Contribution of Calcium-activated Neutral Protease to the Degradation Process of Ischemic Heart

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Heart muscle, as well as other tissues, contains a large amount of calcium-activated neutral protease (CANP), or calpain, which belongs to thiol protease and is sensitive to Ca$^{2+}$ ions in concentrations of millimolar or micromolar range. Concerning the pathological process of myocardial degradation in ischemia, we present the following evidence that CANP is involved, especially in the irreversible breakdown of myocardial proteins. We present a summary of those previously published data.

1. Degradation of myofibrillar protein in myocardial ischemia

Ca$^{2+}$ sensitivity of natural actomyosin (NAM) isolated from both the intact left ventricular free wall and an area of acute myocardial infarction (AMI) was analyzed by use of superprecipitation response from 2 to 48 h after left anterior descending coronary artery ligation in the dog. NAM from the intact tissue showed normal superprecipitation and normal Ca$^{2+}$ sensitivity. Four hours after the coronary ligation, Ca$^{2+}$ sensitivity was lowered only in the endocardial half of AMI region; it was markedly decreased both in the epicardial and endocardial halves at 6 h and completely lost at 24 and 48 h. A superprecipitation response was, however, demonstrated in all samples, indicating that both myosin and actin preserved their functions in the course of AMI. With sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), NAM from the AMI region revealed moderate decrease of the tropomyosin-binding subunit of troponin (TN-T) and the Ca$^{2+}$-binding subunit of troponin (TN-C) and drastic reduction of the inhibitory subunit of troponin (TN-I). This resulted in the formation of extra bands of low molecular weights. These results suggest that degradation of troponin subunits occurs relatively early (4 h after coronary artery occlusion) and from the endocardial half of AMI region. This degradation may be caused by one or several proteases that preferentially degrade the regulatory proteins among myofibrillar proteins.

Furthermore, biochemical studies indicate that CANP isolated from bovine heart selectively degrades both TN-T and -I in vitro. 2. Suppression of myocardial protein degradation by protease inhibitor.

CANP activity is inhibited by antibiotics including leupeptin and antipain or a synthetic reagent, NCO-700. Analogues of NCO-700 were synthesized to study the action against CANP. Among these analogues, NCO-700 was the most potent in reducing the size of AMI, produced by coronary artery ligation in rabbits in vivo, although it showed less powerful inhibition of CANP activity in vitro. Thus, NCO-700 may be a promising agent to reduce acute myocardial infarction size, and useful for the clinical studies, because it has no action on cardiac muscle contractility, different from beta antagonists or calcium-entry blockers.

NCO-700 suppressed the activity of both

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calcium-activated neutral protease and cathepsin B isolated from cardiac muscle. A kinetic study using 14C-labelled NCO-700 suggested that it is incorporated into cultured myocardial cells. Under both aerobic and hypoxic conditions, the NCO-700 inhibited peptide release from cultured myocardial cells in a dose-dependent manner. The amino acid release from heart slices of adult rabbit was also blocked under hypoxic and glucose-depleted conditions. These data and the AMI size reducing action of NCO-700 might support the view that NCO-700 sensitive protease(s), possibly calcium-activated neutral protease and/or cathepsin B induce an irreversible proteolysis in the process of myocardial cell degradation.

3. Salvage of ischemic myocardium by NCO-700

The administration (i.v., 20, 40 and 60 mg/kg) of NCO-700 before and/or after coronary ligation significantly reduced the necrotic mass in the rabbit left ventricle. It also prevented creatine phosphokinase loss in the ischemic myocardium up to 3 h but not at 6 h after ligation. The activities of both CANP and cathepsin B in the subendocardial and subepicardial layers of ischemic, marginal or control myocardium were inhibited by NCO-700 administration after coronary ligation. A hemodynamic study using a dog heart-lung preparation demonstrated a dose-dependent coronary dilatation with weak and transient negative inotropic and chronotropic effects. These data suggested that NCO-700 sensitive protease(s) is (are) involved in myocardial cell degradation and that NCO-700 temporarily inhibits it, as would be of a great significance for PTCA therapy.

4. Human platelet aggregation and NCO-700 effect

To clarify the physiological role of CANP in human platelets, we loaded the platelets with a Ca^{2+}-sensitive fluorescent dye, fura-2, and measured the degree of aggregation, cytosolic calcium ion concentration ([Ca^{2+}]), and proteolysis by SDS-PAGE. At physiological concentrations of Ca^{2+} (1 mM) in the incubation medium, [Ca^{2+}] was below 0.5 μM and platelet aggregation was not observed. A Ca^{2+} ionophore ionomycin (0.15 μM) or collagen (50 μg/ml), but not ADP (10 μM), sharply enhanced the [Ca^{2+}].

to near 1 μM and caused the aggregation. A calcium entry blocker, verapamil, completely abolished both the [Ca^{2+}], rise and the aggregation. NCO-700, a membrane permeable inhibitor against cysteine proteases (including CANP) dose-dependently blocked the aggregation but did not change the [Ca^{2+}], transient. SDS-PAGE revealed that filamin, talin, and 70 kD a protein were specifically degraded when platelets were aggregated by ionomycin or collagen and that the proteolysis was not observed when the aggregation was blocked by verapamil or NCO-700. These data provided evidence that Ca^{2+} entry exceeding 0.5 μM is essential, but not sufficient per se, and that activation of cysteine protease, most likely CANP, is involved in the platelet aggregation by collagen or calcium ionophore. Accordingly, the beneficial effect of NCO-700 on AMI during myocardial ischemia might be explained by the antiplatelet action.

5. Miscellaneous findings and conclusion

During isolation procedure of CANP from human heart, we identified two new calcium-activated neutral hydrolases. One is an esterase, of molecular weight 300,000 which, required mM concentration of Ca^{2+}, hydrolyzed Ac-Tyr-OEt H₂O (optimal pH at 7.0). The other is an amidase, molecular weight 70,000 which, also required mM concentration of Ca^{2+}, hydrolyzed a synthetic substrate for chymotrypsin, suc-Leu-Leu-Val-Tyr-MCA (optimal pH at 7.2). Both enzymes did not degrade casein or myofibrillary proteins (myosin, actin, troponin and tropomyosin). Their activities were not inhibited by exogenous protease inhibitors, leupeptin, antipain, monooiodoacetic acid and chymostatin, while the amidase activity was selectively blocked by the endogenous inhibitor of CANP. Thus, their characteristics are different from chymotrypsin or CANP and they seem to be new hydrolases in the human heart.

At present, both the physiological and pathological significance of these hydrolases are not clear. It would be reasonable to assume their contribution in the progression of myocardial cell breakdown in ischemia, when intracellular Ca^{2+} concentration goes up, in collaboration with CANP or Ca^{2+}-sensitive phospholipases.
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