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Neutral Metalloendopeptidase Associated With the Smooth Muscle Cells of Pregnant Rat Uterus

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Abstract The pregnant rat uterus contains a membrane-bound metalloendopeptidase that is biochemically and immunologically similar to kidney enkephalinase (E.C.3.4.24.11). The uterus enzyme readily cleaved specific neutral endopeptidase substrates and oxytocin as well as the synthetic elastase substrate, Suc(Ala)3-pNA, yet did not digest native elastin. Using specific inhibitors, the uterus endopeptidase was identified as a metallopeptidase and not a serine protease, having an absolute requirement for zinc and perhaps calcium for maximal activity. The uterus endopeptidase cross-reacted with polyclonal antiserum to kidney microvillar endopeptidase and a monoclonal antibody to common acute lymphocytic leukemia antigen. Immunohistochemical localization of the enzyme in a 17 day pregnant uterus indicated that the enzyme was localized on the smooth muscle bundles of the myometrium and the endometrial epithelium. Total enzyme activity was 25 times higher in the late-term pregnant uterus (17th day of pregnancy) than in the nonpregnant uterus. Enzyme levels dropped rapidly prior to parturition and within 4 days after delivery the enzyme activity had returned to control levels. Inhibition of NEP in uterine strips with phosphoramidon resulted in a marked potentiation of oxytocin-induced contractions. Our results suggest that the uterine endopeptidase may have an important role in regulating uterine smooth muscle cell contraction during the latter stages of pregnancy through its action on oxytocin and perhaps other biologically active peptides.

Key words: neutral endopeptidase, uterus contractions, oxytocin, kidney enkephalinase, uterus enzyme

The activity of the uterus during pregnancy depends on the relationship between factors designed to keep the uterus at rest and factors that facilitate contractions. The uterus in situ does not contract spontaneously, but only in response to stimulation. This stimulation is normally governed during pregnancy and parturition by circulating hormones; the most powerful of these is oxytocin which is approximately 2,500 times more potent than PGF2 [1].

Studies relevant to the normal release of oxytocin at parturition are compounded by a number of difficulties: the pulsatile nature of oxytocin release [2,3], the short half-life of oxytocin in the blood [4], and the possibility of an oxytocinase in human blood (and not in some animals) that degrades the hormone [5]. Most studies suggest that circulating oxytocin increases at the onset of labor [6], but investigators are still unable to distinguish whether oxytocin release is important in initiating parturition or whether it merely facilitates labor.

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In the rat [7] and the human [8] there is an enormous (100-fold) estrogen-induced increase in the myometrial oxytocin receptors during pregnancy, reaching a maximum just prior to parturition. The myometrium becomes extremely sensitive to oxytocin, such that small increases in the levels of hormone reaching these receptors could be sufficient to initiate labor.

Neutral endopeptidase (NEP E.C. 3.4 24.11), originally called enkephalinase, has been identified in many organs (kidney, lymph nodes, intestines, brain, and placenta) [9–16] and cells (neutrophils, macrophages, epithelial cells, fibroblasts, and endothelial cells) [16–18]. This enzyme is a transmembrane zinc metallopeptidase with the active site exposed on the external surface of the plasma membrane. NEP cleaves biologically active peptides at the alpha-amino side of hydrophobic amino acids [19]. A partial list of substrates includes the enkephalins, substance P, glucagon, bradykinin, FMLP, insulin B chain, endorphins, angiotensin, and oxytocin [16].

The physiological functions of NEP are varied and depend in large part on the tissue where it is found. Several recent studies indicate that NEP

can modulate the effect of peptide agonists, such as substance P, on smooth muscle contraction in the lung [12,20–22] and trachea [23]. These investigators have shown that the selective inhibition of NEP activity results in a marked potentiation of the agonist effect.

In this study we report on the localization of a neutral endopeptidase on the smooth muscle cells of the pregnant rat uterus with the same characteristics as NEP 24.11. The gestational timing and uterine distribution of this enzyme suggests that it may play a role in regulating the amount of biologically active oxytocin that is present during critical stages of pregnancy.

MATERIALS AND METHODS

The following reagents were commercially obtained: Aminopeptidase M, Pierce Chemical Co. (Rockford, IL); Elastin-rhodamine 200 to 400 mesh, Elastin Products Co., St. Louis, MO); Glu-Ala-Ala-Phe-4-methoxy-2-naphthylamide (MNA), Enzyme Systems Products, (Livermore, CA); phenylmethylsulfonyl fluoride (PMSF), phosphoramidon (N-(alfa-rhamnopyranosyloxy-hydroxyphosphinyl)-L-leucyl-L-tryptophan ammonium salt (PPAD), N-Dansyl-D-Alanyl-Glycyl-P-Nitrophenylalanyl-glycine (DAPGN), soybean trypsin inhibitor, and mouse IgG1 (MOPC 21) from Sigma Chemical Co., (St. Louis, MO); N-Succinyl-L-Ala-Ala-Ala-p-nitroanilide (Suc-(Ala)3-pNA), Peninsula Laboratories, Belmont, CA); J5 (Common Acute Lymphophosytic Leukemia Antigen, CALLA) Coulter Clone® monoclonal antibody, Coulter Immunology, (Hialeah, FL); Captopril and antisera to purified human kidney NEP were provided by Dr. A.R. Johnson of The University of Texas Health Center at Tyler. All other chemicals used were of analytical reagent grade.

Enzyme Preparation

The enzyme used for these studies was partially purified from rat uterus following the general techniques established for isolation of NEP [12,24]. Five pregnant (17–18 day) rats were killed with sodium pentabarbital and the uterus was excised. The fetuses were removed and the uterus was washed thoroughly with cold saline. The washed tissue was homogenized in 5 volumes of 0.02 M Tris-HCl buffer, pH 8.0, containing 0.25 M sucrose and centrifuged at 10,000*g* for 10 min to remove cellular debris and nuclei. The pellet was discarded and the supernatant was centrifuged at 100,000*g* for 60 min to obtain a crude membrane pellet. The pellets were pooled, resuspended in 5 volumes of 0.02 M Tris-HCl buffer, pH 8.0 containing 1% Triton X-100, and stirred for 30 min at room temperature. The suspension was centrifuged at 100,000*g* for 30 min and the supernatant was removed and fractionated by chromatography on DEAE cellulose and by FPLC using a mono Q 10/10 column. All the experimental studies were performed using the pooled and concentrated enzyme fractions following chromatography on mono Q. To measure peptidase activity during various stages of pregnancy, timed pregnant rats (3/time) beginning with the 13th day of pregnancy and continuing until the 4th day following parturition were used. The uteri were removed and the Triton X-100 soluble fraction was prepared as above and assayed with Suc-(Ala)3-pNA and DAPGN.

Enzyme Assays

The hydrolysis of Suc(Ala)3-pNA was measured using a modification of the procedure of Bieth et al. [25]. The enzyme sample (50 μl) was mixed in a microtitre plate with 80 μl of 0.2 M Tris-HCl buffer, pH 7.4. The reaction was initiated with the addition of 30 μl of 10 mM Suc(Ala)3-pNA and the increase in the absorbency at 405 nm was followed with a Titertek Multiscan spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme required to convert 0.113 μmole of substrate to product per minute at ambient temperature (22°C). For a negative control, the rate of hydrolysis of Suc(Ala)3-pNA was measured in the presence of the NEP inhibitor, phosphoramidon (10 μM), and the difference in the absorbance was presented as endopeptidase activity.

An NEP-specific single-stage fluorometric assay was used in parallel with the Suc(Ala)3-pNA assay. The DAPGN substrate (3.14 mg) was dissolved in 100 μl of DMSO and added to 10 ml of 0.05 Tris-HCl buffer pH 7.4. The fraction to be analyzed (50 μl) was added in a microtitre plate to 100 μl of substrate and 150 μl of Tris buffer and incubated at 37°C for up to 24 h, depending on the enzyme concentration. Phosphoramidon (10 μM) was preincubated with the sample for 10 min for a negative control. Fluorescence was measured at 562 nm with the excitation wavelength at 342 nm.

The activity of NEP was also measured using the specific two-stage fluorometric substrate (MNA) [24]. The substrate (40 μM) was dis-

solved in 0.01 M MES buffer, pH 6.5 containing 0.1% Triton X-100. The reaction was stopped after 60 min with the addition of PPAD (10 μM). Aminopeptidase M was added and the fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 425 nm.

Oxytocin was labeled by adding 20 μg of oxytocin to 0.5 μCi of ^{125}I in 100 μl of 0.05 M phosphate buffer pH 7.5 containing one iodobead (Pierce). The reaction was incubated on ice for 15 min and most of the free radiolabel separated from the substrate by centrifuging at low speed through a small spin column containing G-25 Sephadex (Isolab, Inc.). The radiolabeled oxytocin was further purified by FPLC chromatography on a C-18 column equilibrated in 0.05% TFA in water and eluted with a linear gradient of 0.05% TFA in acetonitrile, reaching 30% acetonitrile in 35 min. For the enzyme assay, approximately 50 ng of labeled oxytocin (5 μl) was added in a microfuge tube to 100 μl of the sample in Tris buffer pH 8 and incubated at 37°C for the designated time. Two percent TCA (100 μl) was added and the precipitated protein was pelleted by centrifugation. The supernatant was removed and diluted with 700 μl of 0.05% TCA in water and applied to the C-18 column and separated as described above. Activity was expressed as the percent of oxytocin converted to the cleavage product.

Elastase activity was assayed using rhodamine-elastin as described previously [26].

The pH optimum for the Suc(Ala)3-pNA and DAPGN substrates were determined using the following buffers at 0.1 molar concentrations: MES (pH 5.5–6.0), Bis-Tris (pH 6.5–7.5), and Tris (pH 8.0–9.0).

SDS-Polyacrylamide Gel Electrophoresis

SDS polyacrylamide mini gels [27] were run under nondenaturing conditions to seperate the uterus endopeptidase. Following electrophoresis, the gels were incubated for 45 min at room temperature with 0.05 M Tris buffer pH 7.4 containing 2.5% Tween 20 and then sliced into 2 mm slices. Each slice was placed into a microtitre plate well and incubated with 150 μl of 0.05 M Tris buffer pH 7.4 and either 40 μl of the Suc(Ala)3-pNA or 100 μl of DAPGN and the enzyme activity was measured following an overnight incubation at 37°C.

Isoelectric Focusing

Samples of the concentrated endopeptidase (50 μl) were applied to preformed agarose gels (LKB) covering the pH range of 3.5 to 9.5. After focusing for 2 h, 0.5 cm slices were placed in 100 μl of 0.05 M Tris buffer pH 7.4 and assayed for enzyme activity using either ^{125}I-oxytocin, Suc-(Ala)3-pNA, or DAPGN as substrates.

Immunochemistry

For immunoprecipitation studies, 100 μl of the partially purified uterine enzyme was incubated in a microfuge tube with 10 μl of antiserum to human kidney NEP, CALLA, or normal rabbit serum at room temperature for 2 h. To each sample was added 50 μl of protein A sepharose and the incubation was continued for an additional hour. The samples were then centrifuged and the amount of enzyme remaining in solution was determined using Suc(Ala)3-pNA and DAPGN substrates.

Uteri that were to be used for immunoperoxidase microscopy were excised, washed in PBS, sliced into 5 mm pieces, and immediately fixed by immersion in 1% paraformaldehyde in phosphate buffered saline at pH 7.4. After 1 h fixation, the tissues were incubated for 4 h in a solution of paraformaldehyde/O.C.T. (Miles Lab.), followed by an overnight incubation in 100% O.C.T. Blocks of uterus imbedded in 100% O.C.T. were frozen in absolute ethanol maintained at −70°C with dry ice. Cryostat sections 8 μm in thickness were placed on albumin-coated glass slides and incubated with 3% hydrogen peroxide for 20 min followed by incubation in 5% bovine serum albumin in PBS for 1 h (blocking solution). The sections were then incubated overnight at 4°C in the optimal dilution of the appropriate primary antibody. The primary antibodies used in this study were J5 (CALLA) and an irrelevant mouse IgG (MOPC 21). Antibody diluent was the blocking solution. The sections were washed with the blocking solution and then incubated for 30 min with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). All sections were rinsed again with the blocking solution followed by a rinse with 0.5 M Tris, pH 7.6. The sections were then incubated with a 0.1% diaminobenzidine in 0.5 M Tris containing a 0.3% solution of hydrogen peroxide. In addition to the irrelevant primary antibody control, some sections were incubated in diluent only. Glycerol was used to mount the

coverslips and the sections were examined and photographed with an Olympus Vanox microscope.

Uterine Contractions

Uterine strips (3 mm × 20 mm) were taken from 18 day pregnant rats and placed in an ice-cold Krebs-Ringer solution. The strips were mounted in 25 ml organ baths containing Krebs solution at 30°C and bubbled with 95% oxygen-5% carbon dioxide. The strips were allowed to stabilize for 1 h and the passive tension was adjusted to 0.5 g. Oxytocin was added at levels ranging from 10^{-11} to 10^{-7} M. The strips were washed between the addition of different oxytocin levels and allowed to return to a stable baseline. Inhibition of NEP in the uterine strips was accomplished by preincubating the strips with 10^{-8} M PPAD for 10 min prior to adding the oxytocin. Isometric contractions were measured with a Grass transducer (Ft 03), simultaneously digitized and stored in a Macintosh computer. The data was processed with Pacq-Manager software (BioPAC Systems).

RESULTS

Fractionation of the Uterus Endopeptidase

Very little activity was associated with the cell debris following homogenization and this fraction was discarded. The membrane-associated neutral endopeptidase activity was solubilized with Triton X-100 from the 100,000*g* pellet leaving less than 5% of the activity remaining in the particulate fractions.

A typical elution profile from DEAE cellulose is illustrated in Figure 1. The hydrolyses of both the DAPGN and Suc(Ala)3-pNA substrates occurred in the same fractions. The active fractions were pooled, dialyzed against 0.05M Tris buffer pH 8.0, applied to the mono Q column, and eluted with a linear salt gradient (Fig. 2). The enzyme activity eluted as a symmetrical, yet rather broad peak, with the activity against both substrates co-eluting. The active fractions were pooled and concentrated by filtration through a UM-30 membrane for use in the following studies.

Substrate Specificity and pH Optimum

The pH titration curve using the DAPGN and Suc(Ala)3-pNA substrates is illustrated in Figure 3. DAPGN gives a rather broad curve with a pH optimum at 6.5 and a shoulder at pH 7.5,

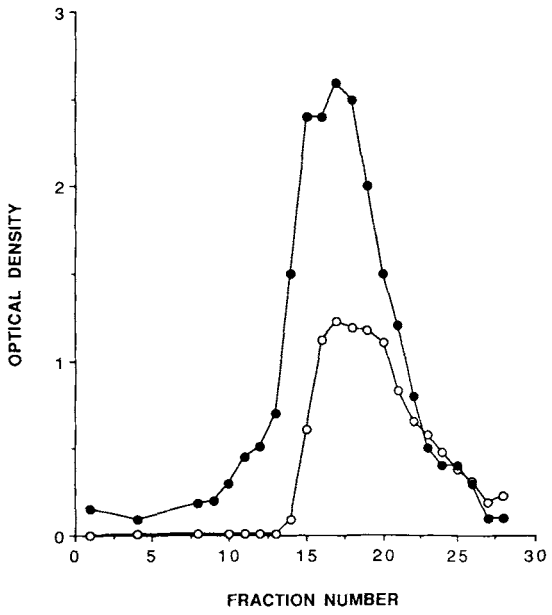

Fig. 1. Chromatographic separation of uterus endopeptidase on DEAE cellulose. The column (2.5 × 25 cm) was equilibrated with 0.02 M Tris buffer pH 8.0 and a linear gradient was developed with 100 ml of Tris buffer containing 0.5 M NaCl. Fraction size was 5 ml. Assay with Suc(Ala)3-pNA is shown in the open circles (-○-), and the DAPGN substrate (OD × 10^{-1}) is shown in the solid circles (-●-).

whereas the optimum for Suc(Ala)3-pNA is more sharply defined at pH 7.5.

The uterus endopeptidase readily hydrolyzed DAPGN, MNA, Suc(Ala)3-pNA, and oxytocin but showed no reactivity against elastin. Reactivity against all three substrates could be completely blocked with 10 μM PPAD. Incubation of oxytocin with the uterus endopeptidase resulted in hydrolysis between the Pro^7-Leu^8 bond [12,28] leaving the radiolabeled heptapeptide which can be readily separated by reverse phase chromatography (Fig. 4). Rate of hydrolysis was linear with time, even with low enzyme levels (Fig. 5). The hydrolysis of oxytocin was also dependent on enzyme concentration, and using the partially purified endopeptidase preparation there was very little evidence of other hydrolysis products. However, if whole tissue homogenates or intact uterine slices were used, the oxytocin [1–7] was further broken down into smaller, unidentified fragments.

Inhibitors and Metal Requirements

Serine protease inhibitors were completely ineffective against the partially purified enzyme (Table I). The reducing agent DTT and the chelating agent EDTA significantly reduced en-

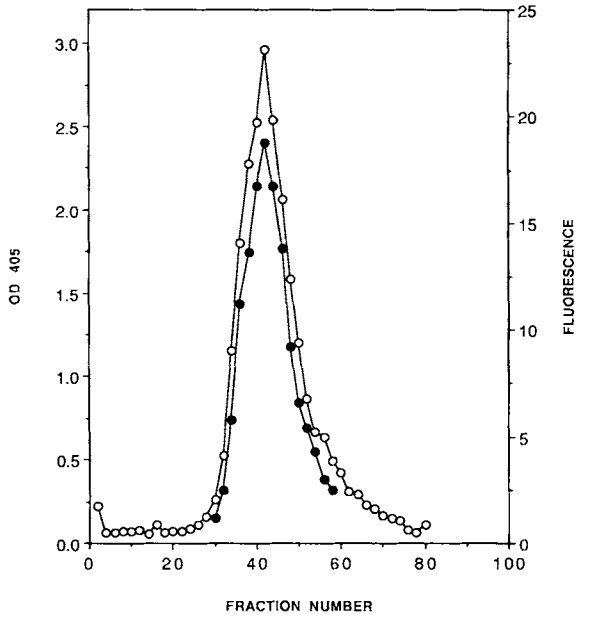

Fig. 2. Fractionation of uterus endopeptidase by FPLC on a mono Q 10/10 column. The column was equilibrated with 0.02 M Tris buffer pH 8.0 and developed with a linear gradient against 0.02 M Tris pH 8 containing 0.5 M NaCl, reaching 100% of the gradient buffer in 82 min. Fraction size was 4 ml. Assay with Suc(Ala)3-pNA is shown in the open circles (-O-) and DAPGN is shown in the solid circles (-●-).

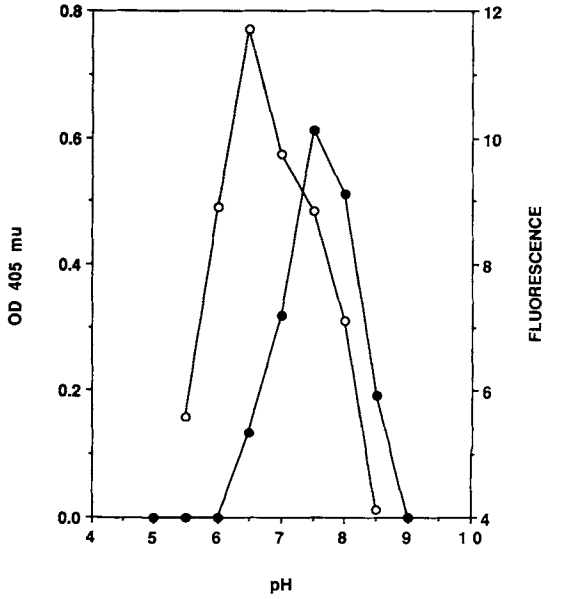

Fig. 3. pH profile of rat uterine enzyme using the synthetic substrates, Suc(Ala)3-pNA (-●-) and DAPGN (-O-). The buffers used were MES (5.0–6.5), Bis-Tris (7.0–7.5), and Tris (8.0–9.0). Reaction time was 1 h at 37°C. Values shown are the mean of two determinations.

zyme activity at levels of 10^{-4} M but the most pronounced inhibitory effect was obtained with low levels of thirophan and PPAD (10^{-6} M). Removal of metal ions from the enzyme by dialysis against 6 mM EDTA resulted in a loss of all activity against both substrates (Table II). Chelated-metal ion complexes were removed from the enzyme preparation by the use of a small Sephadex G-25 spin column. Assay of the enzyme in the presence of 0.1, 1, or 10 mM calcium did not recover appreciable activity. Addition of 1.0 mM zinc in the presence of 1.0 mM calcium resulted in 90% recovery of enzymatic activity. When 1 mM calcium was added together with 1 mM manganese or cobalt, the recovered enzyme activity was 5% and 12%, respectively.

Physical Properties

The uterine endopeptidase focused at two major bands with isoelectric points of 6.2 and 5.7 (Fig. 6). Both isomers hydrolyzed DAPGN and oxytocin as well as Suc(Ala)3-pNA.

The molecular weight estimate from enzymatic activity following SDS PAGE indicated the endopeptidase activity was associated with a high molecular weight protein with an estimated molecular mass of 90 kDa (Fig. 7). Activity against both substrates co-eluted from the gel. Immunoprecipitation of the uterus endopeptidase with antibodies to kidney NEP or CALLA

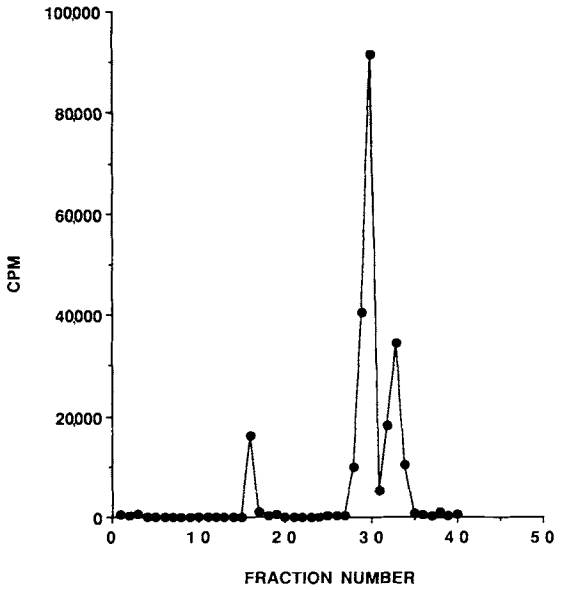

Fig. 4. Analysis of the enzymatic cleavage of ^{125}I-oxytocin by partially purified uterine endopeptidase using reverse phase chromatography on a C-18 column. Intact oxytocin elutes in fraction 33 and the product following the loss of 2 residues from the C terminal end elutes in fraction 30. Incubation time was 2 h and fraction size was 1 ml.

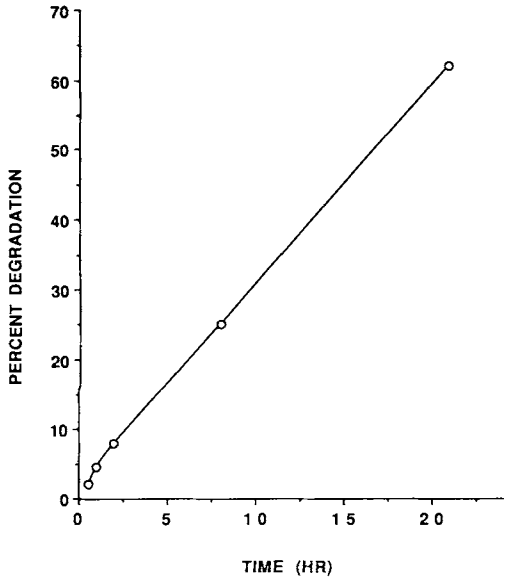

Fig. 5. ^{125}I-oxytocin degradation by partially purified uterine endopeptidase as a function of time. The oxytocin concentration was approximately 10^{-6} M in the reaction mixture and was incubated at 37°C for 2 h.

decreased the uterus endopeptidase activity with increasing amounts of antibody, while normal rabbit serum was without effect (Fig. 8). Both substrates (Suc(Ala)3-pNA and DAPGN) were inhibited to approximately the same degree when precipitated with one level of anti-kidney antiserum (Fig. 8 inset).

Immunohistochemical studies were performed with sections of uterus removed from time-pregnant rats on day 0, day 17, day 20, and 4 days postpartum. The uterus from a virgin rat stained with anti-NEP antibody showed no immunoreactivity when compared to the same uterus stained with irrelevant antibody. In the 17 day pregnant uterus, however, the NEP immunostaining was very strong (Fig. 9a,b). The endometrium was negative except for the epithelium, which stained positive. In addition, both the longitudinal and the circular smooth muscle layers of the myometrium were strongly positive. The 17 day control was negative (Fig. 9c). The 20 day pregnant uterus had already lost much of the anti-NEP reaction but was still notably positive when compared to its control. By 4 days post-partum we were unable to distinguish between sections stained with the anti-NEP antibody and the controls stained with the irrelevant antibody.

Endopeptidase Activity During Development

The endopeptidase activity of timed-pregnant rats during different stages of pregnancy is illustrated in Figure 10. Highest activity was observed between days 15 and 19, with a peak around the 17th day of pregnancy. A marked decrease in the enzyme activity was evident by day 20, 1 day prior to parturition. The enzyme levels continued to fall until the 4th day postpartum, where they reached a low value similar to a control virgin rat uterus (0.26 U). Subsequently, the study was repeated using the DAPGN substrate as well as Suc(Ala)3-pNA and an identical

TABLE I. Inhibition of Rat Uterine Endopeptidase*

Compound	Concentration (mM)	Percent inhibition	
		SAPNA	DAPGNA
SBTI	1.0 (mg/ml)	0	0
PMSF	1.0	0	0
DFP	1.0	0	0
Captopril	0.001	0	0
DTT	0.1	58	12
2-mercaptoethanol	1.0	47	nd
EDTA	1.0	93	79
	0.1	83	39
PPAD	0.001	98	80
Thirophan	0.001	97	83

*The enzyme was preincubated for 15 minutes at 37°C with each inhibitor. Two substrates were utilized: SAPNA [Suc(Ala)3-pNA)] and DAPGN. The buffers used were 0.1 M Bis-Tris pH 7.4 for the SAPNA substrate and 0.1 M MES pH 6.5 for the DAPGN substrate. Inhibition is expressed relative to a value of 100 for 4 mU on enzyme. SBTI, soybean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; DFP, di-isopropyl phosphofluoridate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; PPAD, phosphormidon; nd, not determined. Each value is the average of 2 determinations rounded off to the nearest whole No.

TABLE II. Effect of Metals on Endopeptidase Activity*

Metal (mM)	% Activity
0	<1
Ca 0.1	<1
Ca 1.0	<1
Ca 10.0	<1
Ca 0.1 + Zn 1.0	64
Ca 1.0 + Zn 1.0	92
Ca 1.0 + Mn 1.0	5
Ca 1.0 + Co 1.0	12

*The partially purified uterine enzyme preperation was dialyzed free from metals and the apoenzyme was preincubated with the metals for 10 minutes prior to incubation with the substrate, Suc(Ala)3-pNA in 0.1 M Tris buffer pH 7.5 at 37°C for 2 h.

pattern of endopeptidase activity was obtained for both substrates.

NEP Modulation of Uterine Contractions

Experiments were conducted to determine whether the uterine endopeptidase modulated the strength of the oxytocin response on uterine contraction. In Figure 11 we have plotted the force times rate of contractions of a typical uterine strip in response to various levels of oxytocin

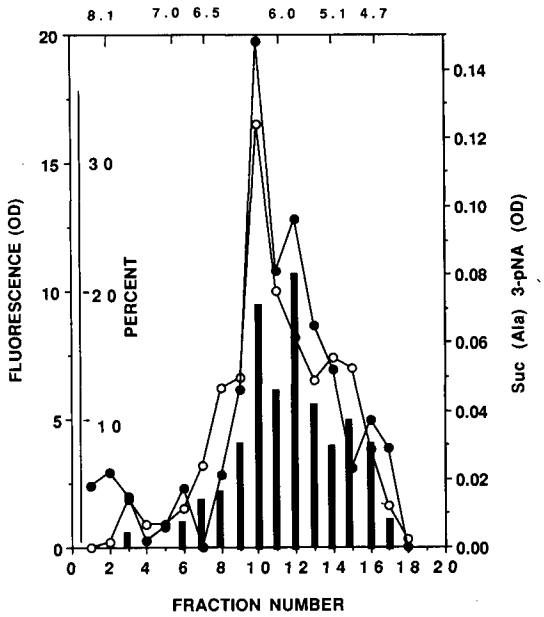

Fig. 6. Isoelectric focusing of uterine endopeptidase on a pH gradient of 3.5 to 9.5. Enzymatic activity was determined using oxytocin, Suc(Ala)3-pNA (-○-), and DAPGN (-●-). The percent degradation of oxytocin is illustrated by the solid bars. The location of isoelectric points standards are shown at the top. Each gel slice was 0.5 cm starting from the origin, pH 9.5.

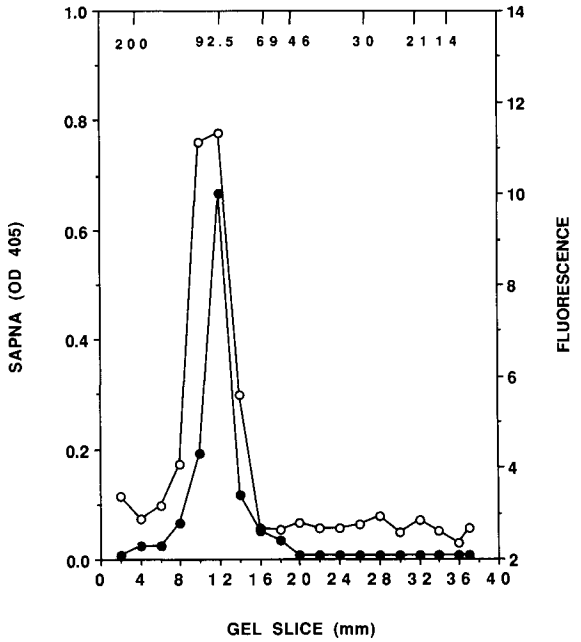

Fig. 7. SDS PAGE gels illustrating the molecular weight of uterine endopeptidase as determined by Suc(Ala)3-pNA (-●-) and DAPGN substrates (-○-). The mobility of molecular weight standards is indicated at the top.

before and after PPAD is added to the organ bath. Inhibition of endogenous NEP activity with PPAD resulted in a marked potentiation of the oxytocin stimulation as indicated by the response curve shifting to the left by an order of magnitude. The magnitude of the response varied from 10- to 100-fold between different strips and different animals.

DISCUSSION

Our original intention of isolating the elastase responsible for postpartum remodeling of the rat uterus elastin was complicated due to the presence of a specific elastase inhibitor, as well as extreme variations in enzyme levels [26]. We find now that part of this variation stems from the presence of a membrane-bound endopeptidase that cleaves the Suc(Ala)3-pNA substrate that was employed to detect the elastase in those experiments. The uterine endopeptidase that we have identified in these studies appears to be identical with NEP (E.C.3.4. 24.11) that has been described in a wide variety of mammalian tissues. The uterine enzyme is a membrane-bound metallopeptidase as evidenced by its solubility only in buffers containing detergents and an absolute requirement for zinc and perhaps

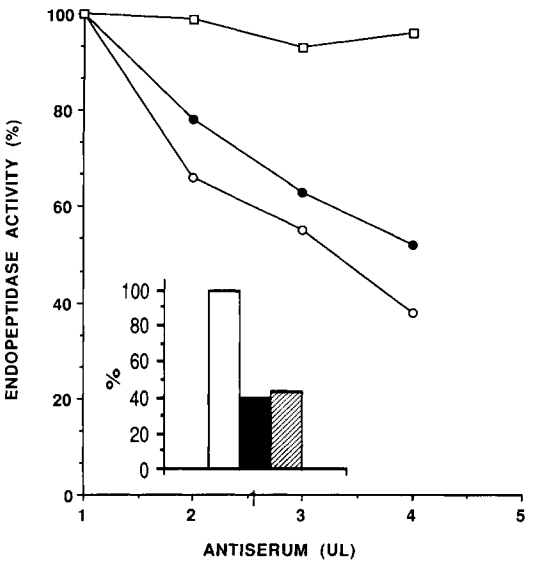

Fig. 8. Immunoprecipitation of uterine peptidase with varying amounts of anti-CALLA antibody (-●-) and anti-human kidney NEP antibody (-○-). Control nonimmune serum is indicated by squares (-□-). The substrate is Suc(Ala)3-pNA. **Inset** shows the effect of adding one level of anti-human kidney NEP antibody to the uterine endopeptidase using Suc(Ala)3-pNA (solid bar) and DAPGN (hatched bar) as the substrates. The open bar represents the control nonimmune serum. Activity is expressed as the percent of enzyme activity remaining in solution following addition of the anti-serum. Each value is the average of two determinations.

some calcium for maximal activity. Phosphoramidon and thiorphan, both recognized NEP inhibitors, were potent inhibitors of the uterine peptidase, while serine protease inhibitors are ineffective. The molecular weight of the enzyme (90 K) coincides with the molecular weight of other NEPs from different sources including the rat kidney. Three compounds that are known substrates for NEP were utilized by the uterine enzyme: DAPGN, MNA, and oxytocin. The concentration of radiolabeled oxytocin used in these assays was approximately 5×10^{-6} M which is considerably higher than the normal circulating levels of plasma oxytocin which can reach 5×10^{-11} M. Since the enzyme was not pure, meaningful kinetic studies could not be performed.

The variation in the pH optimum for enzyme activity, depending on the substrate, has been shown previously for neutral endopeptidases [24,29,30]. The dansyl fluorescence substrate has an activity maximum at pH 6.5, and the p-nitroanilide substrate is maximum around pH 7.5. The two synthetic analogs of the biological substrates probably make different contacts with the surface of the enzyme and these sites could be influenced differently by changes in pH.

The rat uterine peptidase exhibited cross-reactivity with both the polyclonal antibody to human kidney endopeptidase and the monoclonal J5 antibody (CALLA). This is not surprising since it has been shown that the J5 antigen has 100% amino acid homology with human NEP [31]. Great similarity between rat and human NEP has been shown [32] with only 6 nonconservative changes in the 742 amino acids of the rat and human enzymes.

The immunohistochemical studies indicated that during the period of strongest NEP reactivity (17–18 days pregnant) the enzyme in the myometrium was predominantly associated with the longitudinal and circular smooth muscle layers. This would be expected if NEP is actively envolved in oxytocin metabolism, since in the rat the oxytocin receptors are located on the plasma membrane of the smooth muscle cells [33]. The reason for the localization of NEP on the epithelial layer of the endometrium is not known. The difficulty we had in maintaining cellular integrity of the uterine tissue, and at the same time retaining immunological reactivity, prevented a more detailed localization of the peroxidase reaction. This is not the first evidence of NEP being associated with smooth muscle cells, as studies by Sekizawa et al. [23] have shown the presence of NEP in the smooth muscle layer of ferret trachea. In the nonpregnant rat uterus there was no evidence of the endopeptidase by immunostaining, suggesting that under normal circumstances uterine smooth muscle cells express very low levels of the enzyme. Corresponding changes in enzyme activity of the uterine NEP were observed during the course of pregnancy. Activity was expressed per uterus and not on a weight basis, since there is very little cellular hypertrophy in the uterus during pregnancy and a tremendous increase in connective tissue. Expressing activity per mg wet weight gave a 5-fold maximum increase in NEP activity. Very low prepregnancy levels were recorded which increased steadily until 2 or 3 days prior to parturition. Enzyme levels rapidly declined at that point, reaching baseline levels by 3 days after delivery. This agrees very well with immunoperoxidase staining data and is a very different pattern from that seen with collagenase, which is involved in collagen catabolism in the postpartum involuting uterus [34]. A

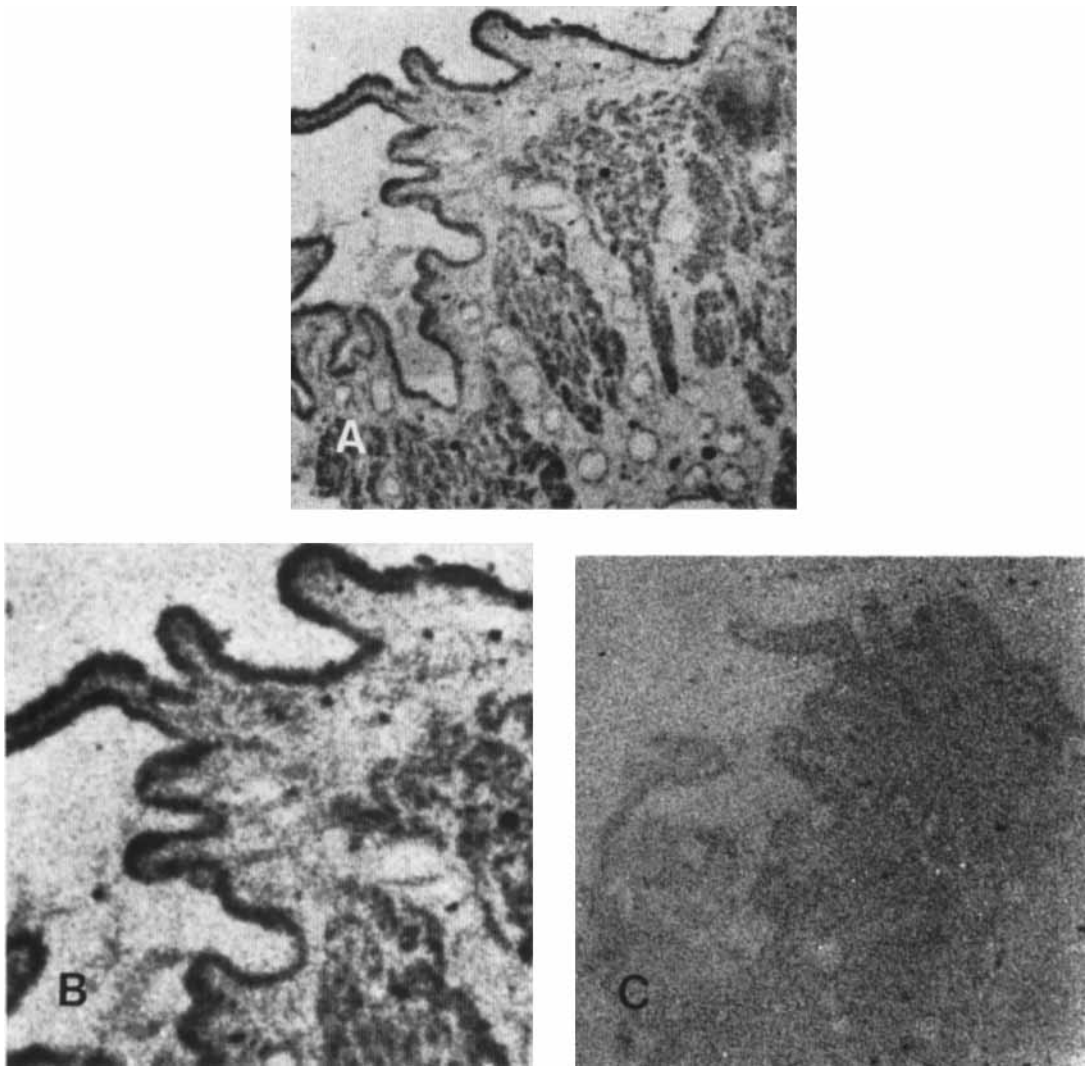

Fig. 9. Immunoperoxidase staining for NEP in rat uterus. Day 17 pregnant uterus viewed at ×112.5 magnification (**A**) and the same section at a magnification of ×245 (**B**). **C** illustrates the same 17 day pregnant uterus at ×112.5 magnification reacted with irrevalent antibody.

small molecular weight (19 K) neutral metalloproteinase has also been described in the involuting rat uterus that appears to be involved in proteoglycan degradation and like collagenase is present in highest levels postpartum [35,36]. This enzyme requires zinc and calcium yet is not inhibited by phosphoramidon, distinguishing it from NEP. Two somewhat higher molecular weight metalloproteases were extracted from the rabbit uterus [37] which were also present in highest concentrations four days postpartum. These enzymes are apparently involved in the remodeling of the uterine connective tissues following parturition.

The uterine NEP also cleaves Suc(Ala)3-pNA which was originally considered a rather specific elastase substrate [25]. We were initially worried that an aminopeptidase was being carried along with NEP during the purification procedures and could be responsible for the hydrolysis of this substrate. This is highly unlikely however, since activity against NEP substrates and Suc(Ala)3-pNA co-chromatographed during ion exchange chromatography, SDS PAGE, isoelectric focusing, and immunoprecipitation. Hydrolysis of Suc(Ala)3-pNMA by nonelastolytic enzymes has been reported by others in rheumatoid synovial fluid [38], human bile [39], and

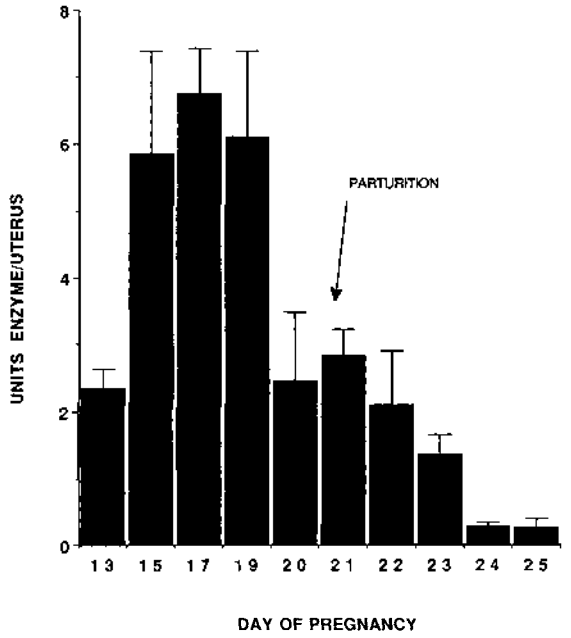

Fig. 10. Activity of the uterus endopeptidase at various times during pregnancy. Activity is expressed as total units of activity/uterus using Suc(Ala)3-pNA as the substrate. Each value is the mean of three uteri with the standard deviation.

serum [40]. Two metalloproteases partially purified from rabbit myometrium and, distinct from elastase, have been reported to hydrolyze Suc-(Ala)3-pNA [37]. Suc(Ala)3-pNA is a synthetic substrate analogous to the hydrophobic region of the signal sequence on the precursors of secreted polypeptides. Based on the concept that endopeptidases and signal peptidases can be identical with each other [41], it has been shown that a partially purified rat kidney endopeptidase which hydrolyzes Suc(Ala)3-pNA into Suc(Ala)2 and Ala-pNA [42,43] has the ability to cleave the prehuman placental lactogen into its mature form. Similar Suc(Ala)3-pNA hydrolysis was reported [44] using microsomal kidney endopeptidase. Under our conditions the uterine NEP was able to produce a direct color reaction with Suc(Ala)3-pNA and an aminopeptidase was not required to liberate p-NA. We have also observed that when purified human kidney NEP was incubated with Suc(Ala)3-pNA the substrate was rapidly cleaved, liberating p-NA without requiring a second stage amine peptidase.

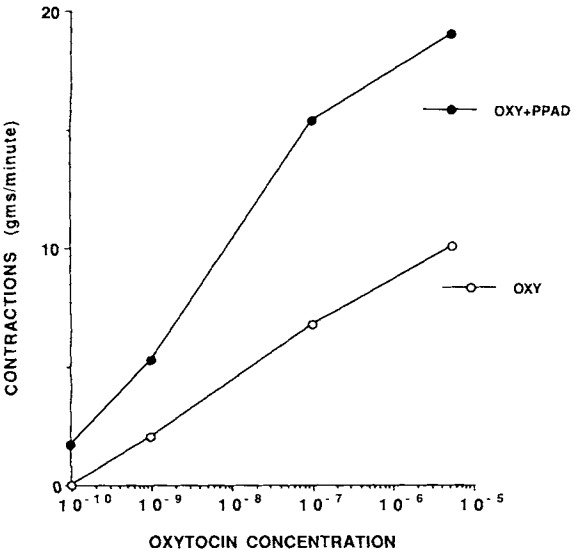

Fig. 11. Effect of PPAD on oxytocin-induced contractions of a 17 day pregnant rat uterine strip. The control strip is indicated with open circles (-○-) and the same strip receiving 10^{-6} M PPAD with the solid circles (-●-).

The role of the uterine NEP that we have identified is unknown, but probably does not have a direct effect on connective tissue remodeling. This is indicated for one thing by the rapid decline in enzyme levels several days prior to parturition. This would suggest a regulatory role, such as inactivating biological peptides, which has been the proposed role of most neutral endopeptidase that have been characterized so far. A logical peptide substrate would be oxytocin, since we have shown that this hormone functions as a substrate for uterine NEP and is known to have such an important role in uterine physiology. It was evident from the *in vitro* studies that inhibition of uterine NEP resulted in a tissue that was capable of contracting in response to much lower levels of agonist. Our studies also indicated that uterine NEP reaches maximum levels just as oxytocin receptors are reported to be rapidly increasing. The marked fall in NEP activity shortly before parturition may allow the receptors to bind sufficient oxytocin to initiate uterine contractions. It is possible that the increase in NEP is crucial for maintaining reduced oxytocin levels on smooth muscle cell membranes, thereby preventing premature uterine contractions during the critical period prior to parturition. The role of estrogen or progesterone in regulating NEP during gestation is not known. The increase in NEP between days 13 and 18, and the rapid decline just prior to parturition, seem to coincide well with the changing titer of serum progesterone. It is also during this period, prior to parturition, that increasing estradiol levels result in increasing numbers of oxytocin receptors [45].

A recent report strengthens the concept of an important role for metalloenzymes in regulating uterine contractions [46]. These authors found that rats exposed to high levels of lead exhibited marked increases in spontaneous uterine contractions and related this observation to increased frequency of preterm labor in areas polluted with heavy metals.

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