CALPAIN IS ACTIVATED BY β-ADRENERGIC RECEPTOR STIMULATION UNDER HYPOXIC MYOCARDIAL CELL INJURY

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This study was undertaken to investigate the correlation between hypoxic cell injury and protease activity, as well as the effects of α1- and β-adrenergic blocking agents and calcium antagonists during hypoxia. Cell death during hypoxia rose to 80% after 6h. Calpain activity increased to 4 units during hypoxia, much higher than the 0.7 units seen in aerobic condition at 6h. This activity was markedly inhibited by calpain-specific inhibitor I (n-acetyl-leucyle-leucyle-norleucinal). α1-adrenergic blocking agents did not affect calpain activity and cell death under hypoxia. On the other hand, β-adrenergic blocking agents and calcium antagonists suppressed the calpain activity and decreased cell death during hypoxia. These β-adrenergic blocking agents and calcium antagonists also inhibited intracellular calcium-influx during hypoxia.

These results suggest the β-adrenergic receptor stimulation activates adenylate cyclase activity and enhances calcium-influx during hypoxia. The elevated intracellular calcium concentration then stimulates calpain activity under hypoxia. These results prove that β-adrenergic blocking agents and calcium antagonists prevent protein degradation during hypoxic cell injury.

MANY investigations1,2 have recently shown the relationship between intracellular calcium overload and cell injury. Calcium ions have important functions in excitation-contraction coupling, intracellular signal transduction, and cell metabolism with other cations3,4. The free calcium concentration in extracellular fluid is in the millimolar range and is 1000-fold higher than that of the cytosol. Several mechanisms are resposible for maintaining this large electrochemical gradient and preventing intracellular calcium overload in normal cells5,6. Loss of ionic hemeostasis, including intracellular calcium control, may exacerbate injury by depressing mitochondrial energy production and activating cytosolic protease and phospholipase. Considerable interest has focused on calpain, also known as calcium-activated neutral protease (CANP), as a cause of hypoxic myocardial injury9. Calpain is a typical intracellular cystine protease of higher animals. It was first extracted from rat brain in 196410 but the biological function of this protease has not yet been clarified. Two types of calpain differentiated by their calcium sensitivities have been indentified in mammals9. Calpain-1 requires a micromolar order of calcium, while calpain-2 requires a millimolar order of calcium for activation.

Recently, we showed that free fatty acid and prostacyclin were released from isolated heart during hypoxia11. We have also reported that phospholipase A2 was activated during hypoxia and that this required a high

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concentration of calcium ions\textsuperscript{12} However, it is not yet clear how calcium-mediated phospholipase A\textsubscript{2} and calpain are activated under hypoxia.

In this study, we attempted to investigate the correlation between hypoxic cell injury and intracellular protease activity, in particular to determine the mechanism of increased calpain activity in cultured myocytes under hypoxia.

**MATERIALS AND METHODS**

**Reagents**

Calpain-inhibitor I (N-acetyl-leucyl-leucyl-norleucinal; Ac-Leu-Leu-Nle-al) and calpain inhibitor II (N-acetyl-leucyl-leucyl-methioninal; Ac-Leu-Leu-Met-al) were obtained from Nakarai Chemical Co., Kyoto, Japan. Propranolol (ICI Pharma, Tokyo), metoprolol (CIBA-GIGY, Osaka), nifedipine (BAYER, Tokyo), diltiazem (Tanabe, Osaka), bunazosin HCl (Eizai, Tokyo), and prazosin HCl (Pfizer, Tokyo) were generously supplied to us. cAMP assay kit was purchased from Amersham International (U.K.)

**Cell culture**

Neonatal rats were anesthetized in ether and the hearts quickly removed and placed into sterilized modified Krebs-Henseleit and HEPES buffer (NaCl 8.3 g/L, KCl 0.42 g/L, CaCl\textsubscript{2} 0.34 g/L, KH\textsubscript{2}PO\textsubscript{4} 0.19 g/L, MgSO\textsubscript{4} 0.17 g/L, HEPES 11.92 g/L) adjusted to pH 7.4.

Blood was carefully washed out and excised ventricles were cut into 1 to 2 mm cubes and then placed in phosphate-buffered saline (NaCl 8.0 g/L, Na\textsubscript{2}HPO\textsubscript{4} 1.5 g/L, KH\textsubscript{2}PO\textsubscript{4} 0.29 g/L, K\textsubscript{2}HPO\textsubscript{4} 0.2 g/L) containing 0.2% trypsin. The hearts were dissociated using a magnetic stir bar at a slow speed (100 to 150 rpm) for 8 min at 37°C\textsuperscript{13} Cells from the first two combined treatments were discarded, and the sequence was repeated 5 times until almost all tissue was dissociated. Liberated cells were pooled in cold Eagle’s modified minimum essential medium (MEME) salts from Sigma containing 0.06% yeast extract (wt/vol), 0.25% lactalbumin hydrolysate (wt/vol), 400 U/ml penicillin G and streptomycin (200 μg/ml) with 10% fetal bovine serum\textsuperscript{14} and centrifuged gently enough (800 to 1000 rpm) to pellet the cells.

The supernatant was poured off and swirled gently to disperse the pelleted cells in 10 ml of MEME. Isolated cells were then plated into 10 cm plastic tissue culture dishes at 37°C in humidified air with 5% CO\textsubscript{2} and maintained at pH 7.4\textsuperscript{15}

**Cell Hypoxogenation**

Cells were cultured at a density of 4 × 10\textsuperscript{7}/dish and the MEME medium was changed to 95% N\textsubscript{2}, 5% CO\textsubscript{2} humidified Krebs-Henseleit, HEPES buffer at 37°C\textsuperscript{12} The culture dishes were then exposed to a 95% N\textsubscript{2}, 5% room air atmosphere at 37°C for 6h for hypoxic treatment. After this incubation, 1 ml of each buffer was collected and used as the enzyme-containing buffer sample for the protease activity assay.

**Assay of protease activity**

Protease proteolytic activity was measured using α-casein as the substrate at a final concentration of 0.5% in 1 ml of reaction mixture containing 5 mM CaCl\textsubscript{2}, 10 mM 2-mercaptoethanol, 10 mM Na\textsubscript{2}SO\textsubscript{4} and 100 mM Tris acetate buffer (pH 7.4)\textsuperscript{16} The reaction was initiated by adding enzyme. The mixture was then incubated for 30 min at 37°C and the reaction was terminated by the addition of 2 ml of tri-chloroacetic acid at a final concentration of 2.5%. After centrifugation (2000 × g for 4 min.), the absorbance of the supernatant at 280 nm was measured with a spectrophotometer (model U-2000, Hitachi, Tokyo, Japan). One unit of protease activity was defined as the amount of enzyme per 1 × 10\textsuperscript{6} cells that increased the absorbance by 0.1 at 280 nm during incubation under these conditions.

In these preliminary experiments, we determined that calpain activity in culture medium paralleled the intracellular calpain activity.

**Cell viability**

After cell hypoxogenation for from 1 to 6h, the cell death ratio was determined from the number of myocytes per 200 cells which did not exclude 0.023% trypan blue within 2 min\textsuperscript{17} The time which was required for counting the numbers of cells was not long and, therefore, the cytotoxic effect of trypan blue appeared to be insignificant in this study.
Fig. 1. Effect of hypoxia on myocardial cell viability. Myocardial cells were treated under aerobic (95% air, 5% CO₂) and hypoxic (95% N₂, 5% CO₂) conditions. (A) Protease activity during hypoxia. Enzyme activity was determined by using 0.5% a-casein as the substrate with 5 mM CaCl₂ at pH 7.4, 37°C for 30 min. (B) Cell viability was determined as described in Materials and Methods. Hypoxia (●), normoxia (□) Points represent means ± SE. *p < 0.001, compared with normoxia.

Measurement of Ca²⁺ flux

Cells were prepared in 35-mm petri dishes as described above. To determine the Ca²⁺ influx, cells were washed with Hanks' balanced salt solution with 1.26 mM calcium and incubated in 0.7 ml of Hanks' balanced salt solution (containing 1.26 mM) and 1 μCi of ⁴⁵Ca²⁺ during hypoxia at 37°C. The cells were then placed on ice. Thereafter, they were scraped from the dishes, filtered through a 0.45 Millipore filter, washed twice with 3 ml of Hanks' balanced salt solution, and counted in 10 ml of Aquasol.

Statistical analysis

Statistical analysis was performed using the Wilcoxon method and by the chi-square method for cell viability. The criterion of significance was a probability (p) value of 0.05 or less. All the data are expressed as mean ± SEM.

RESULT

Myocardial cell injury and protease activity under hypoxia

The cell death was 7% at 1 hour-hypoxia and 80% 6 hour-hypoxia. There was no increase in the cell death during aerobic in-
cubation (Fig.1b). Protease activity was elevated in the course of hypoxia compared with the aerobic state through 6h (Fig. 1a). Calpain-specific inhibitors were added to dishes in order to specify the protease activated during hypoxia. The protease activity during hypoxia was strongly inhibited by calpain-inhibitor I (Ac-Leu-Leu-Nle-al; this agent completely inhibits calpain-1 and calpain-2 activity) (Fig. 2a), but calpain-inhibitor II (Ac-Leu-Leu-Met-al, this agent only inhibits calpain-2) did not reduce its activity (Fig. 2a). Calpain-inhibitor I also diminished cell death in 4-, 5- and 6-h hypoxia samples compared with non-hypoxic samples (Fig. 2b). However, calpain-inhibitor II had no effect on hypoxic cell injury protection, at least during the 6-h hypoxia (Fig. 2b).

Effects of \(\beta\)-adrenergic blocking agents on protease activity and cell injury

Propranolol and metopronolol significantly prevented hypoxic cell injury during 60—360 min of hypoxia (Fig. 3b). Metoprolol markedly suppressed cell death during 60—360 min hypoxia. In these conditions, the calpain activity (protease activity) was significantly inhibited by both agents between 60 and 360 min (Fig. 3a).

Effects of calcium channel-blocking agent on protease activity and cell injury

Nifedipine significantly inhibited calpain activity during 60 to 360 min of hypoxia. But diltiazem did not inhibit calpain activity between 240 and 360 min of hypoxia (Fig. 4a). Under these conditions diltiazem did not prevent hypoxic cell injury. On the other
hand, nifedipine significantly prevented cell death between 60 and 360 min of hypoxia (Fig. 4b).

Effects of α₁-adrenergic blocking agents on protease activity and cell injury
As mentioned above, β-adrenergic blocking agents and calcium channel blockers were effective in preventing hypoxic cell injury. On the other hand, α₁-adrenergic blocking agents could not inhibit protease activity, nor prevent cell death during hypoxic incubation from 60 to 360 min (Fig. 5a,b).

Calcium influx under hypoxia
Calcium-influx gradually increased during hypoxia (60—360 min). Metoprolol significantly suppressed this calcium entry into cells, but prazosin did not. On the other hand, nifedipine markedly inhibited calcium-influx during hypoxia (Fig. 6).

Intracellular cAMP levels under hypoxia and effect of isoproterenol on calpain activity
The data described above suggest that β-adrenergic receptor stimulation activates calpain during hypoxia. To confirm this hypothesis, we determined intracellular cAMP levels under hypoxia. As shown in Fig. 7a, cAMP levels increased during 6h hypoxic incubation. We also determined whether and β-adrenergic agonist stimulates calpain activity. Isoproterenol (1 μM) was
added to dishes and incubated in aerobic conditions for up to 6h. It significantly stimulated calpain activity at 1h incubation, and the activation was continued up to 6h (Fig. 7b).

DISCUSSION

We measured the activity of protease which leaked from cytosol, probably due to destruction of the cell membrane, during hypoxegenation. Our results indicate that calcium-activated neutral protease contributes to hypoxic myocyte injury.

In previous experiments\(^5,6,18\) the interaction between myocardial injury and calcium overload was noted. It was also demonstrated that the activation of calpain was one of the causes of irreversible hypoxic cell injury following the increase in intracellular calcium concentration\(^19,20\). Our study showed evidence linking the gradual activation of calpain and hypoxic myocyte injury. Protease activity and myocardial cell injury increased, at least up to 6h, in the course of hypoxegenation, but was negligible in aerobic states. In the ischemic and/or hypoxic state, many other leupeptin-sensitive protease, such as cathepsins-B, L, and H, may contribute to proteolysis.\(^8\) We focused on calpain activity during hypoxic myocardial injury in this experiment. Using calpain-specific inhibitors we demonstrated that the protease activated under hypoxia is calpain-1. Two types of calpain have been identified in mammals.\(^8\) They are differentiated by their calcium sensivities in that calpain-1 requires a micromolar order of calcium, while calpain-2 requires a millimolar order of calcium for its activation. We attempted to clarify the relationship between hypoxic cell damage and calpain activation using the newly synthetized calpain-inhibitor I (Ac-Leu-Leu-Nle-al) and calpain-inhibitor II (Ac-Leu-Leu-Met-al). Our data suggest that proteolysis in hypoxic cell injury is mediated by calpain-1, but calpain-2 did not appear to be related to cell injury, at least during 6h of hypoxegenation.

There is an endogenous specific inhibitor of calpain in cells, and it should also be present in hypoxic conditions.\(^21,22\) This endogenous inhibitor of calpain is generally assumed to be a cytosolic protein\(^23\) and when this inhibitor is bound to the membrane, it may regulate intracellular functions, as does protein kinase C, by limiting proteolysis.\(^24\) Under hypoxic conditions, the endogenous inhibitor may bind to activated calpain and protect the cell from injury. However, current experiments suggest that this mechanism cannot be controlled by endogenous inhibitor alone in later hypoxic periods. The effect of the added selective calpain-1 inhibitor in our study may support this hypothesis.

It has been reported that Ca\(^{2+}\)-influx increases and Ca\(^{2+}\)-efflux decreases through the plasma membrane during hypoxia.
route of increased Ca\(^{2+}\)-influx through the plasma membrane has not been established, but it does not appear to occur through the voltage-dependent calcium channels. Although the Na\(^+\)/Ca\(^{2+}\) exchange system has been suggested as a likely route, this has not clearly been demonstrated experimentally. In our experiments, calcium-influx increased under hypoxia and was suppressed by \(\beta\)-adrenergic blocking agents and calcium antagonists. However, \(\alpha_1\)-adrenergic blocking agents had no effect on it. We did not add \(\beta\)-adrenergic stimulatory agents such as norepinephrine, but \(\beta\)-adrenergic blocking agents were effective in preventing cell damage and protease activation during hypoxia. It is reported that endogenous catecholamines exist in cell preparations. Hence, we may assume that the isolated cardiac cells used in this experiment contained endogenous catecholamines. These data suggest that \(\beta\)-adrenergic receptor stimulation activates calpain during hypoxia. To confirm this hypothesis, we determined intracellular cAMP levels under hypoxia. During 6h-hypoxia, cAMP levels increased. We also determined the direct effect of \(\beta\)-adrenergic agonist on calpain activity. Isoproterenol was added to dishes and incubated in aerobic conditions for up to 6h. It significantly stimulated calpain activity.

In summary, hypoxic rat myocyte injury is related to the activation of calpain-1 during 6h of hypoxegenation. Hypoxic cell injury was reduced by \(\beta\)-adrenergic blocking agents and calcium antagonists. These results suggest that \(\beta\)-adrenergic receptor stimulation increases calcium-influx, and that calpain-1 is activated during hypoxia.

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REFERENCES


4. TANAKA T, SOBUE K, UEKI N: Calcium-binding proteins and their physiological function: evolution aspects in structure and function. Protein, Nucleic Acid, Enzyme 1986; 31: 1784


15. OTSUKA Y, TANAKA H: Purification of a new calcium activated protease (low calcium requiring form) and comparison to high calcium requiring form. Biochem Biophys Res Commun 1983; 111: 700


18. PAGE E, POLIMENI PI: Ultrastructural changes in the ischemic zone bordering experimental infarcts in rat left ventricles. Am J Pathol 1977; 87: 81


20. MURACHI T: Calpain and calpstatin. Trends


