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Downregulation of Calpastatin in Rat Heart after Brief Ischemia and Reperfusion[1]

Yoshihide Sorimachi,*,[2] Kazuki Harada,* Takaomi C. Saido,† Tomio Ono,† Sei-ichi Kawashima,† and Ken-ichi Yoshida*,[3]

*Department of Legal Medicine, Yamaguchi University School of Medicine, 1144 Kogushi, Ube, Yamaguchi 755; and †Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113

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The activities of calpain and its endogenous inhibitor, calpastatin, were measured in the soluble fraction of perfused rat heart after ischemia for 5-20 min and reperfusion for up to 30 min. The method for *m*-calpain measurement was modified: washing of the DEAE-cellulose column with 0.18 M NaCl instead of 0.15 M NaCl increased the *m*-calpain activity 12.5-fold. Ischemia for 20 min followed by reperfusion for 30 min did not affect the *m*-calpain activity but decreased the calpastatin activity. *m*-Calpain was enriched in the nucleus-myofibril fraction but was not further translocated on ischemia-reperfusion. μ-Calpain was below the limit of detection on immunoblotting or casein zymography, but its mRNA was substantially expressed, as detected on Northern blotting. Casein zymography also revealed a novel Ca^{2+}-dependent protease without the typical characteristics of μ- or *m*-calpain. The immunoblotting of myocardial fractions showed that calpastatin was proteolyzed on ischemia-reperfusion. The calpastatin proteolysis was suppressed by a calpain inhibitor, Ac-Leu-Leu-norleucinal. Calpastatin may sequester calpain from its substrates in the normal myocardium, but may be proteolyzed by calpain in the presence of an unidentified activator in the early phase of calpain activation during ischemia-reperfusion, resulting in the proteolysis of calpastatin and then other calpain substrates.

Key words: calpain, calpastatin, ischemia, myocardium, reperfusion.

Calpain, a ubiquitous neutral protease, is activated by μM Ca^{2+} (μ-calpain) or mM Ca^{2+} (*m*-calpain) *in vitro*. Either μ- or *m*-calpain (*1, 2*) is thought to be involved in the myocardial injury during anoxia (*3*), and ischemia-reperfusion (*4*). Previously, we reported that 60 min ischemia increased the calpain activity (*5*) and decreased the calpastatin activity in the perfused rat heart (*6*), although the underlying mechanisms remain undetermined. Recently, we also found that calspectin (fodrin), a well known substrate for calpain, is proteolyzed on brief (10-20 min) ischemia followed by reperfusion (3-30 min) (*7*). A calpain inhibitor inhibited the proteolysis and contractile dysfunction during reperfusion, suggesting the involvement of calpain in the proteolysis and reperfusion injury (*7*). However, the changes in the activities in calpain and calpastatin on brief ischemia and reperfusion remain to be studied.

In this study, we re-examined the assay conditions for the calpain activity, and determined the activities of calpain and calpastatin during brief ischemia and reperfusion. Secondly, we examined the μ-calpain expression in rat heart by immunoblotting, casein zymography, and Northern blotting. Finally, we investigated the mechanism of the change in calpastatin activity.

MATERIALS AND METHODS

Perfusion Procedure—Male Wistar rats weighing 200-250 g were perfused by the Langendorff procedure as we previously described (*5-8*). The hearts were perfused initially for 10-20 min with a modified Krebs-Henseleit (KH) solution comprising 124 mM NaCl, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.5 mM glucose, and 2.0 mM sodium pyruvate, pH 7.4, gassed with 95% O_2-5% CO_2, at a constant perfusion pressure of 80 cm H_2O at 37°C. Global ischemia was produced by incubating the hearts for 20 min at 37°C without perfusion. After the ischemia, some hearts were reperfused for 3-30 min with the oxygenated KH buffer. Other hearts were perfused with either 10 or 100 μM Ac-Leu-Leu-norleucinal (ALLNal, Nacalai Tesque), or its solvent, dimethyl sulfoxide (DMSO, 0.1%), for 5 min before ischemia and during reperfusion.

Subcellular Fractionation—The subfractionation procedure was performed as we previously described (*8*). The

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[2] Present address: Department of Legal Medicine, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602.

[3] To whom correspondence should be addressed. Tel: +81-836-22-2233, Fax: +81-836-22-2232, E-mail: kyoshida@po.cc.yamaguchi-u.ac.jp

Abbreviations: ALLNal, Ac-Leu-Leu-norleucinal; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol, bis(2-aminoethyl)-*N,N,N',N'*-tetraacetic acid; KH solution, Krebs-Henseleit solution; TE buffer, Tris-EGTA buffer.

frozen hearts were homogenized in sucrose-Tris-EGTA (STE) buffer comprising 0.34 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EGTA, 5 mM NaN_3, 10 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 150 nM pepstatin A with a Polytron homogenizer four times each for 30 s at the maximum speed. The homogenate was centrifuged at $1,000\times g$ for 30 min. The supernatant was centrifuged at $100,000\times g$ for 60 min.

Determination of m-Calpain Activity—To isolate *m*-calpain, the $100,000\times g$ supernatant was applied to a DEAE-cellulose column (2.0 ml packed volume) equilibrated with 50 mM NaCl in Tris-EGTA (TE) buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EGTA, 5 mM NaN_3, and 10 mM β-mercaptoethanol, as we described previously (*5, 6, 8*). The column was washed twice with 3.0 ml of TE buffer containing 50 mM NaCl, and then twice with 5.0 ml of TE buffer containing 0.18 M NaCl. μ-Calpain is eluted with 0.18 M NaCl if it exists (*9*). *m*-Calpain was eluted from the column twice with 2.0 ml of TE buffer containing 0.40 M NaCl. In an experiment to determine the optimum conditions for the isolation of calpain, we also performed stepwise elution from the DEAE-cellulose column. As previously reported (*5, 6*), *m*-calpain activity was measured as the release of trichloroacetic acid-soluble peptides from azocasein (Sigma) in an assay mixture (1.5 ml) comprising 20 mM Tris-HCl, pH 7.4, 2 mg/ml azocasein, 20 mM β-mercaptoethanol, 1% Triton X-100, 0.75 ml sample, and Ca^{2+} at a final concentration of 1 mM for 60 min at 25°C. The Ca^{2+} concentration was calculated using Ca^{2+}-EGTA buffer (*10*). One unit of calpain activity was defined as an increase in A_{440} of 1.0 per hour. Protein concentrations were determined by the method of Bradford (*11*) using bovine serum albumin (BSA) as a standard.

In Vitro Proteolysis of Calpastatin—The $100,000\times g$ supernatant (2.0 mg/ml) in STE buffer was incubated with purified porcine kidney *m*-calpain (Chemicon Int., Temecula, USA; 0.5-100 μg/ml) in the presence of 5 mM $CaCl_2$ for 20 min at 25°C and then subjected to immunoblotting.

Electrophoresis and Immunoblotting—SDS-polyacrylamide gel (7.5%) electrophoresis and immunoblotting were performed according to Laemmli *et al.* (*12*) and Towbin *et al.* (*13*), respectively, with some modification (*7, 8*). To detect limited proteolysis of calpastatin, 10% gels were used. A monoclonal antibody (1D10A7) against calpain recognizes the inactive and active forms of the large subunits of both μ- and *m*-calpains as reported previously (*14*). A polyclonal antibody to calpastatin was raised by injecting rabbits with a peptide synthesized according to the structure of calpastatin, as we previously reported (*15*). The same amount of protein of each fraction and of either calpain or calpastatin was subjected to immunoblotting to obtain linearity between the amount of sample applied and the intensity x area of the protein band on the blot. Calpain or calpastatin was quantified with an image analyzer (Atto, Densitogragh) as we previously reported (*7, 8*).

Casein Zymography—The 0.18 M NaCl and 0.40 M NaCl eluates from the DEAE-cellulose column of the soluble fraction of rat heart were concentrated with Centriplus concentrators (Amicon). The 0.18 M NaCl eluate (45 μg) and 0.40 M NaCl eluate (10 μg) of the heart (control and ischemia-reperfusion), and those of the lung and erythrocytes of rats were subjected to non-denaturation gel electrophoresis, followed by incubation of the casein-containing gels with either $CaCl_2$ (8 mM), EGTA (1 mM), or $CaCl_2$ plus 100 μM ALLNal by the method of Raser *et al.* (*16*). The same proportions (as to the whole volume of the fraction) of the 0.18 M NaCl and 0.4 M NaCl eluates were subjected to the electrophoresis.

Determination of Calpastatin Activity—We partially purified *m*-calpain and μ-calpain, respectively, from bovine lung and human erythrocyte lysates by DEAE-cellulose and phenyl-Sepharose column chromatographies. The $100,000\times g$ supernatant of the heart was boiled for 5 min and then centrifuged for 10 min at $10,000\times g$ to remove denatured proteins. After incubation of the heat-treated sample (10 μg/ml) with either *m*-calpain or μ-calpain (0.2 unit/ml), calpain activity was determined as described above. Calpastatin activity (inhibition rate, %) was defined as (calpain activity minus calpain activity with calpastatin)/calpain activity, as described previously (*6*).

Northern Blotting—Total cellular RNA of rat heart and lung was purchased from Sawaday Technology (Tokyo). RNA was separated on a 1% formaldehyde agarose gel and then transferred to a Biodyne B membrane (Pall). A 0.24-9.5 kb RNA ladder (Life Technologies) was used as size markers. Hybridization was carried out at 65°C in Rapid-hybridization buffer (Amersham), as described by the manufacturers. After hybridization, the membrane was washed with 2×SSC/0.1% SDS buffer at 55°C. A specific probe for the μ-calpain large subunit was obtained by screening an adult rat brain λgt10 library. The cDNA fragment corresponding to nucleotide residues 2070-2917 of the large subunit of μ-calpain cDNA (*17*) was used as a probe. The cDNA was labeled with a random 9-mer (Takara) and [α-^{32}P]dCTP (Amersham).

Statistics—The statistical significance ($p<0.05$) was evaluated by ANOVA with posthoc analysis according to Fisher.

RESULTS

To re-evaluate the conditions for the calpain assay, we performed immunoblotting of the stepwise eluates from a DEAE-cellulose column to which the soluble fraction of a control heart had been applied. As shown in Fig. 1, the antibody that recognizes the large subunits of both μ-calpain (80 kDa) and *m*-calpain (77 kDa) of various rat tissues

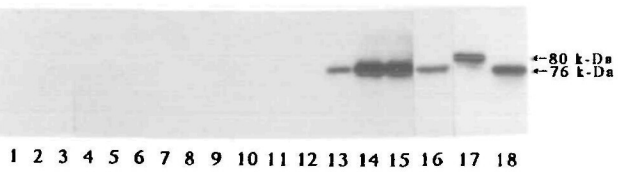

Fig. 1. **Profile of calpain immunoreactivity in the stepwise eluates of the rat heart soluble fraction from a DEAE-cellulose column.** The immunoreactivity was detected by immunoblotting with an antibody that reacts with the large subunits of *m*- and μ-calpains. The eluates (1.0 ml) with 0.05, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.25, 0.30, and 0.40 M NaCl were run in lanes 1-15, respectively, while the $100,000\times g$ supernatant was run in lane 16. As controls, partially purified μ-calpain and *m*-calpain were run in lanes 17 and 18, respectively. There was no calpain immunoreactivity in the fractions eluted with NaCl concentrations between 0.10-0.15 M NaCl. *m*-Calpain was detected in the eluates with NaCl concentrations of 0.25 M and above.

(*14*) reacted only with the 77 kDa band of the soluble fraction of rat heart. Figure 1 also shows that there was no calpain immunoreactivity in the fractions eluted with NaCl concentrations between 0.10–0.15 M NaCl, where μ-calpain is expected to be eluted if any (*9*). In addition, *m*-calpain (77 kDa) was eluted with a NaCl concentration of 0.25 M and above.

To further explore the expression of μ- and *m*-calpains in the rat heart, we performed casein zymography, using rat erythrocyte and lung lysates as positive controls for μ- and *m*-calpains, respectively. Figure 2 shows that the 0.40 M NaCl eluate from the DEAE-cellulose column gave an intense caseinolytic band of *m*-calpain for the heart and lung. However, no band was observed for the 0.18 M NaCl eluate of the heart with the same mobility as the fast band of the 0.18 M NaCl eluate of erythrocytes. Notably, both the 0.18 and 0.40 M NaCl eluates of the heart contained a slow mobility protease, as found in the erythrocytes. This band and the *m*-calpain band decreased on incubation of the gels with either EGTA or a calpain inhibitor (100 μM ALLNal). Since the 0.18–0.40 M NaCl eluate has not been shown to contain μ-calpain, the protein level of μ-calpain with known characteristics is negligible in the rat heart. In addition, there was no difference between the controls and ischemia (20 min)-reperfusion (30 min) in the extent of the caseinolysis by *m*-calpain or that by the slow protease.

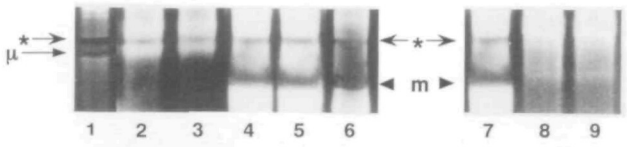

Fig. 2. **Casein zymography of the rat heart soluble fraction.** The 0.18 M NaCl eluates (lanes 1–3) of an erythrocyte lysate (lane 1), and control (lane 2) and ischemia-reperfused hearts (lane 3), and 0.40 M NaCl eluates (lanes 4–6) of control (lanes 4) and ischemia-reperfused hearts (lane 5), and lung (lane 6) were subjected to non-denaturation gel electrophoresis, followed by incubation of the casein-containing gels either with $CaCl_2$ (lanes 1–7), EGTA (lane 8), or $CaCl_2$ plus 100 μM ALLNal (lane 9). Caseinolytic activity appears as dark bands, indicated by arrows, in the grayish background, although we cannot show the difference in the darkness due to the caseinolysis and that due to the low protein contamination in the background (the lower portion of lanes 1–3). μ, *m*, and * denote the bands of μ-calpain, *m*-calpain, and the protease showing slow mobility, respectively. Note that the image has been reversed with the image analyzer to make it clearer.

To observe expression of the μ-calpain gene, we performed Northern blotting of the same amount of mRNA from the heart and the lung. The lung expresses a substantial amount of the μ-calpain protein (*9*). Figure 3 shows that the rat heart expresses a substantial level of μ-calpain mRNA, although it was only 18.1% of the level in the rat lung.

Figure 4 shows the elution profile of calpastatin with the DEAE-cellulose column. Although most calpastatin was eluted with 0.05–0.16 M NaCl, a substantial amount of it was detected in the 0.16–0.17 M NaCl eluate. On the basis of this finding, we decided to wash the DEAE-cellulose column with 0.18 M NaCl, expecting better removal of calpastatin contaminating the *m*-calpain sample. As expected, the *m*-calpain activity in the 0.40 M NaCl was 12.5-fold higher after washing of the column with 0.18 M NaCl (2.5 U/mg protein) than after washing with 0.15 M NaCl (0.20 U/mg protein), that was used in the previous studies (*5, 6, 9*). In addition, the volume used for the washing also affected the activity: washing with 10 ml or more of 0.18 M NaCl-containing TE buffer gave higher activity than washing with 8 ml or less of the buffer (data not shown). Hereafter, we modified the conditions for the *m*-calpain assay: elution with 0.40 M NaCl after washing of the DEAE-cellulose column with 10 ml of TE buffer containing 0.18 M NaCl.

As shown in Fig. 5, there was no change in the specific activity of *m*-calpain on ischemia for 20 min with or without reperfusion for 30 min. As the activation of calpain

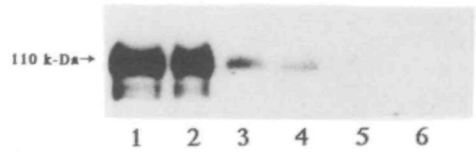

Fig. 4. **Profile of calpastatin immunoreactivity in the stepwise eluates of the rat heart soluble fraction from a DEAE-cellulose column.** The immunoreactivity was detected by immunoblotting using an anti-calpastatin antibody. The eluates with 0.05 M NaCl (4.0 ml), 0.15 M NaCl (6.0 ml), 0.16, 0.17, 0.18, and 0.19 M NaCl (2.0 ml each) were run in lanes 1–6, respectively. Some calpastatin was eluted with 0.16–0.17 M NaCl.

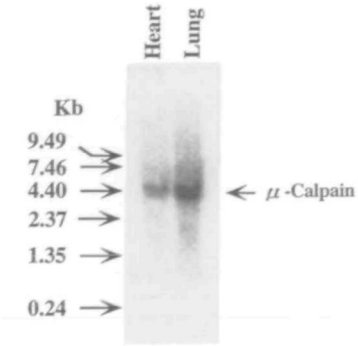

Fig. 3. **Northern blotting of rat heart with rat μ-calpain cDNA.** Twenty micrograms of total cellular RNAs prepared from rat heart and rat lung were subjected to Northern blotting analysis with the cDNA as a probe, as described under "MATERIALS AND METHODS."

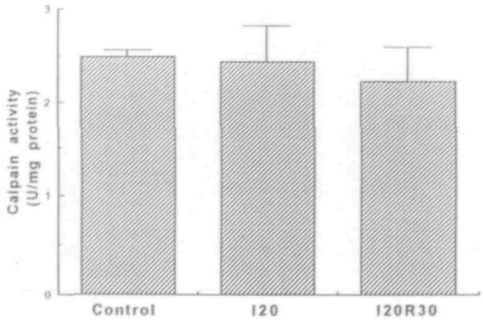

Fig. 5. ***m*-Calpain activity in the heart after ischemia or ischemia-reperfusion.** Calpain activity (units/mg protein; mean ± SE, $n=3$) in the 100,000 × *g* supernatant was not significantly different in a control heart, a heart after 20 min ischemia (I20), and a heart after ischemia (20 min)-reperfusion (30 min) (I20R30).

is thought to be associated with its translocation from the cytosol to the membrane or its autolysis (*1, 2*), we investigated these possibilities by immunoblotting of the subcellular fractions using an anti-calpain antibody. We showed previously that the 1,000×*g* pellet is the nucleus-myofibril fraction, and the 100,000×*g* pellet is the membrane fraction, while the 100,000×*g* supernatant fraction is the cytosolic fraction (*18, 19*). Figure 6 shows that ischemia (20 min)-reperfusion (30 min) did not significantly change the *m*-calpain distribution in the soluble fraction (56.0% of total calpain in the control heart), the nucleus-myofibril fraction (34.7%), or the membrane fraction (9.3%) (*8*). Figure 6 also shows that there is neither a native molecule (80 kDa) nor autolytic fragments (78 and 76 kDa) of *μ*-calpain in any fraction. We showed previously that the sizes of the autolytic fragments of *μ*-calpain are distinct from that of the large (77 kDa) subunit of *m*-calpain (*20*).

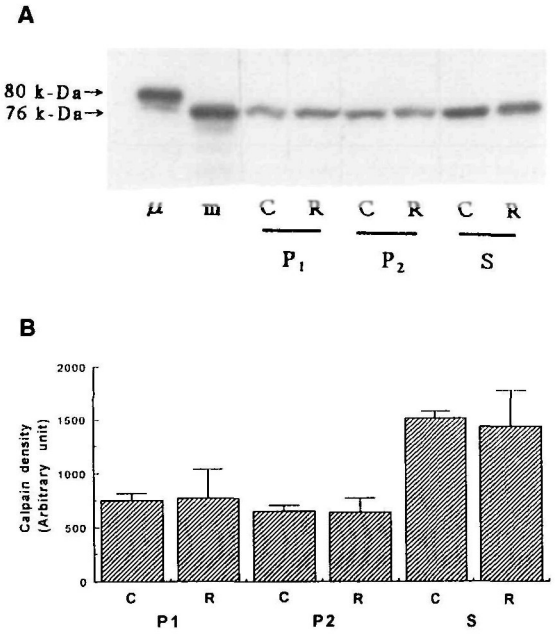

Fig. 6. **Distribution of calpain in rat heart subcellular fractions before and after ischemia-reperfusion.** The calpain immunoreactivity was not different in the 1,000×*g* pellets (P1), 100,000×*g* pellets (P2), and 100,000×*g* supernatants (S) of a control heart (C) and a heart after ischemia (20 min)-reperfusion (30 min) (R). Panel A is a representative immunoblot of the fractions with an anti-*m*- and *μ*-calpain antibodies. The same amount of protein was applied for each fraction. Panel B shows the amount of calpain (mean±SE, $n=3$) determined from the immunoblots.

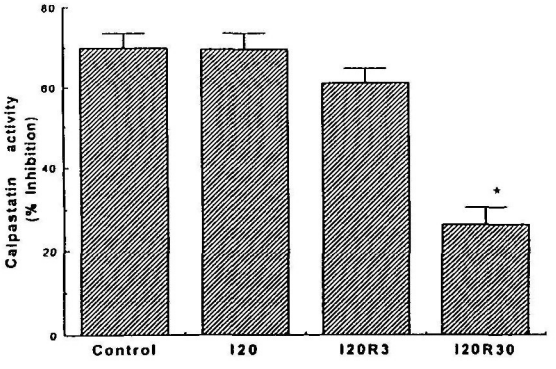

Fig. 7. **Calpastatin activity of the rat heart after ischemia or ischemia-reperfusion.** Reperfusion for 30 min (I20R30) but not for 3 min (I20R3) after ischemia for 20 min decreased the calpastatin activity, as compared with in the control (*$p<0.05$), in the 100,000×*g* supernatant (mean±SE, $n=3$), as measured against *m*-calpain. Ischemia for 20 min (I20) did not affect the activity.

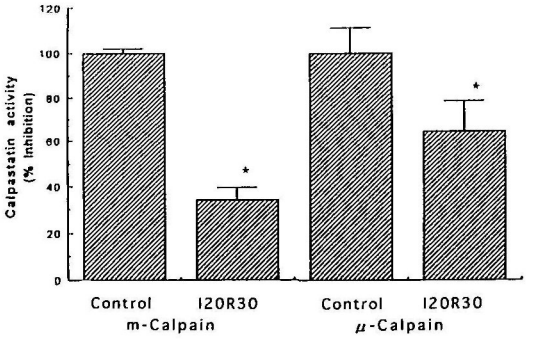

Fig. 8. **Comparison of calpastatin activity against *m*-calpain and that against *μ*-calpain in the heart before and after ischemia-reperfusion.** The inhibitory activity of the 100,000×*g* supernatant (mean±SE, $n=3$) toward *m*-calpain and that toward *μ*-calpain were both lower in the heart after ischemia (20 min)-reperfusion (30 min) (I20R30), as compared with in the control heart (*$p<0.05$).

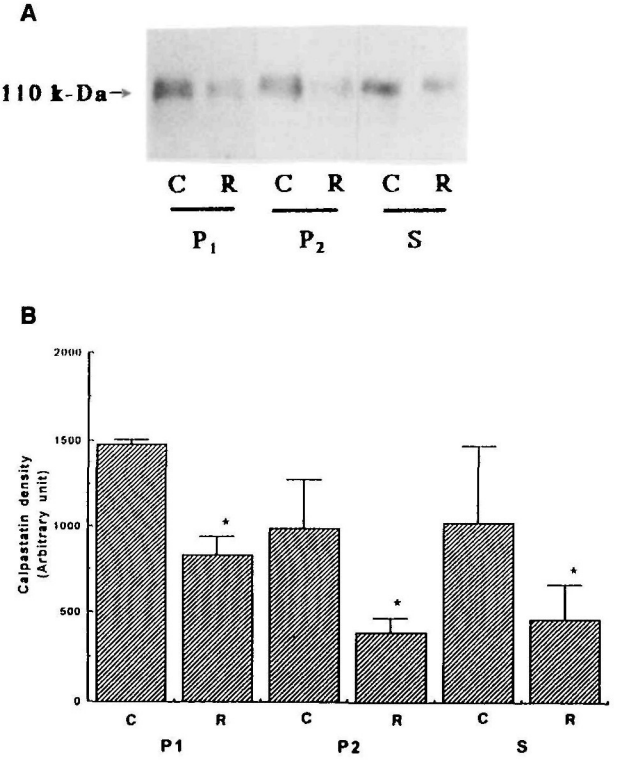

Fig. 9. **Distribution of calpastatin in rat heart subcellular fractions before and after ischemia-reperfusion.** Calpastatin immunoreactivities in the 1,000×*g* pellet (P1), 100,000×*g* pellet (P2), and 100,000×*g* supernatant (S) fractions were reduced after ischemia (20 min)-reperfusion (30 min) (R), compared with in the control heart (C) (* $p<0.05$), as shown in a representative immunoblot with an anti-calpastatin antibody (Panel A), and on its quantification (Panel B; mean±SE, $n=3$). The same amount of protein was applied for each fraction.

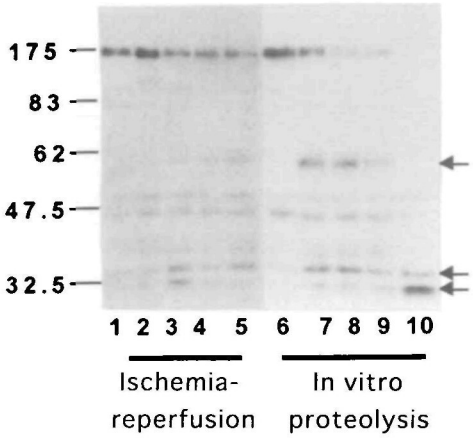

Fig. 10. **Comparison of the proteolysis of calpastatin during ischemia-reperfusion and the *in vitro* proteolysis with *m*-calpain.** The soluble fraction of a control heart (lane 1), and hearts after 20 min ischemia followed by 0, 3, 10, and 30 min reperfusion (lanes 2, 3, 4, and 5, respectively), and the soluble fraction (2 mg/ml) incubated *in vitro* with *m*-calpain at 0, 0.5, 1.0, 10, and 100 μg/ml (lanes 6, 7, 8, 9, and 10, respectively) in the presence of 5 mM Ca^{2+} for 20 min were subjected to immunoblotting analysis using an anti-calpastatin antibody.

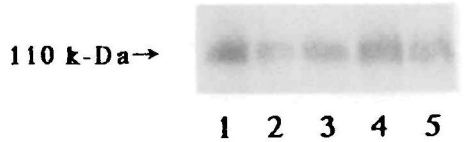

Fig. 11. **Effect of a calpain inhibitor on calpastatin proteolysis.** Lane 1: control; lane 2: ischemia (20 min)-reperfusion (30 min) (I20R30); lanes 3–5: ischemia-reperfusion in the presence of 10 μM ALLNal (lane 3), 100 μM ALLNal (lane 4), or 0.1% DMSO (lane 5). ALLNal suppressed calpastatin proteolysis in the 100,000 × *g* supernatant after ischemia-reperfusion.

We then measured the calpastatin activity in the soluble fraction. As shown in Fig. 7, reperfusion for 30 min but not for 3 min following ischemia for 20 min decreased the inhibitory activity of calpastatin toward *m*-calpain. It was reported that the dephosphorylation of calpastatin attenuates its inhibitory activity toward *m*-calpain, but enhances its inhibitory activity toward μ-calpain *in vitro* and in the perfused heart (*21, 22*). However, Fig. 8 shows that ischemia (20 min)-reperfusion (30 min) decreased the inhibitory activity of calpastatin toward both μ-calpain and *m*-calpain. These findings suggest that the decrease in the inhibitory activity of calpastatin is not due to a change in its phosphorylation state.

We then examined whether the reduced activity of calpastatin is due to either proteolysis or translocation by means of immunoblotting. Figure 9 shows that calpastatin immunoreactivity was decreased in all fractions after ischemia-reperfusion, indicating the proteolysis but not translocation of calpastatin. Figure 10 shows that both the proteolysis of calpastatin *in vitro* with excessive calpain and the proteolysis in the ischemia-reperfused heart generated fragments with the same molecular masses. Notably, calpastatin was proteolyzed rapidly after reperfusion, as calspectin and ankyrin were degraded, as we reported previously (*7, 23, 24*). Moreover, Fig. 11 shows that the calpain inhibitor, ALLNal, but not its vehicle, DMSO, inhibited the proteolysis of calpastatin on ischemia-reperfusion, as it inhibited the proteolysis of calspectin and ankyrin (*7, 23, 24*). Thus, it was shown that calpastatin is proteolyzed by calpain in the heart on ischemia-reperfusion.

DISCUSSION

In this study, we modified the conditions for calpain isolation: washing the column with 0.18 M NaCl instead of 0.15 M NaCl. This modification greatly increased the *m*-calpain activity, possibly through elimination of the contamination by calpastatin of the *m*-calpain sample, as compared with in the previous studies (*5, 6, 9*). *m*-Calpain activity in the soluble fraction remained unaltered after ischemia for 20 min and reperfusion for up to 30 min (Fig. 5), consistent with the zymography data (Fig. 2). These findings also show that *m*-calpain is not activated irreversibly during ischemia-reperfusion, through its autolysis or dissociation of the large and small subunits, that results in inactivation of calpain (*25*). Consistent with the proposed involvement of calpain in the turnover of the myofibril proteins (*1*) that comprise about 60% of the total proteins in the myocardium (*8*), we found an abundance of *m*-calpain in the nucleus-myofibril fraction but no translocation after ischemia-reperfusion (Fig. 6). Alternatively, the abundance of *m*-calpain in the particulate fractions may be due to the cell fractionation with a hypotonic buffer, as reported previously (*26*).

Rat heart appears to express the μ-calpain protein minimally, because the antibody that reacts with the large subunits of both μ- and *m*-calpains of rat tissues (*8*) did not detect μ-calpain in the 0.18 M NaCl eluate from the DEAE-cellulose column (Fig. 5), in which μ-calpain is eluted if it exists (*8*). Additionally, casein zymography revealed a fast mobility protease that is likely to be μ-calpain in the 0.18 M NaCl eluate of erythrocytes but not in that of the heart (Figs. 2 and 3). By contrast, a slow protease was found in the 0.18 M NaCl eluate of the heart as well as in the erythrocytes. The slow protease in the heart was inhibited by either Ca^{2+} chelation or a calpain inhibitor, ALLNal. However, it is unlikely that the slow protease is μ-calpain because it was similarly found in the 0.18–0.40 M NaCl eluate (Fig. 2). Unexpectedly, the rat heart expressed a low but substantial level of μ-calpain mRNA as compared with the lung (Fig. 3), that expresses the μ-calpain protein substantially (*9*). The discrepancy between the expression of the mRNA and protein of μ-calpain may be due to either the extremely rapid turnover or a translation defect.

Calpastatin activity remained unchanged during ischemia, but it was reduced after 30 min reperfusion after ischemia (Fig. 6). Calpastatin was downregulated through its limited proteolysis by calpain because a calpain inhibitor (ALLNal) inhibited the calpastatin proteolysis after ischemia-reperfusion (Figs. 9 and 10). The limited proteolysis of calpastatin not after ischemia but after reperfusion is consistent with the proteolysis of calspectin (fodrin) (*7*) and ankyrin (*23, 24*), other calpain substrates. Recently, we found that brief forebrain ischemia followed by reperfusion downregulates calpastatin through limited proteolysis in the hippocampus, except in the CA2 region (*27*). The

CA2 region is resistant to ischemic insults (*27*), showing the protective role of calpastatin in reperfusion injury. This study showed that the calpastatin proteolysis during postischemic reperfusion is rapid (Fig. 10), which is consistent with our finding that calpastatin is more preferentially proteolyzed than calspectin (fodrin) in cultured cells (*28*).

Together with the finding in the ischemic brain and the results of this study, it is tempting to speculate that (1) Ca^{2+} influx during an early phase of reperfusion (*6, 29*) induces calpain activation that is not detected as an increase in its activity under the present experimental conditions. (2) The activated calpain degrades calpastatin, reducing its inhibition of calpain. (3) Then, the calpain activity prevails over the calpastatin activity, leading to the proteolysis of other substrates such as calspectin (fodrin) and ankyrin. In other words, calpastatin may sequester calpain from its substrates in the normal myocardium, but calpain may become activated during an early phase of ischemia-reperfusion, resulting in the proteolysis of calpastatin and then of other substrates. How does calpain proteolyze calspectin (fodrin) (*7*) and calpastatin in the absence of apparent activation of *m*-calpain? We speculate that calpain is activated in the heart in the presence of activators, such as phospholipids (*30*) or activator proteins (*31*), during ischemia-reperfusion, but that calpain dissociates from the activators during the isolation procedure for calpain determination. We must also consider the variation in the spatial and temporal distributions of calpain, calpastatin, and calpain substrates, although we could not obtain any positive evidence with the subfractionation procedure employed in this study.

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