Age-dependent Degradation of Calpastatin in Kidney of Hypertensive Rats*

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Hypertensive rats from the Milan strain show a significant decrease in calpastatin activity as compared with normotensive control animals. Calpastatin deficiency is age-related and highly relevant in kidney, heart, and erythrocytes and of minor entity in brain tissue. In normotensives the changes during aging in the levels of calpastatin activity and mRNA are consistent with an increase of calpastatin protein. In hypertensive rats such a relationship during aging is not observed, because a progressive accumulation of mRNA is accompanied by a lower amount of calpastatin protein as compared with control rats. Together with the low level of calpastatin in kidney of hypertensive rats, a progressive accumulation of an active 15-kDa calpastatin fragment, previously shown to represent a typical product of calpain-mediated calpastatin degradation, is also observed. Evidence for such intracellular proteolysis by Ca2+-activated calpain is provided by the normalization of the calpastatin level, up to that of control animals, in hypertensive rats treated with drugs known to reduce both blood pressure and intracellular Ca2+ influx. Further evidence is provided by the disappearance, in these conditions, of the 15-kDa calpastatin fragment. These data allow the conclusion that calpastatin degradation is a relevant part of the overall mechanism for regulating calpain activity.

Calpain activation is a multistep process involving transient conformational changes as well as irreversible limited autoproteolysis. The first event is ascribed to a calcium-induced molecular transition initiated by the binding of Ca2+ to the calmodulin-like domains of the catalytic and small calpain subunits and subsequently transferred to the thiol proteinase-like domain II, promoting a rearrangement of the active site (1–6). The calpain active site becomes accessible, and the enzyme is ready for autoproteolysis and is probably active also on exogenous substrates. This reversible phase is thus followed by a rapid autoproteolysis affecting the N-terminal region of both calpain subunits. The resulting digested enzyme is considered to be the true active form of the cells on the basis that its calcium requirement was reduced to values close to the physiological range. However, some evidence indicates that the autoproteolyzed calpain may also represent a transient product of an auto-inactivation process, typical of almost all proteinases (7, 8).

Activation of calpain occurs at the inner face of the plasma membrane, but the digested proteinase form is released and recovered as soluble enzyme (9). This calpain form is potentially dangerous, having almost completely lost its dependence from a high concentration of Ca2+ and being freely active in the cytosol. Furthermore, it is preferentially recognized by calpastatin, the natural inhibitor of the proteinase (10, 11). Taken together, these observations support the conclusion that within the cells autoproteolysis represents a rough regulation of calpain through two steps. The first stage promotes the removal of the enzyme from its preferential site of action, and the second one involves the inhibition in the cytosol by calpastatin. Further autoproteolysis can then produce inactive fragments of calpain. This is consistent with the assumption that calpastatin can play an essential role inhibiting activated calpain in the soluble fraction of the cells.

The preferential interaction of calpastatin with the autoproteolyzed calpain form explains why intracellular calpain can become active even in the presence of an excess of calpastatin as we have observed in human erythrocytes enriched with calcium (11). The inhibitor molecule cannot interact with native calpain at the basal cytosolic concentration of Ca2+ because the formation of the enzyme-inhibitor complex requires a conformationally modified calpain that is produced, in the absence of specific cofactors, only at a higher [Ca2+], reached probably only transiently or in pathological conditions. On the basis of these observations, it can be concluded that calpastatin binds only active calpain forms, regardless if they are autoproteolyzed or not.

It has been reported that when calpastatin is associated to calpain, it is not fully resistant to the proteinase digestion but is slowly degraded (10, 12). It has been also demonstrated previously that both in vivo and in vitro μ-calpain seems to be involved in the inhibitor fragmentation, generating free active inhibitory domains, whereas m-calpain digests calpastatin producing inactive peptides (13–15). The different kinds of degradation accomplished by the two calpains is consistent with the hypothesis that fragmentation of calpastatin by μ-calpain, the first step of the inhibitor digestion process, corresponds to a local increase in active calpastatin species. The subsequent calpastatin inactivation could be the charge paid by cells to prevent further activation of calpain.

Accumulation of intracellular active calpain species could be responsible for the rapid degradation of calpain targets such as cytoskeletal proteins, neurofilaments, and transmembrane car-

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We have reported previously that erythrocytes from hypertensive rats and kidney of Milan strain contain much less calpastatin activity as compared with cells from normotensives (23). In the case of hypertensive rats, inhibitor levels are reduced significantly also in kidney (24). In the present study we have used the “calpastatin-deficient” tissues of these hypertensive animals to analyze the in vivo proteolysis of this inhibitor molecule.

We have shown that calpastatin deficiency in kidney, heart, and erythrocytes of Milan hypertensive rats is a function of aging. Further evidence indicates that in adult animals the loss of calpastatin can be reversed almost completely by a 7-day treatment with an antihypertensive drug affecting those mechanisms that control intracellular calcium homeostasis (25). The normalization of inhibitor levels observed under these conditions suggests that calpastatin deficiency is caused by a direct degradation by calpain.

EXPERIMENTAL PROCEDURES

Materials—Leupeptin, phenylmethylsulfonyl fluoride, and Ca2+-ionophore A23187 were purchased from Sigma. Diethylaminoethyl cellulose resin (DE53) was obtained from Whatman. Human erythrocyte calpain was purified as reported previously (9). One unit of calpain activity is defined as the amount causing the production of 1 μmol of acid-soluble NH₃ groups in the conditions reported previously (26). The anti-calpastatin monoclonal antibody 35.23, which recognizes the N-terminal region of calpastatin, was produced as described in Ref. 27.

Isolation of Calpastatin from Kidney, Heart, Erythrocytes, and Brain by Ion Exchange Chromatography—Samples of kidney (2 g), heart (1 g), and brain (1.5 g) from both normotensive and hypertensive rats of the Milan strain were minced, homogenized in a Potter Elvejem homogenizer, and lysed by sonication (four bursts of 10 s each at 0 °C) in 3 volumes of 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.1 mg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Samples of erythrocytes from both normotensives and hypertensive rats were lysed by sonication in the same conditions reported above. The disrupted cell suspensions from each sample were centrifuged at 100,000 × g for 15 min, and the clear supernatant (crude extract) was heated at 100 °C for 3 min and centrifuged at 100,000 × g for 10 min. The soluble material (heated extract) was collected and loaded onto a DE53 column (5 ml) equilibrated in 50 mM sodium borate buffer, pH 7.5, containing 0.1 mM EDTA and 0.5 mM 2-mercaptoethanol (buffer A). The absorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.35 M, and the amount of calpastatin activity was measured in the eluted fractions using human erythrocyte calpain and human acid denatured globin as substrate (28) in the presence of 1 mM human acid denatured globin as substrate (28) in the presence of 1 mM human acid denatured globin as substrate (28) in the presence of 1 mM 2-mercaptoethanol. Each fraction (0.5 ml) was tested for the presence of inhibitors of calpain.

Identification of Calpastatin Species by SDS-Polyacrylamide Gel Electrophoresis—Equal amounts of kidney heated extract from normotensive and hypertensive rats, prepared as described above and corresponding to 600 μg of protein contained in crude extract, were separated on SDS-polyacrylamide gel electrophoresis (12%). At the end of the electrophoretic run, the gel was washed with buffer A containing 20% methanol to remove any remaining SDS. After 20 min, the gel was transferred to buffer A without methanol and stirred gently for additional 20 min; then the gel lanes containing calpastatin were cut in 2-mm sections and submitted to electrophoresis on a 3.5% polyacrylamide/7 M urea gel. The gel lanes were subsequently sliced, and the radioactivity was quantified in a scintillation counter. To minimize tube-to-tube variation each experiment was carried out in triplicate.

Calpastatin Digestion in a Reconstructed System—Rat kidney calpastatin (10 units) was incubated in 0.1 ml of 50 mM sodium borate buffer, pH 7.5, in the presence of 100 μM Ca2+ (33) with 10 units of native (80 kDa) or autoproteolyzed calpastatin (75 kDa plus 18 kDa) (11). In this reconstructed system we used purified rat erythrocyte calpain that, although belonging to the μCL family, catalyzes proteins both from classical and nonclassical calpain substrates (26). This enzyme has been selected because it is equally sensitive to all calpastatin forms regardless of their origin.

Identification of anti-calpastatin monoclonal antibody 35.23, which recognizes the N-terminal region of calpastatin, was produced as described in Ref. 27.

RESULTS

Levels of Calpastatin Activity during Aging in Normotensive and Hypertensive Rats—In kidney of normotensive rats (Fig. 1), calpastatin activity increases progressively with aging, reaching at 150 days values approximately three times higher than those of 30-day-old animals. On the contrary, in kidney of hypertensive rats, calpastatin activity remains almost unchanged at levels corresponding to those of 30-day-old normotensive animals; this results in 3–4 times lower levels in 150-day-old animals as compared with those of control rats. For comparison, we measured calpastatin levels also in other tissues (Fig. 1). Although in heart and erythrocytes the behavior of the inhibitor activity is similar to that observed in kidney, in brain, which contains the highest levels of calpastatin, we observed no significant differences between normotensive and hypertensive rats.

Levels of Calpastatin mRNA in Normotensive and Hypertensive Rats at Different Ages—The amount of native calpastatin

1 The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.
protein in kidney extracts, measured by immunoblot analysis (Fig. 2A) increases with age only in normotensive animals, whereas in hypertensive rats it remains at lower levels regardless of the age of the animal. The ratio between calpastatin activity, calculated from Fig. 1, and the corresponding amount of protein determined from Fig. 2A (see "Experimental Procedures") is almost identical in all animals, indicating that in normal and hypertensive rats calpastatin has similar specific activity and inhibitory efficiency (Fig. 2B).

Levels of Calpastatin mRNA in Normotensive and Hypertensive Rats at Different Ages—We also analyzed the expression of calpastatin in kidney of normotensive and hypertensive rats at different ages. As shown in Fig. 3, after 30 amplification cycles by PCR, although the staining intensity of GAPDH transcripts is almost the same in all the samples considered, calpastatin mRNA increases progressively with aging in both normotensive and hypertensive animals. Furthermore, this increase is much more consistent in normotensive rats; in fact, comparing Fig. 3, A and B, although old rats of both strains display similar amounts of calpastatin transcript, in young normotensive rats, unlike hypertensive animals, this band is very faint. Because of the fact that the fluorescence analysis shown in Fig. 3 is not really quantitative, we have chosen the method described by Chelly et al. (39) to obtain more satisfactory results. This procedure utilizes the co-expression of an internal standard (GAPDH) and 5’-32P-labeled primers to detect the amount of product present at various amplification cycles.

As shown in Fig. 4, in kidney of normotensive and hypertensive 30-day-old rats, the amounts of the internal marker (GAPDH) and calpastatin, expressed at increasing amplification cycles, follow straight parallel lines, intercepting the y axis at values comparable with the amount of the original material. With this method we determined the quantitative levels of mRNA in kidney of normotensive and hypertensive rats, which are reported in Table I, together with the corresponding amount of inhibitor activity. During aging in normotensive rats, mRNA and protein increase in comparable amounts,
reaching the maximal level at 150 days. A difference in the behavior has been observed in kidney of hypertensive rats, in which the level of mRNA in younger animals is four times higher than that in normotensive rats of the same age and undergoes only a small increase during aging. To obtain more information about these differences in the expression of calpastatin, we compared the amount of calpastatin mRNA with the level of calpastatin protein (see Table I). This ratio is almost constant in kidney of control rats, whereas in hypertensive animals it is very high in younger rats and further increases as a function of age. These data are indicative of an imbalance between levels of mRNA transcript and accumulation of the inhibitor protein. This imbalance could be ascribed to an increased rate of calpastatin degradation caused by the concomitant calpain activation resulting from an increase in intracellular free [Ca\(^{2+}\)] (40). The calpain-mediated calpastatin digestion is then responsible for the reduced amount of calpastatin available for calpain inhibition.

Identification of the Active 15-kDa Calpastatin Fragment in Kidney of Normotensive and Hypertensive Rats—In previous experiments using LAN-5 cells, we demonstrated that calpastatin digestion following calpain activation induced by an increase in intracellular [Ca\(^{2+}\)] is accompanied by the formation of the active 15-kDa calpastatin fragment, which does not contain the N-terminal region (15). To investigate whether the calpastatin deficiency observed in kidney of hypertensive rats is caused by calpain-mediated inhibitor digestion, we followed in a reconstructed system both the formation of the active 15-kDa fragment and the loss of calpastatin activity. When purified kidney calpastatin is incu-
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Fig. 7. Effect of antihypertensive treatment on arterial pressure and kidney calpastatin activity of normotensive and hypertensive rats. 150-day-old normotensive and hypertensive rats were treated with Lisinopril for 7 days (see "Experimental Procedures"). N, untreated normotensive rats; NT, treated normotensive rats; H, untreated hypertensive rats; HT, treated hypertensive rats. A, systolic blood pressure detected on repeated measurements by a plethysmographic method (see "Experimental Procedures"). B, the levels of kidney calpastatin activity measured after ion exchange chromatography as described under "Experimental Procedures" and in the legend to Fig. 1. Inset, the levels of kidney calpastatin protein determined by immunoblot as described under "Experimental Procedures" and in the legend to Fig. 5. The values reported are the means ± SD of four different experiments.

In light of these observations, we measured the amount of the 15-kDa fragment in kidney of normotensive and hypertensive rats at different ages. As shown in Fig. 6, the kidneys of normotensive rats contain low levels of the 15-kDa calpastatin fragment. However, in hypertensive rats this fragment is already detectable in 30-day-old animals and increases ~3-fold in 150-day-old rats. Even if the amount of total calpastatin activity is higher in kidney of normotensive rats, the level of the 15-kDa calpastatin fragment ranges from 1 to 3%. However this calpastatin fragment represents in kidney of younger and adult hypertensive rats from 6 to 20% of total calpastatin activity, respectively. Thus, the identification of large amounts of this calpastatin form in tissues of hypertensive rats further supports the in vivo calpain-mediated digestion of its inhibitor protein, producing an imbalance in the regulation of the calcium-dependent proteolytic system.

Expression of Calpain Isozymes in Kidney of Normotensive and Hypertensive Rats—The indication for an increased degradation of calpastatin by calpain in hypertensive rats posed the question of a different level of calpain isozymes in kidney of hypertensive rats. To answer this question, we thus determined the expression and activity of both m- and m-calpain in kidney of 150-day-old normotensive and hypertensive rats, and no difference between the two strains of animals has been observed (data not shown). These data allow the conclusion that increases in calpastatin degradation can presumably be caused by an augment of intracellular free [Ca2+]i, leading to calpain activation.

Restoration of Normal Levels of Calpastatin by Antihypertensive Treatment—This hypothesis was tested by the administration to both strains of adult animals of an antihypertensive drug (Lisinopril, an angiotensin I-converting enzyme inhibitor) known to promote the normalization of Ca2+ homeostasis (41). After a week of therapy, the blood pressure of normotensive rats is not modified significantly (Fig. 7A), whereas that of hypertensive rats decreases from 150 to ~90–95 mm Hg. Furthermore in treated hypertensive rats a 3–4 times increase in calpastatin activity was observed (Fig. 7B); no significant effect could be detected in normotensive animals. As shown in 7B, inset, immunoblot analysis reveals that an increase in the protein amount parallels that of biological activity. As expected, the normalization of the calpastatin level was found to be accompanied by the restoration of normal small levels of the 15-kDa active calpastatin fragment (Fig. 7C).

DISCUSSION

Calpain regulation is accomplished by a number of posttranscriptional and post-translational modifications of the nat-
ural inhibitor protein calpastatin (13, 42, 43). An alternative exon splicing at the level of 1-domain seems necessary to produce tissue-specific calpastatin molecules (44). In addition, the number of repetitive domains present in the calpastatin molecule was found to range between four in the high M<sub>i</sub> form and one in the lowest M<sub>i</sub> form (32). At a molecular level calpastatin protein undergoes structural modifications determining its specificity and inhibitory efficiency (13, 45) as well as its intracellular localization.

This is particularly evident in neuroblastoma cells (46), in which calpastatin is normally present in a phosphorylated and aggregated form localized in a specific position close to the nucleus. After hydrolysis of these phosphate groups by a protein phosphatase, the dephosphorylated inhibitor diffuses into the cytosol and acquires a significant increase in its inhibitory efficiency (45). These sequential events are triggered by an increase in intracellular [Ca<sup>2+</sup>]<sup>+</sup>. Hence, the intracellular mechanism for the regulation of calpastatin inhibitory activity and functional localization seems to be represented by a reversible phosphorylation and dephosphorylation process.

An additional down-regulation process can be attributed to the prolonged activation of the calcium-dependent proteolytic system resulting in a decrease in calpastatin activity (13, 14). The proteins involved in this process are the calpain family or members of other thiol proteinase families such as caspasases (47, 48). During this down-regulation process mediated by calpain, we have observed that both in cultured tumor cells and in vitro reconstructed systems during calpastatin degradation both 15-kDa active fragments or inactive peptides are produced (15).

In this paper we provide evidence indicating that this degenerative process is responsible for the calpastatin deficiency occurring in Milan adult hypertensive rats. In fact, we have observed that the levels of calpastatin protein and activity are similar in young normotensive and hypertensive rats, but they become four times lower in old hypertensive animals. Erythrocytes display the highest defect in calpastatin, probably because they are unable to synthesize new protein to compensate calpastatin decay. Kidney and heart are affected also, whereas in brain no significant difference in calpastatin activity between normotensive and hypertensive rats is observed (26).

It can be concluded that the calpastatin deficiency observed in hypertensive rats is a primary event which causes they are unable to synthesize new protein to compensate for the calpastatin deficiency observed in hypertensive rats, and (iii) treatment with angiotensin I-converting enzyme inhibitors has no effect on the expression of calpastatin (25). Taken together these observations demonstrate that the calpastatin deficiency observed in hypertensive rats is the result of an increase in calpastatin degradation by calpain, most presumably activated by an increase in [Ca<sup>2+</sup>]<sup>+</sup>.

The model reported in Scheme 1 describes the cascade of events occurring in hypertensive rats; specifically, it is proposed that an initial interaction of calpastatin with the 75-kDa calpain form released from its site of activation. In this complex, calpastatin undergoes degradation to the 15-kDa active form together with inactive fragments. After a further increase in [Ca<sup>2+</sup>]<sup>+</sup>, cytosolic calpain can directly acquire the active conformation rapidly inhibited through the formation of a complex with calpastatin, which becomes in this associated form susceptible to a slow proteolytic degradation.

It is well known that in essential hypertension, calcium homeostasis is affected by the result of the increase of this metal ion in different tissues (40), in all of which calpain activation is thereby promoted. In these conditions, the degradation of calpastatin and decrease in its inhibitory efficiency result in a profound imbalance of the proteolytic system, leaving calpain without its natural regulator.

It can be concluded that the calpastatin deficiency observed in hypertensive rats can be regarded as one of the risk factors responsible for tissue damage that is typical of essential hypertension. Because of similarities between the genetically determined hypertension in rats of Milan strain and essential hypertension in humans, it can be postulated that also in these patients calpastatin deficiency is a potential risk for kidney failure and heart attack.

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