractile function, expressed as a percentage of the baseline value, was significantly greater in NCO-700-treated dogs as than in control dogs: -14.3 ± 10.6 vs -48.9 ± 7.2 ($p < 0.05$) at 15 min, 10.8 ± 10.3 vs -31.1 ± 9.0 ($p < 0.05$) at 30 min, 42.5 ± 10.1 vs -16.4 ± 9.1 ($p < 0.005$) at 1 h, and 47.5 ± 8.3 vs -14.9 ± 9.4 ($p < 0.001$) at 2 h. The data suggest that the protease inhibitor markedly improved contractile function in stunned myocardium by inhibiting intracellular protease activity.

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IT is well established that reperfusion after a brief episode of myocardial ischemia lasting less than 20 min does not lead to myocardial necrosis¹ but is associated with a delayed recovery of contractile function. This condition, which is referred to as myocardial stunning², is a mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage, and is distinct from myocardial infarction³. Various mechanisms have been proposed for myocardial stunning, including generation of oxygen-derived free radicals, calcium overload,

and excitation-contraction uncoupling due to sarcoplasmic reticulum dysfunction.

Bis [ethyl (2R, 3R) -3 -[(S)-methyl -1 -[4 - (2, 3, 4 -trimethoxyphenyl)methyl] piperazin-1-ylcarbonyl] butyl-carbonyl] oxiran-2-carboxylate] sulfate (NCO-700) is a novel inhibitor of protease such as calcium-activated neutral protease (CANP)⁴ and cathepsin⁵. Protease induces degradation of myofibrils when the concentration of calcium in the myocardium is elevated during ischemia and reperfusion⁶. Furthermore, it has been suggested that calcium-dependent protease mediates major sources of free radicals by conversion of xanthine dehydrogenase to xanthine oxidase⁷. In vivo studies have shown that NCO-700 reduces the infarct size due to coronary artery ligation in rabbit⁴ and in-

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hibits the degradation of myofibrillar proteins induced by myocardial ischemia in dog^{8,9}. However, the role of protease in myocardial stunning is still unclear¹⁰. Therefore, the purposes of this study were to determine whether or not postischemic myocardial dysfunction can be attenuated by pretreatment with NCO-700, and to explore the role of protease in the canine model of stunned myocardium.

MATERIALS AND METHODS

Experimental Preparation

All experimental studies were performed according to the Guidelines for Animal Experimentation, based on the Rules and Regulations of the Animal Research Committee of Kurume University School of Medicine. Mongrel dogs of either sex, weighing 12–25 kg, were anesthetized with sodium pentobarbital (30 mg/kg). The dogs were then ventilated with a tidal volume of 10–20 ml/kg at a respiratory rate of 15–18 breaths/min using an oxygen-flushed anesthesia machine (Model K-1, Igarashi Ika Kogyo) and a ventilator (Harvard Apparatus). Arterial blood gases were maintained within normal physiologic limits and the body temperature was kept constant using a heating pad. A thoracotomy was performed in the fifth left intercostal space of each dog under sterile conditions and the heart was suspended in a pericardial cradle. Polyethylene catheters were placed in the right atrium through the right atrial appendage for drug infusion. A segment of the left anterior descending (LAD) coronary artery was gently dissected free from the surrounding tissue and a pulsed Doppler flow velocity probe was placed around the LAD artery distal to a balloon occluder. A micromanometer was placed in the femoral artery to monitor aortic pressure. A Doppler ultrasonic wall-thickening probe (10-MHz) was positioned in the center of the region to be rendered ischemic, and a second probe was placed in the region of the left circumflex (LCx) coronary artery. An integrated measuring system based on pulsed Doppler (Model VF-1, Crystal Biotech, Inc.) simultaneously recorded myocardial thickening, electrocardiogram, and LAD blood flow velocity and continuously recorded these variables on an

eight-channel recorder (RECTI-HORIZ-8K, San-ei). Aortic pressure was measured simultaneously.

Experimental Protocol

NCO-700 (a generous gift from Nippon Chemiphar Research Institute) was dissolved in normal saline solution. The 6 treated dogs received a single bolus infusion of NCO-700 (20 mg/kg) starting 1 min before reperfusion. Thereafter, they received a continuous infusion of NCO-700 for 30 min after reperfusion (20 mg/kg). The 6 control dogs received an equivalent volume of the vehicle (normal saline) under the same protocol.

The LAD coronary artery was occluded for 15 min and then reperfused. Restoration of blood flow was confirmed by Doppler flow probe measurements. Hemodynamic and wall thickening data were obtained 5 min before coronary occlusion, 10 min after occlusion, and at 15, 30, 60, and 120 min after the reperfusion. At the end of the study, the dogs were given a lethal dose of potassium chloride. The heart was removed and cut into 1.0 cm-thick slices in a plane parallel to the atrioventricular groove for 2,3,5-triphenyltetrazolium chloride staining. The absence of any myocardial infarction was confirmed in all dogs. Animals which developed lethal arrhythmia during coronary occlusion or following reperfusion were excluded from the present study.

Measurement of Myocardial Function

Regional myocardial function was assessed using a pulsed Doppler epicardial probe according to the method of Zhu et al.¹¹ Briefly, the pulsed Doppler technique uses a single epicardial transducer to determine systolic myocardial wall displacement by digitally integrating the velocity of myocardial layers which pass through a range-gated sample volume. The beginning and end of systole were determined at the Q wave on the electrocardiogram, and at the dicrotic notch of the aortic pressure tracing, respectively. The net systolic thickening was defined as the maximal systolic increase in the wall thickness from the end-diastolic value. When paradoxical wall thinning (dyskinesis) persisted for 50% or more of systole, the maximal extent of wall thinning was subtracted from wall thickening to give a net

Fig. 1. Representative recordings of transmural wall thickening measured sequentially by pulsed Doppler techniques under baseline conditions, during coronary occlusion, 30 min, 1 h, and 2 h after reperfusion. Systolic wall thickening is measured from the Doppler signal as the distance in millimeters between end-systole and end-diastole. Under baseline conditions, thickening fraction was calculated with a simplified formula as $[1.4 \text{ mm (systolic thickening)} / 7 \text{ mm (range gate depth)}] \times 100 = 20\%$. ECG: electrocardiogram.

systolic thickening. Transmural myocardial function was assessed as a percent systolic thickening fraction (ThF) = (transmural net systolic thickening/end-diastolic wall thickness) $\times 100$ (%). If no paradoxical systolic thinning was not observed until 10 min after coronary occlusion, the dog was excluded from this study because of failure to develop ischemia. Representative recordings of transmural wall thickening at various time points are illustrated in Fig. 1.

Statistical Analysis

Values are expressed as mean $\pm$ SE. The control and treated dogs were compared using the two-tailed Student's *t* test for unpaired data. Differences were considered to be significant when the probability was less than 0.05.

RESULTS

Excluding Dogs From The Study

Of the 23 dogs initially anesthetized for the acute experimental protocol, 11 dogs were excluded for the following reasons. Five dogs showed no paradoxical systolic

thinning during ischemia. One dog died of ventricular tachycardia during ischemia. Four of the control dogs and 1 of the treated dogs died of ventricular fibrillation following reperfusion. Thus, analysis of data was carried out in 6 control and 6 treated dogs. There was no significant difference between the control and the treated dogs with respect to the incidence of arrhythmias during coronary artery occlusion or subsequent reperfusion. However, the low incidence of ventricular fibrillation and the relatively small sample sizes in our study preclude definitive conclusions.

Hemodynamics and Wall Thickening in Control and NCO-700-Treated Dogs

The hemodynamic data in the control and NCO-700-treated dogs are presented in Table I. At baseline, occlusion, and reperfusion, there were no significant differences between the control and treated groups with respect to heart rate, systolic or diastolic arterial pressure, and rate-pressure product. Systolic ThF in the LAD artery region is presented as an absolute value in Table I and as a percentage of the baseline

TABLE I HEMODYNAMIC AND WALL THICKENING DATA IN CONTROL AND NCO-700 TREATED DOGS

	Baseline	Occlusion	Reperfusion			
			15 min	30 min	1 h	2 h
Heart Rate (beats/min)						
Control	146±3	149±3	146±5	146±6	147±5	149±5
NCO-700	148±5	148±3	149±6	151±6	149±6	147±4
Systolic Arterial Pressure (mmHg)						
Control	127±4	126±4	127±4	127±4	128±4	127±5
NCO-700	126±4	128±4	127±4	126±3	125±4	128±5
Diastolic Arterial Pressure (mmHg)						
Control	83±5	82±4	82±5	82±5	82±4	83±5
NCO-700	83±3	81±4	82±4	82±4	82±3	84±4
RPP						
Control	18.6±0.5	18.8±0.5	18.9±1.1	18.7±0.6	18.7±0.7	18.8±0.7
NCO-700	18.7±0.9	19.0±0.9	18.8±0.6	19.1±1.1	18.7±1.0	18.8±0.9
%ThF(LAD)						
Control	23.3±1.1	-15.8±1.5	-11.2±1.5	-7.0±1.8	-3.8±2.2	-3.4±2.2
NCO-700	22.1±1.8	-16.2±1.6	-2.4±2.1 ^a	3.3±2.3 ^a	8.9±1.9 ^b	10.1±1.6 ^c
%ThF(LCx)						
Control	17.9±1.1	18.7±1.0	18.2±1.2	18.2±1.2	16.8±1.1	16.8±0.8
NCO-700	17.7±1.2	18.7±1.3	17.4±1.5	17.1±1.2	16.2±1.1	16.2±1.1

Data are means±SE. RPP: rate-pressure product (heart rate×systolic blood pressure/1,000), % ThF (LAD): percent thickening fraction in left anterior descending coronary artery territory, % ThF (LCx): percent thickening fraction in left circumflex coronary artery territory. ^a $p<0.01$, ^b $p<0.005$, ^c $p<0.001$ vs control.

Fig. 2. Systolic thickening fraction in the ischemic-reperfused region after coronary occlusion (Occl) and at selected times after reperfusion in control (dashed line) and treated (continuous line) groups. Thickening fraction is expressed as a percent of percentage of the baseline value measured before coronary occlusion. * $p<0.05$, ** $p<0.005$, *** $p<0.001$ vs control.

value in Fig. 2. Baseline systolic ThF averaged $23.3 \pm 1.1\%$ in the control and $22.1 \pm 1.8\%$ in the treated dogs (NS). The extent of paradoxical systolic thinning during occlusion was comparable in the control and NCO-700-treated dogs (-15.8 ± 1.5 vs $-16.2 \pm 1.6\%$, NS). In the control dogs, although there was a gradual improvement in the contractile function following reperfusion, as compared with baseline values, the previously ischemic region remained still dyskinetic 2 h after reperfusion. In the NCO-700-treated dogs, the recovery of function after reperfusion was substantially greater (Fig. 2). ThF, expressed as a percentage of the baseline values, was -14.3 ± 10.6 in the treated dogs vs -48.9 ± 7.2 in the control ($p<0.05$) at 15 min after reperfusion, 10.8 ± 10.3 vs -31.1 ± 9.0 ($p<0.05$) at 30 min, 42.5 ± 10.1 vs -16.4 ± 9.1 ($p<0.005$) at 1 h, and 47.5 ± 8.3 vs -14.9 ± 9.4 ($p<0.001$) at 2 h after reperfusion. Similar differences were observed when the statistical analysis was performed using the absolute values of ThF (Table I). In the LCx region, there were no significant differences in ThF be-

tween the two groups throughout the entire experiment (Table I).

DISCUSSION

The present study demonstrates that a protease inhibitor, NCO-700, markedly attenuates postischemic contractile dysfunction. After 2 h of reperfusion, previously ischemic myocardium had recovered to approximately one-half of its baseline systolic function in dogs that had been treated with NCO-700, but remained dyskinetic in untreated control dogs. These observations suggest that intracellular protease plays an important role in the pathogenesis of regional postischemic myocardial dysfunction caused by a 15 min coronary occlusion followed by reperfusion. This finding supports the results of a recent study in which leupeptin, a cysteine-protease inhibitor, had a protective effect against myocardial stunning in Langendorff-perfused hearts¹⁰

Although the pathogenesis of myocardial stunning has not been fully determined, oxyradical generation, calcium overload, and excitation-contraction uncoupling have each been proposed as most likely contributing factors.³ Oxygen species can cause enzyme inactivation and lipid peroxidation during ischemia and reperfusion. This could induce damage to the sarcolemma and the sarcoplasmic reticulum, resulting in an increased transsarcolemmal calcium influx and a cellular calcium overload, especially under conditions of re-flow. An impaired calcium homeostasis could in turn lead to excitation-contraction uncoupling. Indeed, several studies have demonstrated that oxygen free radical scavengers, such as superoxide dismutase and catalase, significantly enhance the recovery of the stunned myocardium, and suggest that cytotoxic oxygen metabolites, including the superoxide anion, hydrogen peroxide and hydroxyl radical, play an important role in the genesis of myocardial stunning.¹²⁻¹⁴ Moreover, Bolli et al., using electron paramagnetic resonance spectroscopy, demonstrated that the myocardial production of free radicals begins during coronary occlusion and further increases after reperfusion in canine stunned myocardium.¹⁵ In a subsequent study¹⁶ they found that superoxide dismutase plus catalase suppressed

the production of free radicals, indicating that these radicals were derived from the univalent reduction of oxygen.

The xanthine oxidase inhibitor allopurinol has also been shown to inhibit uric acid production during ischemia and reperfusion, and to produce a marked improvement in the functional recovery of stunned myocardium. These data suggest that xanthine oxidase is a source of free radicals that contribute to postischemic dysfunction in the dog.¹⁷ In this regard, McCord proposed that elevated cytosolic calcium concentration activates a protease which is capable of converting xanthine dehydrogenase to xanthine oxidase during ischemia.⁷ Concomitantly, an elevated concentration of AMP, due to the depletion of ATP during ischemia, is catabolized to adenosine, inosine, and hypoxanthine. The increased hypoxanthine serves as a purine substrate for the xanthine dehydrogenase or xanthine oxidase, leading to further free radical generation in reoxygenated tissues.⁷ Therefore, the results of the present study suggest that the beneficial effect of the protease inhibitor NCO-700 on myocardial stunning may be associated with the inhibition of the conversion of xanthine dehydrogenase to xanthine oxidase in microvascular endothelial cells, which thereby reduces oxygen free radical generation, since this agent has been shown to scavenge free radicals generated from the xanthine-xanthine oxidase system.¹⁸ However, since we did not attempt to examine the direct effect of NCO-700 on either the xanthine dehydrogenase-xanthine oxidase system or on free radical formation in the present study, further studies are required.

It is also possible that NCO-700 directly protects against the increase in intracellular protease activity, such as of CANP⁴ and cathepsin⁵, in endothelial and myocardial cells, if intracellular concentrations of calcium increase during ischemia and reperfusion. A previous study used endothelial cell monolayers to demonstrate that intracellular calcium significantly increased during hypoxia and decreased to reach the normal value of resting cells during the reoxygenation.¹⁹ Furthermore, in a recent study using nuclear magnetic resonance spectroscopy which directly measured serial changes in the intracellular concentration of calcium in per-

fused heart that had been subjected to 20 min of ischemia followed by reperfusion, the intracellular concentration of calcium significantly increased about three fold at 15–20 min and was significantly elevated during the first 5 min after reperfusion, which reflected an increase in the concentration of intracellular calcium of $1 \mu\text{M}$.²⁰ On the other hand, CANP has been shown to require more than μM concentrations of calcium for maximum activity.²¹ These data suggest the possibility that transient calcium overload may increase intracellular CANP activity, leading to myocardial contractile dysfunction. Therefore, the present study and others¹⁰ suggest that the preventive effect of protease inhibitor on myocardial stunning may be closely associated with the inhibition of CANP in tissue cells.

Cathepsins have been shown to be released from lysosomes into cytoplasm during ischemia,²² degrading myofibrillar proteins of cardiac muscle.⁵ Since NCO-700 is a potent inhibitor of cathepsin,⁵ cathepsin may also play a role on myocardial stunning. However, in a very recent study Matsumura et al proposed that lysosomal protease does not play an important role in stunning.¹⁰ Thus, although CANP is more likely to contribute to the stunning process, the precise mechanism of the role of CANP in myocardial stunning awaits further investigation.

Collateral flows during ischemia may result in large differences in recovery of contractile function. Previous studies by Bolli et al. using microspheres technique indicated that the presence of hypokinesia during ischemia was associated with rapid recovery after reperfusion, but there was no relation between ischemic and postischemic dysfunction when dyskinesia was present during occlusion, implying that functional recovery in stunned myocardium was closely coupled to collateral flow.²³ In this study, wall thickening measurements were obtained 10 min after coronary occlusion according to the method of Bolli et al. Wall thinning was not defined as paradoxical (dyskinetic) unless it persisted throughout at least 50% of the duration of systole. Transmural ThF measured 10 min after coronary occlusion in control dogs averaged $-15.8 \pm 1.5\%$ (Table I). This value is consistent with the results of previous studies,¹⁷ excluding dogs which

showed a lack of dyskinesia during the 15 min coronary occlusion. Accordingly, the influence of collateral flow during ischemia on regional myocardial function may be relatively lower in this study.

Transient myocardial ischemia is frequently observed in patients with unstable angina or acute myocardial infarction with early recanalization which occurs spontaneously or is produced by thrombolytic therapy and coronary angioplasty.^{24,25} These clinical situations may induce myocardial stunning and left ventricular dysfunction.^{2,26} The present observations suggest that NCO-700 may be able to prevent postischemic myocardial dysfunction under such clinical conditions. When compared with the free radical scavengers like such as superoxide dismutase and catalase, NCO-700 offers the advantages of entering the intracellular space. Therefore, NCO-700 may be suitable for greater clinical application.

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