Kininase II–Type Enzymes

Their Putative Role in Muscle Energy Metabolism

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Because of the importance of bradykinin in improving heart function in some conditions or in enhancing glucose uptake by skeletal muscle, we investigated kininases in these tissues. In P_3 fraction of the heart and skeletal muscles, angiotensin I–converting enzyme (ACE) and neutral endopeptidase 24.11 (NEP) are the major kininases, as determined first with specific substrates and second with bradykinin. ACE activity was highest in guinea pig heart (2.7 ± 0.07 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$) but decreased in other species in this order: dog atrium, rat heart, dog ventricle, and human atrium. The specific activity of NEP was lower: 0.45 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$ in cultured neonatal cardiac myocytes and varying between 0.12 and 0.05 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$ in human, dog, rat, and guinea pig heart. In the skeletal muscle P_3, ACE was most active in guinea pig and rat (1.2 and 1.1 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$, respectively) but less so in dog (0.09 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$). NEP activity was higher in dog P_3 (0.28 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$) but lower in rat and guinea pig (0.19 and 0.1 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$, respectively). Continuous density gradient centrifugation enriched NEP activity in dog and rat (from 0.3 to 1.0 and 0.49 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$, respectively). Immunoprecipitation with antiserum to purified NEP proved the specificity of the rat enzyme. Bradykinin (0.1 mmol/l) was inactivated in the presence and absence of inhibitors by rat skeletal muscle NEP, as measured by high-performance liquid chromatography. Here, 36% of the activity was caused by NEP and 19% by ACE. In radioimmunoassay (bradykinin 10 nmol/l), 46 and 55% of kininase in rat and dog skeletal muscle P_3, respectively, was due to ACE; 36 and 28%, respectively, was due to NEP. Aside from these enzymes, an aminopeptidase in rat P_3 also inactivates bradykinin. Thus, in conclusion, heart and skeletal muscle membranes contain kininase II–type enzymes, but their activity depends on the species. *Diabetes* 45 (Suppl. 1):S34–S37, 1996

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ACE, angiotensin I–converting enzyme; HPLC, high-performance liquid chromatography; M199, medium 199; NEP, neutral endopeptidase 24.11; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

Bradykinin and kallidin (Lys[1]-bradykinin) have a variety of actions besides affecting blood pressure; these actions have been the subject of many investigations through the years. For example, kinins antagonize smooth muscle proliferation (1). In the heart, angiotensin I–converting enzyme (ACE; kininase II) inhibitors can prolong the half-life of bradykinin (2–5) and potentiate its local actions in vitro on the isolated rat and guinea pig atria (6) (R.D Minshall, E.G. Erdös, S. Vogel, et al., unpublished data). In skeletal muscles, bradykinin is a factor that enhances the effect of insulin on glucose uptake, according to Dietze and colleagues (7,8).

To establish the functions of bradykinin in an organ, first the enzymes that terminate its actions should be characterized. When this characterization is known, then the application of specific inhibitors can prolong the half-life of kinins. Historically, the name kininase II was given to a renal and plasma enzyme that cleaved the COOH-terminal dipeptide of bradykinin (9); its identity with the ACE was shown somewhat later (10). Then, a structurally different enzyme, neutral endopeptidase 24.11 (NEP; enkephalinase, neprilysin, CD10, CALLA), was found to cleave the same bond in bradykinin in vitro and in vivo as ACE (11) and to release Phe[8]-Arg[9]; thus it acted as a kininase II–type enzyme. Of course, these enzymes have more activity than that because they hydrolyze different peptide bonds in other substrates (12).

The goal of the present investigations was to establish the activity of kininase II–type enzymes that break down bradykinin in tissues, where kinins can affect energy metabolism.

RESEARCH DESIGN AND METHODS

Laboratory animals. Hearts and hind-limb skeletal muscles (semimembranosus/semitendinosus, gastrocnemius, and biceps femoris) from adult male rats, dogs, and female guinea pigs were obtained after the animals were killed with halothane and rapidly exsanguinated. Neonatal rats (2–3 days old) were obtained from pregnant rats. The protocol for this project was approved by the University of Illinois Animal Care and Use Committee.

Chemicals, reagents, and enzymes. Unless otherwise stated, the chemicals, reagents, and enzymes were purchased from Sigma (St. Louis, MO).

Tissues. The tissue was placed in ice-cold 0.25 mol/l sucrose, 20 mmol/l Tris buffer, pH 7.5, homogenized by using a Polytron (three times for 30 s each), and filtered through four layers of cheesecloth. The filtrate was then homogenized and subsequently used for differential or density gradient centrifugation. In differential centrifugation, the homogenates were fractionated at 1,000*g* for 10 min (P_1), 10,000*g* for 25 min (P_2), and 100,000*g* for 60 min (P_3) at 4°C.

For density gradient separation, the homogenate first was centrifuged at 100,000*g* for 60 min. The pellet was then resuspended in 2 ml sucrose (0.25 mol/l), layered over a 20–50% continuous sucrose gradient, and

centrifuged at 100,000*g* for 1 h. The fractions (10–13/1.5 ml) were collected, diluted 1:10, and centrifuged at 100,000*g* for 1 h. The pellets were suspended in 500 μl of the original buffer and either immediately assayed or stored at −70°C.

Culture of neonatal rat cardiac myocytes and skeletal muscle myoblasts. Neonatal rats were anesthetized with halothane and killed by cervical dislocation. Myocytes were isolated by the aseptic technique according to the method of Sadoshima with minor modifications (13). The hearts were rapidly removed and placed in ice-cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing 40 U/ml sodium heparin, 4 mmol/l glucose, and 25 mmol/l HEPES. The hearts from eight litters (~100 pups) were washed three times with PBS, and the atria and aortas were removed and discarded. The ventricles were minced with scissors into 1- to 3-mm^3 fragments, which were then washed with PBS by gently stirring in a 37°C water-jacketed Erlenmeyer flask for 10 min. The tissue was then enzymatically digested five times for 10 min each with 10 ml PBS containing 0.1% trypsin, 0.1% collagenase (type IV), 15 μg/ml deoxyribonuclease I, and 1% chicken serum. The liberated cells were collected by centrifugation at 200*g* and resuspended in PBS containing 20% calf serum. The pooled washed cells were preplated in T-75 cell culture flasks in medium 199 (M199), a supplemented medium. The nonadherent cells were harvested after they were incubated at 37°C for 60 min in a humidified incubator with 5% CO_2 in air. The cells were counted and resuspended in M199-supplemented media containing 0.1 mmol/l 5-bromo-2′-deoxyuridine (to inhibit cell division and thereby control other cell growth). The suspension was then aliquoted onto 0.1% gelatin-coated plates for evaluation. Myocyte monolayers typically began vigorously contracting 24–48 h after plating.

Skeletal muscle myoblasts (L8 cell line) were obtained from American Type Culture Collection (Rockville, MD) and grown in culture as recommended. In brief, the myoblasts were grown to confluency on 10- or 15-cm diameter dishes precoated with 0.1% gelatin in a 4:1 mixture of Dulbecco's modified Eagle's medium and M199 containing 10% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Confluent cultures of cardiac myocytes and L8 skeletal muscle myoblasts were washed three times with PBS and scraped from the dish. The collected cells were immediately pelleted by centrifugation at 800*g* × 10 min, and the pellet was sonicated to disrupt the cells.

Enzyme assays. NEP activity (14–16) was measured in a fluorometric assay with Glut-Ala-Ala-Phe-4-methoxy-2-naphthylamine from Enzyme Systems (Livermore, CA) as substrate. Phosphoramidon (1 μmol/l) was used as a specific NEP inhibitor. The protein concentration was determined according to Bradford (17) with bovine serum albumin (fraction V) as standard.

Na^+-K^+-ATPase activity was used as a marker for plasma membranes. The activity was determined according to the method of Penney (18) with ouabain as a specific ATPase inhibitor. ACE activity was measured by a radiometric assay with [^{3}H]hippuryl-glycyl-glycine substrate from Amersham (Arlington Heights, IL) (19). Of its inhibitors, enalaprilat was a gift from Merck (West Point, PA) and captopril was a gift from Bristol-Myers Squibb (Princeton, NJ).

Immunoprecipitation was carried out by using antiserum to purified rat NEP and protein A (20). Antiserum was donated by Dr. L.B. Hersh of the University of Kentucky.

High-performance liquid chromatography (HPLC). To study bradykinin metabolism, membrane fractions (P_3) from rat skeletal muscle were incubated at 37°C in the 2-*N*-morpholinoethanesulfonic acid buffer, pH 6.5, and 0.25 mmol/l bradykinin in the presence or absence of the NEP inhibitor phosphoramidon (1 μmol/l) or the ACE inhibitor captopril for varying lengths of time. The reaction was stopped with 20 μmol/l 10% trifluoroacetic acid, samples were centrifuged at 16,000*g* for 3 min, and supernatant fractions were used for HPLC analysis in a Waters Automated Gradient System. Peptide hydrolysis products were separated on a Bondapak C_{18} reverse-phase column in 40 min, with a 10–40% increasing linear gradient of acetonitrile in 0.05% trifluoroacetic acid in water, and were detected at 214 nm. The amount of bradykinin hydrolyzed that was inhibited by phosphoramidon (1 μmol/l) was taken as NEP activity; similarly, ACE activity was inhibited by captopril (1 μmol/l).

Radioimmunoassay (RIA) of bradykinin. Skeletal muscle membrane-enriched fractions from the dog and rat hydrolyzed bradykinin (10 nmol/l) in the presence or absence of the following: 0.1 μmol/l enalaprilat (an ACE inhibitor), 0.1 μmol/l phosphoramidon (an NEP inhibitor), or 0.1 mmol/l bacitracin (an aminopeptidase inhibitor). In brief, 20 or 50 μl of muscle membranes (P_3) was incubated at 37°C with 10 nmol/l bradykinin in 1 ml of 0.1 mol/l Tris buffer, pH 7.4, containing 0.1 mol/l NaCl. Aliquots (100 μl) from the reaction were removed, usually after 15 min, and hydrolysis was stopped with 4 ml ethanol. After the ethanol was evaporated under a stream of nitrogen, the sediment was resuspended in 1 ml of 0.1 mol/l Tris buffer, pH 7.4, containing 0.2% gelatin and 0.1% neomycin sulfate. Sample aliquots (10–100 μl) were then measured in duplicate for bradykinin content using a RIA as described previously (21), with [^{125}I-Tyr^8]bradykinin from Du Pont-NEN (Boston, MA).

RESULTS

Heart

ACE. The apparent concentration of ACE in the heart tissue varies greatly according to the species. In agreement with data published previously (22,23), the activity in the human and rat heart was on the low side compared with that in other organs such as the lung. In the rat heart, the activity was 278 (range 252–304) nmol · h^{-1} · mg $protein^{-1}$ in the high-speed membrane-enriched P_3 fraction, and in a single specimen it was 158 nmol · h^{-1} · mg $protein^{-1}$ in the human cadaver heart atrium. The values were higher in the dog heart: 583 ± 196 and 177 ± 54 nmol · h^{-1} · mg $protein^{-1}$ (mean ± SE) in the left atrium and ventricle, respectively (n = 3 each). The activity in the ventricle of guinea pig heart was the highest compared with other species: 2,698 ± 74 nmol · h^{-1} · mg $protein^{-1}$ (n = 3).

NEP. In general, the values for relative NEP activity were closer and lower in the heart samples tested than those for ACE. The value in the P_3 of guinea pig ventricle, expressed as nmol · h^{-1} · mg $protein^{-1}$, was 73 ± 28 (n = 5); in the rat heart homogenate, it was 54 (range 40–68); and in neonatal rat cardiac myocytes after 7 days in culture, it was 454 (range 372–535). The corresponding NEP values in the dog heart (n = 3) were 93 ± 9, 103 ± 30, 79 ± 9, and 64 ± 13 nmol · h^{-1} · mg $protein^{-1}$ for left atrium, right atrium, left ventricle, and right ventricle, respectively. In the single human cadaver heart, P_3 from atrial tissue had activities of 117 and 87 nmol · h^{-1} · mg $protein^{-1}$ in the left ventricle.

Skeletal muscle

ACE. The ACE activity was very high in the P_3 fraction of guinea pig and rat skeletal muscle: 1,171 ± 153 and 1,120 ± 514 nmol · h^{-1} · mg $protein^{-1}$, respectively (n = 3 each). In contrast, homogenized embryonic rat skeletal muscle cells, L8 myoblasts, were less active (175 ± 40 nmol · h^{-1} · mg $protein^{-1}$ [n = 4]). Dog skeletal muscle P_3 fraction had low activity (91 ± 26 nmol · h^{-1} · mg $protein^{-1}$ [n = 9]), but when the 100,000*g* pellet of the homogenized dog muscle was layered on top of a sucrose gradient, ACE in the top three fractions was more concentrated. ACE activity from dog skeletal muscle in fraction II was 300 ± 61 nmol · h^{-1} · mg $protein^{-1}$ (n = 5).

NEP. With the exception of the dog, the apparent NEP activity concentration was lower in the P_3 fraction of the skeletal muscles of laboratory animals than that of ACE. This value was 284 ± 33 (n = 11), 187 ± 49 (n = 9), and 102 (range 92–112) nmol · h^{-1} · mg $protein^{-1}$ in the dog, rat, and guinea pig, respectively. After the density gradient centrifugation, the activity in fraction II was 302 ± 51 (n = 5) and 486 ± 146 (n = 4) nmol · h^{-1} · mg $protein^{-1}$ in the dog and rat, respectively. When freshly prepared dog muscle preparations were tested on the same day instead of being stored frozen overnight, the NEP activity was 1,000 ± 108 nmol · h^{-1} · mg $protein^{-1}$ (n = 3). In one experiment, fractions I–III from rat P_3, collected on the top after continuous density gradient centrifugation, had 84% of the total Na^+-K^+-

TABLE 1
Inhibition of bradykinin metabolism by skeletal muscle membranes established by RIA

Inhibitor	Concentration (mol/l)	Rat (% inhibition)	Dog (% inhibition)
Enalaprilat	10^{-7}	64	55
Phosphoramidon	10^{-7}	36	28
Bacitracin	10^{-4}	24	5

Bradykinin concentration: 10 nmol/l. Results represent the means of two experiments done in duplicate.

ATPase activity (considered to be a membrane marker). The same fractions had high ACE and NEP levels as well. Rat skeletal muscle cells, L8 myoblasts, also contained NEP, and in the crude homogenate of the cells this value was 33 ± 3 nmol · h^{-1} · mg $protein^{-1}$ (n = 5).

Immunoprecipitation. Antiserum to purified rat renal NEP and protein A (20) precipitated 76 and 71% of the activity, respectively, in the suspended P_3 fraction of rat hind-limb muscle, showing that authentic NEP is present in the membrane-enriched fraction of the homogenized skeletal muscle.

Bradykinin metabolism

HPLC. To evaluate the contributions of various membrane peptidases that cleave bradykinin, specific inhibitors at concentrations of 1 μmol/l were used. Rat skeletal muscle P_3 in two different preparations cleaved bradykinin (100 μmol/l) at rates of 137 and 242 nmol · h^{-1} · mg $protein^{-1}$. Captopril inhibited the reaction 19% (range 17–20%), phosphoramidon 36% (range 30–42%), and the combination of captopril and phosphoramidon 50% (range 49–51%).

RIA. To establish the contributions of enzymes of skeletal muscle membranes to bradykinin inactivation at a bradykinin concentration nearer to a physiological one, inhibition studies were repeated by using an RIA for assaying bradykinin. Table 1 summarizes the results. In both rat and dog skeletal muscle membrane fractions, the ACE inhibitor enalaprilat inhibited over half the activity. The inhibitor of NEP, phosphoramidon, was less active, inhibiting 36% in rat and 28% in dog P_3 fraction. Bacitracin, an inhibitor of aminopeptidases, blocked 24% of bradykinin inactivation in the rat but only 5% of that by dog P_3 fraction.

DISCUSSION

The results of these preliminary studies showed, somewhat unexpectedly, that membrane-enriched fractions of skeletal and heart muscles contain two kininase II–type enzymes. ACE activity was especially high in skeletal muscles obtained from rat and guinea pig. In the heart, the relative ACE activity was also higher than NEP activity; the highest value was obtained with the guinea pig heart tissues. The apparent NEP concentration in the skeletal muscles, as established in the assay systems used, was generally lower than that of ACE, with the exception of the dog.

Although both ACE and NEP are zinc metallopeptidases, containing two histidines and one glutamic acid to bind the metal cofactor (24–26), the two proteins have no significant homology. They are plasma membrane–bound enzymes, but ACE is inserted into the membrane at its COOH-terminal end by a hydrophobic anchor peptide (24), whereas NEP is attached to the bilayer through its NH_2-terminal signal peptide (26). ACE has a single peptide chain with two active domains, which are called, because of their position in the peptide chain, the N and C domains (24,27,28). The two domains, although they have a high degree of homology around the active sites, differ in chloride sensitivity, inhibition pattern, and some of the substrates cleaved (24). Very recently, we reported that a variant ACE, consisting of a single active N domain, can be released intact by proteases and, because of its structure, appears to be more resistant to enzymatic cleavage than the C domain of either ACE or NEP (29,30).

Both NEP and ACE cleave bradykinin at the Pro^7-Phe^8 bond, but in general, they differ in substrate specificity. ACE cleaves COOH-terminal dipeptides and protected NH_2- or COOH-terminal tripeptides (12,31), whereas NEP hydrolyzes peptides at the NH_2 end of hydrophobic amino acids (32). Both are widely distributed in various organs and cell types and are uniformly present in the microvilli of epithelial brush borders (31,32). In addition, NEP is identical with CD10, the common acute lymphoblastic leukemia antigen (CALLA), a marker for lymphoblasts (33,34). Although both enzymes cleave a variety of peptides in vitro, their action is more restricted in vivo. ACE cleaves angiotensin I and bradykinin in vivo and, at some sites, enkephalins and substance P as well (12,31). Bradykinin, atrial natriuretic peptide, substance P, and enkephalins (12,32) are the most frequently mentioned substrates of NEP in vivo or in situ. For example, in a recent study, the contribution of bradykinin to myocardial blood flow in rat was enhanced by administering the NEP inhibitor phosphoramidon (35). Aside from normal tissues and lymphoblasts, some malignant tumor cells also express NEP in high concentrations (16,36).

The NEP activity measured in the membrane preparation of rat was due to the presence of authentic NEP; this was shown by immunoprecipitation by specific anti-NEP antiserum and inhibition by phosphoramidon. It is unlikely that the majority of either ACE or NEP activity originated from blood vessels or nerve endings because myoblast cells of rat also contained both enzymes.

Bradykinin hydrolysis by skeletal muscle membranes was inhibited somewhat differently when determined at relatively high (0.1 mmol/l) or low (10 nmol/l) concentrations using HPLC or RIA. At a lower bradykinin concentration, enalaprilat inhibited more than phosphoramidon. The differences in the ratios of the kininase activities of the enzymes may be due to differences in the K_m values of the substrate of bradykinin. For example, bradykinin with human ACE has a much lower K_m (31) than with NEP (14).

A putative aminopeptidase as a kininase was detected in the rat. Aminopeptidase inactivates bradykinin by cleaving Arg^1, as it was first detected by using erythrocytes as source of the enzyme (37). This enzyme, by now called aminopeptidase P, is present in some organs, such as the rat lung, in high concentrations (38); however, in skeletal muscles, under our conditions of assay, it plays only a secondary role compared with ACE and NEP.

The tissues studied may release kinins locally, because they contain kallikrein. For example, rat skeletal muscle liberates a kallikrein identical to the urinary kallikrein (39). Contraction of cat skeletal muscle releases a kinin, and ACE inhibition or lowering of the pH increases the kinin concentration in venous effluent of cat muscle (40). Kinins so liberated can affect glucose metabolism, in part by releasing prostaglandins (7).

In conclusion, both cardiac and skeletal muscle mem-

branes contain kininases that can terminate the actions of bradykinin, thus determining its metabolic functions. The apparent concentration of kininases, together with the ratios of ACE versus NEP activities, varies a great deal and depends on the species tested. Thus, in studying bradykinin's paracrine actions (for example, in enhancing glucose uptake), it is advisable to use a combination of inhibitors of the enzymes that are most active in human or in the laboratory animals being investigated.

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