Proteinase and Proteinase-Inhibitor Activities of Rat Uterine Myometrium during Pregnancy and Involution

By ERNST-GÜNTER AFTING, MARIE LOUISE BECKER and JOHN S. ELCE*
*Institut für Toxikologie und Biochemie der Gesellschaft für Strahlen- und Umweltforschung und
Biochemisches Institut der Universität Freiburg, D7800 Freiburg i. Br., West Germany*

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A supernatant fraction was prepared from rat uterine myometrium by homogenization, sonication and centrifugation. In this supernatant the protein concentration and the activities of an acid proteinase, an acid phosphatase and a proteinase inhibitor were measured. From the fibrous sediment, after washing with 0.5% Triton X-100 and with water, an actomyosin-containing solution was obtained by extraction with 0.6M-NaCl, and in this extract the protein concentration and a neutral proteinase activity were measured. The myometrial wet weight and the activities of the acid proteinase, acid phosphatase and proteinase inhibitor increased by factors of 3–15 during pregnancy and decreased to the same or a greater extent during involution. The amount of protein extracted with 0.6M-NaCl increased by a factor of only 2.3 and the neutral proteinase activity remained essentially constant during pregnancy and involution. The pH optimum of the neutral proteinase, and its pattern of activity compared with those of the lysosomal enzymes, show that the neutral proteinase is not of lysosomal origin. Actomyosin is degraded by the neutral proteinase activity *in vitro*. Since actomyosin is rapidly broken down only after parturition, the action of the neutral proteinase activity on actomyosin, if this occurs *in vivo*, must be regulated in some way. The proteinase-inhibitor activity measured in the first supernatant varied in a manner which suggested that it could be involved in this control.

Most tissue protein is subject to continued turnover, but the mechanisms of this turnover and its regulation, although much reviewed, are not understood (Goldberg & Dice, 1974; Reich *et al.*, 1975; Goldberg & St. John, 1976; Ribbons & Brew, 1976; Kolata, 1977). Extracellular protein is taken up and hydrolysed in the lysosomes of certain cells, but the role of lysosomes in degradation of intracellular material has only recently been demonstrated (Dean, 1975; Dean & Barrett, 1976). Although the final stages of the hydrolysis of any protein molecule probably take place within a lysosome, the mechanisms whereby lysosomes select intracellular proteins for degradation are not clear (Ballard, 1977). One of several possible mechanisms is that an intracellular protein suffers a preliminary extra-lysosomal limited proteolysis, which renders the fragments more susceptible to lysosomal uptake (Canonica & Bird, 1970; Schiaffino & Hanzlikova, 1972). A specific proteinase cannot exist for every protein, but several non-lysosomal protein-degrading systems have been observed (Schimke & Katunuma, 1975; Dean & Barrett, 1976; Katunuma & Kominami, 1977) which

may be involved in intracellular proteolysis in special cases.

Pennington (1977) has summarized the many reports of skeletal-muscle proteinases, but little precise information is available about proteinases of smooth muscle. The involuting rat uterus shows a rapid loss of wet weight and has been recognized as an excellent model for study of controlled protein degradation (Harkness & Moralee, 1956; Woessner, 1965). Measurement of DNA in the involuting rat uterus (Afting & Elce, 1978) had shown that about half of the loss of wet weight is due to hypertrophy of the cells, which implies an intracellular proteolysis, and it seems likely that both lysosomal and non-lysosomal proteinases are involved. Goodall (1965) and Woessner (1965) described an acid proteinase of the uterus, which might be located either in the smooth-muscle cells or in the macrophages, cells which appear in large numbers in the myometrium immediately after parturition (Parakkal, 1969). In addition, several neutral proteinases, presumably not lysosomal, have been reported in uterus and other smooth muscle. A soluble Ca^{2+} -dependent proteinase (Puca *et al.*, 1977) and a Ca^{2+} -dependent collagenase (Jeffrey *et al.*, 1971) have been found in the uterus, in addition to a serine proteinase, not

Abbreviation used: ATPase, adenosine triphosphatase.

* Present address: Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada.

Ca^{2+} -dependent (Notides *et al.*, 1973; Katz *et al.*, 1976), which was solubilized completely only in 1.5M-KCl and was induced by hormone treatment of ovariectomized rats. In rat intestinal muscle, Beynon & Kay (1977) described a non- Ca^{2+} -dependent trypsin-like neutral proteinase, and Woodbury *et al.* (1978) have reported the amino acid sequence of the group-specific proteinase. Several different substrates have been used for assay of these proteinases, which were not directly relevant to a possible role in uterine involution. However, Roth *et al.* (1976) and M. Roth & E.-G. Afting (unpublished work) studied a non- Ca^{2+} -dependent neutral proteinase in pig uterus, and showed that actomyosin was a substrate for this enzyme. The hydrolysis of uterine actomyosin with parallel loss of ATPase activity that was observed is consistent with a possible physiological function of this neutral proteinase in uterine involution.

Although efforts to purify this proteinase from pig uterus continue, it was important to pursue studies of possible regulation of the proteinase activity. The activity of muscle proteinases may be regulated by compartmentation (Pennington, 1977), but changes in the amount of proteinase, conceivably under hormonal control, or changes in the amount of proteinase inhibitors could also be involved. The tension in the uterine muscle seems also to be important in control of involution (Csapo *et al.*, 1965; de Mattos *et al.*, 1967).

The results reported here concern variations of wet weight and of two lysosomal enzyme activities, in comparison with measurements of the activities of a neutral proteinase and a proteinase inhibitor, in rat uterine myometrium during pregnancy and involution *post partum*.

Experimental

Materials and methods

Bovine serum albumin was obtained from Behringwerk, Marbury, Germany, and casein-Hammarsten and haemoglobin were from Merck, Darmstadt, Germany. Azocoll was purchased from Calbiochem, Giessen, Germany. Proteinase B from yeast was kindly provided by Dr. H. Holzer, Freiburg, Germany. All other reagents were of analytical grade and obtained from commercial sources.

Wistar rats (200–250 g, with timed pregnancies) were obtained from Ivanovas, Kisslegg, Germany, and maintained on Altromin (Altrogge, Lage-Lippe, Germany) and water *ad libitum*. The rats were killed by decapitation and drained of blood; the uteri were excised and separated from adhering tissue and from the cervix. The uterine contents of pregnant animals were removed, and the uteri slit open lengthways so that the epithelial layer and some of the endometrium could be scraped off with a scalpel. Needham &

Cawkwell (1956), working with pig uterus, and Wakid (1960), with rat uterus, stated that scraping left mainly myometrium. Histological sections of our scraped rat uterus preparations showed, however, that a neat removal of the endometrium was not possible, and a small amount of the basal layer of the endometrium sometimes remained (Bloom & Fawcett, 1975) according to the vigour of the scraping process. The tissues were frozen as soon as possible in liquid air and stored at -80°C . Uteri from animals pregnant in only one horn or having less than six offspring were rejected.

Extraction of the myometrium

All extraction operations were performed in tubes kept on crushed ice or in centrifuges at 4°C . The whole myometrium, prepared as described, was weighed and then minced finely with scissors while thawing from -80°C , and the mince was well mixed. After samples were taken for DNA analysis (Afting & Elce, 1978), approx. 0.5 g of tissue (or all the available tissue from smaller uteri) was mixed well with 5.0 ml of 0.05M-veronal/HCl, pH 7.4, containing 10mM- β -mercaptoethanol in a Sorvall 50ml steel centrifuge tube. The mixture was homogenized three times for 30s each, with cooling in ice for 15s between each homogenization, at maximum speed with a Sorvall Omnimix apparatus, by using the blade attachment and adaptor that fits the 50ml steel centrifuge tube. This was followed by sonication for $4 \times 15\text{s}$, by using the microtip of a Branson S110 sonifier, operated at setting 5 (about 75W). Cooling of the Sorvall tube in ice was very important during the sonication. The mixture was centrifuged in an SS34 rotor on a Sorvall machine at 12000g (r_{av} , 7.5cm) for 10min, leaving a supernatant which was decanted and is referred to as the 'first supernatant'. The fibrous residue was washed once in 5.0 ml of 0.05M-veronal/HCl, pH 7.4, containing 10mM- β -mercaptoethanol and 0.5% (w/v) Triton X-100 by resuspending the pellet, homogenizing for 30s with the Omnimix apparatus and centrifuging as before, and then washed once again similarly in 5.0 ml of water. The final fibrous residue was homogenized for 30s in 5.0 ml of 0.05M-veronal/HCl, pH 7.4, containing 5mM- β -mercaptoethanol and 0.6M-NaCl, and the mixture left at 0°C for 16–20h overnight. The mixture was then centrifuged as above and the supernatant, the 0.6M-NaCl extract, decanted.

Assays

Acid phosphatase (EC 3.1.3.2) activity in the first supernatant was determined at pH 5.0 with β -glycerophosphate as described by Appelmans *et al.* (1955), the phosphate released being measured by the method of Fiske & SubbaRow (1925).

The neutral proteinase activity in the 0.6M-NaCl extract was assayed with casein as substrate

(Laskowski, 1955) from the rate of formation of trichloroacetic acid-soluble Lowry-positive material, which is said to contain free amino acids only (McLaughlin & Bosmann, 1976). The incubation mixture (in a final volume of 0.7 ml in Eppendorf 1.5 ml micro test tubes) contained 0.5 ml of a solution of casein (10 mg/ml) in 0.05 M- $\text{Na}_2\text{B}_4\text{O}_7$ /1 M-NaCl, adjusted to pH 8.0 with HCl, and a portion of enzyme solution. Duplicate samples of each enzyme solution were incubated at 37°C for 0, 0.5, 1.0 and 2.0 h, and reaction was stopped by addition of 0.5 ml of 10% (w/v) trichloroacetic acid. After 15 min on ice, the tubes were centrifuged at 4000 rev./min in a bench centrifuge for 3 min, and 0.5 ml portions of the supernatant layers were taken, neutralized with 0.25 ml of 1 M-NaOH, and assayed by the method of Lowry *et al.* (1951). The A_{750} was plotted as a function of time, to confirm the linearity of the assay in each case, and the line of best fit drawn by eye.

For rates of change of absorbance of up to 0.15 A_{750} unit/h, the assay was linear for up to 2 h with respect to time and the initial velocity was proportional to the quantity of enzyme. The rates of change of A_{750} in incubation mixtures containing either casein alone or enzyme solution alone were negligible. With stock solutions of casein of concentrations greater than 5 mg/ml, the initial velocities were independent of the casein concentration.

The acid proteinase activity in the first supernatant was determined at 37°C by the method of Anson (1938), with a solution of haemoglobin (12 mg/ml) in 0.2 M-sodium citrate, pH 3.3, as substrate.

One unit of acid or neutral proteinase activity is defined as that amount of enzyme that caused a rate of change of A_{750} of 0.1 absorbance unit/h under the described incubation and test conditions. The units refer to the 0.5 ml of trichloroacetic acid supernatant taken for the Lowry *et al.* (1951) assay, and not to the total volume of the trichloroacetic acid supernatant, which is 1.2 ml.

The proteinase-inhibitor activity of the first supernatant was measured by its effect on the activity of yeast proteinase B. Yeast proteinase B was assayed with Azocoll as described by Saheki & Holzer (1974) and the units of inhibition were expressed as units of proteinase inhibited (Betz *et al.*, 1974).

Protein was measured as described by Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Treatment of the data

The mean and standard deviation of data from groups of three or more animals were calculated, and comparisons between data for two groups were made by use of Student's *t* test. The results are plotted in the Figures $\pm$ standard errors of the means.

The results of measurements of all the parameters

were expressed in terms of units per total myometrium.

Results and Discussion

Extraction and properties of the neutral proteinase

The purpose of the work was to study variations in activity of the neutral proteinase and of the proteinase inhibitor as a function of time of pregnancy and involution. Since it has been found difficult to purify the corresponding activities from pig uterus (E.-G. Afting, unpublished work), the neutral proteinase (in the 0.6 M-NaCl extract) and the inhibitor (in the first supernatant) from the rat uterus were measured without further purification.

The method of extraction of the neutral proteinase from the rat myometrium was based on published methods for preparation of actomyosin from smooth muscle, with the addition of a sonication step, by using a washing procedure in low-ionic-strength buffer with Triton X-100 (Sobieszek & Bremel, 1975), but the final extraction was performed with 0.6 M-NaCl. The amount of neutral proteinase activity in the final extract was in the range of 60–120 units per uterus, and a further extraction of the fibrous residue with 0.6 M-NaCl could produce a further 10–20 units. If β -mercaptoethanol was omitted from the extraction procedure, the amount of neutral proteinase activity obtained varied from 0 to 40 units per uterus. Clear data were not obtained on the effects of sonication, because the success of the extraction depended both on the efficiency of the Sorvall Omnimix apparatus in homogenizing the tissue, and on the efficiency of the sonication, both of which were difficult to quantify, but the yields of activity without sonication varied from 25 to 100% of those with sonication. Owing to the presence of inhibitor activity, no estimate of the neutral proteinase activity originally present in the whole uterine homogenate could be made. The yield was not affected by storage of the myometrium at -80°C and the extracted activity was stable for some weeks at 0°C and -20°C .

Since the neutral proteinase activity was measured in a crude extract, precise enzymological studies could not be carried out. The enzyme preparation catalysed hydrolysis of casein, and of native and heat-denatured uterine actomyosin, with a pH optimum from 7.0 to 8.7. Amino acid-*p*-nitroanilides were not hydrolysed, and lactate dehydrogenase was not affected, by incubation with the rat uterine neutral proteinase (Beynon & Kay, 1977). With casein as substrate, the activity was inhibited 88% by 0.1 mM- HgCl_2 , 74% by the soya-bean trypsin inhibitor (0.4 mg/ml), and 16% by 0.1 mM-phenylmethanesulphonyl fluoride. Hydrolysis of casein was not affected by the presence of 50 mM- Ca^{2+} or of 10 mM-EDTA. It appears that the neutral proteinase activity may be a mixture of enzymes, but does not appear to contain an enzyme

similar to the more soluble, Ca^{2+} -dependent neutral thiol proteinases from other tissues, including skeletal, cardiac and uterine muscle (Jeffrey *et al.*, 1971; Reville *et al.*, 1976; Puca *et al.*, 1977; Phillips & Jakabova, 1977). This Ca^{2+} -dependent proteinase does not attack actin or myosin, and is also unstable in the presence of Ca^{2+} , whereas the neutral proteinase in the 0.6M-NaCl extract is stable. Katz *et al.* (1976) described a neutral trypsin-like proteinase in uterus which seems to be more soluble than the enzyme studied here, and Beynon & Kay (1977) have reported that a similar enzyme is present in rat intestinal smooth muscle.

The properties of the rat uterine neutral proteinase activity seem in general to be very similar to those of the neutral proteinase activity from pig uterus. The latter activity has been partially purified and more precisely characterized (M. Roth & E.-G. Afting, unpublished work). With the pig uterus preparation, it was shown that the myosin ATPase activity decreased in parallel to the loss of myosin protein on gel electrophoresis, and with a proportional increase in the soluble products of proteolysis.

Variations of the measured activities during pregnancy and involution

The choice of a suitable form for the data presented some difficulties. Some previous work on uterine composition and enzyme activities in the rat has been published in terms of units/g wet wt. of tissue (Woessner, 1965; Michael & Schofield, 1969), but since the wet weight of the uterus varies greatly during pregnancy and involution, the presentation of data in this way can be misleading. Brody (1958) observed that the water content of the human uterus remained constant during pregnancy, but that the protein per cell increased considerably, and recommended the expression of tissue constituents on a per nucleus basis. Since the amount of DNA in the rat uterus was then found to vary considerably during pregnancy and involution (Afting & Elce, 1978), the data in the present paper were calculated as total units per myometrium.

Wet weight and first supernatant protein

The changes in myometrial wet weight and in the total protein present in the first supernatant are shown

Fig. 1. Variation of the myometrial wet weight in g (○) and of protein in the first supernatant in mg per myometrium (●) throughout pregnancy and involution

The rat uterus was excised, scraped to remove the endometrium, and the myometrium that remained was frozen in liquid N_2 . The myometrium was weighed, chopped, homogenized and sonicated as described in the Experimental section. The mixture was centrifuged at 12000g for 10 min, leaving a first supernatant and a fibrous residue. The amount of protein in the first supernatant was measured by the method of Lowry *et al.* (1951). The S.E.M. was too small for most of the points to be shown. The number of experimental animals averaged four per time point before parturition and sixteen per time point afterwards.

in Fig. 1, to illustrate the growth and involution and to permit comparison with previous reports (Harkness & Harkness, 1954; Woessner, 1965; Geyer *et al.*, 1977) and with the subsequent Figures. The myometrial wet weight increased by a factor of about 6.1 at its peak, and at 10 days *post partum* showed a super-involution, that is, the wet weight was significantly less than that of the non-pregnant animals (0.18 ± 0.01 g, cf. 0.32 ± 0.05 g; $P < 0.001$). The protein in the first supernatant increased by a factor of about 12 at its peak and did not show a super-involution at 10 days *post partum*.

Acid proteinase and acid phosphatase

The variations of two lysosomal marker-enzyme activities in the first supernatant, an acid phosphatase (EC 3.1.3.2) and an acid proteinase, are shown in Fig. 2. From the molecular weight of the acid proteinase, its specificity towards several substrates and inhibitors and its pH optimum at 3.3, it appears that this uterine acid proteinase is probably the known lysosomal cathepsin D (EC 3.4.23.5) (Barrett, 1975; Sloane & Bird, 1977; Afting, 1978).

The acid phosphatase activity increased by a factor of 4.5 at its peak at about the time of parturition, and there was no super-involution. The acid proteinase activity showed about the same degree of

increase up to the last day before parturition, but then increased abruptly in days 1 and 2 *post partum*. The different behaviour of the two lysosomal enzyme activities in the days immediately *post partum* suggests that they are derived at least partly from different populations of lysosomes. The peak of acid proteinase activity *post partum* may be derived from lysosomes in macrophages, since these cells are known to increase sharply in number in the myometrium after parturition, and to disappear gradually over the next 14 days (Lobel & Deane, 1962; Parakkal, 1969). At 10 days *post partum* a significant super-involution of the acid proteinase activity had occurred (527 ± 29 units, cf. 411 ± 31 units; $P < 0.001$).

Protein and neutral proteinase activity in the 0.6M-NaCl extract

From the nature of the extraction process (see the Experimental section) the amount of the 0.6M-NaCl-soluble protein is expected to provide a rough estimate of myometrial actomyosin, and the presence of actomyosin as the main component in this extract was qualitatively confirmed by gel electrophoresis by the method of Laemmli & Favre (1973). The 0.6M-NaCl-soluble protein increased by a factor of only 2.3, and the increase took place rather abruptly between days 9 and 13 of pregnancy, with little subsequent increase (Fig. 3). No significant decrease was

Fig. 2. Variation of the acid proteinase (○) and of the acid phosphatase (●) activities in units per myometrium, $\pm$ S.E.M., throughout pregnancy and involution

A first supernatant was obtained from rat myometrium by homogenization, sonication and centrifugation, as described in the Experimental section, and the two activities were measured in samples of this supernatant. The number of experimental animals averaged four per time point before parturition and eight per time point afterwards.

Fig. 3. Variation of the 0.6M-NaCl-soluble protein in mg per myometrium (●) and of the neutral proteinase activity in units per myometrium (○), $\pm$ S.E.M., throughout pregnancy and involution

The fibrous residue obtained by homogenization, sonication and centrifugation of the rat myometrium was washed and then extracted with 0.6M-NaCl as described in the Experimental section. After a further centrifugation, the amounts of protein and of neutral proteinase activity were measured in samples of the 0.6M-NaCl-containing supernatant. The number of experimental animals averaged four per time point before parturition and seven per time point afterwards.

observed until day 3 *post partum*, and a marked super-involution could be seen at day 10 *post partum*. This super-involution of actomyosin and of the wet weight illustrates the fact that the proteolysis of the tissue goes 'too far'. However, the rate of proteolysis must eventually be slowed to normal turnover values when the myometrium returns to its original non-pregnant size at the end of lactation.

The neutral proteinase activity, in marked contrast with the other parameters measured, was essentially constant throughout pregnancy and involution, except that the values in the non-pregnant animals were a little higher than the average (Fig. 3). The constancy of this activity, compared with the great variations in the lysosomal enzyme activities, makes it clear that the neutral proteinase is not located in the lysosomes, as had already been suggested from its pH optimum. Intact lysosomes cannot readily be obtained from smooth muscle to confirm this point by direct assay (Chobanian & Brecher, 1977; Peters *et al.*, 1972). The neutral proteinase is also unlikely to be located in mitochondria, plasma membrane or soluble phase, since the activities of marker enzymes for each of these compartments have been measured and showed an abrupt fall after parturition (Geyer *et al.*, 1977).

It is not known whether the neutral proteinase activity is present in smooth-muscle cells, or in one of the several other types of cell which are found in the myometrium (Bloom & Fawcett, 1975). It does not, however, seem probable that the neutral proteinase activity, which remains essentially constant, should be located either in the macrophages, which increase in number sharply after parturition (Parakkal, 1969), or in the endometrial remnants, since these were present in very variable amounts.

Proteinase-inhibitor activity

The inhibitory activity in the first supernatant was measured by its effect on proteolysis of Azocoll by yeast proteinase B, but it also inhibited proteolysis of actomyosin and casein by the neutral proteinase, although with a 10-fold lower sensitivity. Further studies on the proteinase inhibitor, and a demonstration that the inhibitor derived from the myometrial tissue and not from the serum, have been described by Afting *et al.* (1978).

The activity of the proteinase inhibitor rose earlier than the increases in wet weight and lysosomal activities, and had increased by a factor of 15 at its peak on the day before parturition, a larger increase than any other recorded here, but its activity then

Fig. 4. Variation of a proteinase inhibitor activity in units per myometrium, $\pm$ S.E.M., throughout pregnancy and involution

A first supernatant was obtained from rat myometrium by homogenization, sonication and centrifugation as described in the Experimental section. The inhibitor activity was measured in samples of this supernatant by means of its effect on the hydrolysis of Azocoll by yeast proteinase B (Betz *et al.*, 1974). The number of experimental animals averaged four per time point before parturition and nine per time point afterwards.

by their abrupt fall at parturition (Hashimoto *et al.*, 1968; Yoshinaga *et al.*, 1971), and by the changes in muscle tension (Csapo *et al.*, 1965; de Mattos *et al.*, 1967), are not yet clear, but even these influences must ultimately be expressed at the enzymic level. It is possible to suggest that the proteinase inhibitor acts to limit the activity of the neutral proteinase, and thereby retards the initial step in actomyosin catabolism. Further work must be directed to finding out the subcellular location of the neutral proteinase activity and of the inhibitor, particularly in relation to the location of actomyosin, and at various times during pregnancy and involution. This information may help to explain the function of the neutral proteinase activity in involution of the uterus, and may clarify the role of compartmentation in regulation of the proteolytic activity.

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fell markedly even in animals killed during parturition (Fig. 4). This fall in activity occurred 1–2 days earlier than the fall in the other parameters.

Conclusion

The normal course of pregnancy and involution of the myometrium is shown by the changes in wet weight and supernatant protein (Fig. 1). The changes in activities of the lysosomal marker enzymes (Fig. 2) were roughly parallel to the changes in the wet weight, and served also to confirm that the neutral proteinase was not located in the lysosomes.

The most important findings of this work were that a proteinase inhibitor activity increased earlier and by a larger factor in pregnancy than the other activities measured, and decreased earlier, and that the neutral proteinase remained constant. The acid proteinase, whose measurable activity rose during pregnancy, and the neutral proteinase which remained constant in activity, are not greatly active *in vivo* until after parturition, and the question therefore arises of how proteolysis is inhibited until parturition and then permitted to take part in the involution. Similarly, the rate of proteolysis must be decreased again to normal turnover values when the myometrium has returned to its non-pregnant size.

The roles played by the rising steroid hormone concentrations during pregnancy, and particularly

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