Bradykinin metabolism in the postinfarcted rat heart: role of ACE and neutral endopeptidase 24.11

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Bradykinin metabolism in the postinfarcted rat heart: role of ACE and neutral endopeptidase 24.11. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1769–H1779, 1999.—The role of angiotensin-converting enzyme (ACE) and neutral endopeptidase 24.11 (NEP) in the degradation of bradykinin (BK) has been studied in the infarcted and hypertrophied rat heart. Myocardial infarction (MI) was induced in rats by left coronary artery ligation. Animals were killed, and hearts were sampled 1, 4, and 35 days post-MI. BK metabolism was assessed by incubating synthetic BK with heart membranes from sham hearts and infarcted (scar) and noninfarcted regions of infarcted hearts. The half-life (t1/2) of BK showed significant differences among the three types of tissue at 4 days [sham heart (114 ± 7 s) > noninfarcted region (85 ± 4 s) > infarcted region (28 ± 2 s)] and 35 days post-MI [sham heart (143 ± 6 s) = noninfarcted region (137 ± 9 s) > infarcted region (55 ± 4 s)]. No difference was observed at 1 day post-MI. The participation of ACE and NEP in the metabolism of BK was delayed by preincubation of the membrane preparations with enalaprilat, an ACE inhibitor, and omapatrilat, a vasopeptidase inhibitor that acts by combined inhibition of NEP and ACE. Enalaprilat significantly prevented the rapid degradation of BK in every tissue type and at every sampling time. Moreover, omapatrilat significantly increased the t1/2 of BK compared with enalaprilat in every tissue type and at every sampling time. These results demonstrate that experimental MI followed by left ventricular dysfunction significantly modifies the metabolism of exogenous BK by heart membranes. ACE and NEP participate in the degradation of BK since both enalaprilat and omapatrilat have potentiating effects on the t1/2 of BK.

ACE inhibitors have proven to prolong the survival of postinfarction patients with left ventricular dysfunction (25). Several mechanisms have been proposed to explain this effect, one of which is the prevention of adverse ventricular remodeling postinfarction. Although the inhibition of the production of angiotensin II plays a role in this effect, there is mounting evidence that at least part of the beneficial effects of ACE inhibitors postinfarction are the result of the inhibition of bradykinin (BK) metabolism, which in turn increases nitric oxide and prostaglandin levels (18). Paradoxically, the cardiac metabolism of BK in the acute, subacute, and chronic postinfarction periods has never been measured. Also, the effects of ACE inhibitors on the cardiac metabolism of BK in these settings have never been evaluated.

It has been proposed that a potentiation of the effect of BK by ACE inhibitors in the postinfarction period could be an important mechanism by which ACE inhibitors prevent scar expansion postinfarction (19). The effect of ACE inhibitors on BK metabolism in the area of the necrosis and scar appears then to be particularly important to evaluate. Although ACE inhibitors reduce cardiac collagen production in the postinfarction period (19, 37), which in turn should promote scar expansion, ACE inhibitors might also reduce scar expansion, thus contributing to their beneficial effects. The effect of ACE inhibitors on BK metabolism in the remaining viable myocardium also appears to be important, because there is mounting evidence that BK plays an essential role in preventing chronic left ventricular dilatation postinfarction (19).

BK is the prototype of kinins, a family of powerful bioactive autacoids released from their precursors called kininogens (4, 27). BK exerts its pharmacological activity by activating the B2 receptors, which are widely distributed throughout mammalian tissues (4, 13). In the past several years, there has been a renewed interest in BK because of its cardiovascular effects, consisting mainly of vasodilatory and anti-proliferative effects (18). BK is a short-lived peptide because of its rapid metabolism by different peptidases. In vitro, many enzymes are susceptible to metabolize BK. These enzymes belong mainly to metallopeptidases but also to serine peptidases and proteases, astacin-like metallo-peptidases, and cathepsins (12). The nature of the enzymes involved in the metabolism of BK in vivo and their relative importance depend on the biological medium considered. Recently, we have shown that ACE (peptidyl dipeptidase A, kininase II; EC 3.4.15.1) is the main enzyme responsible for the metabolism of BK, not only in rat and human serum (9) but also at the coronary bed level (11) and in cardiac membrane preparations of normal hearts of different animal species (5). First, ACE metabolizes BK into BK-(1—7) and, in a second step, BK-(1—7) is degraded into BK-(1—5) (12). Besides ACE, other enzymes metabolize BK. Kininase I, a generic name for different plasma and cell mem-
brane carboxypeptidases, is responsible for the metabolism of BK into its active metabolite des-Arg²-BK (12, 32). In the serum as well as in the heart, the kininase I pathway is a minor metabolic pathway of BK, which becomes evident only when ACE is inhibited (5, 9). Finally, we have also demonstrated that neutral endopeptidase 24.11 (NEP, nephrilysin; EC 3.4.24.11) plays an important role in the degradation of BK at the endothelial level. Like the kininase I pathway, the NEP pathway becomes evident only if ACE has been inhibited previously (11). NEP metabolizes BK into BK-(1—7), and then BK-(1—7) is cleaved into BK-(1—4) (12).

A new class of compounds, the vasopeptidase inhibitors, has recently been developed that not only inhibits the activity of ACE but also inhibits the activity of NEP (28, 35). In addition to their protective effect on natriuretic peptides (28, 31), these dual ACE/NEP inhibitors would be expected to increase BK levels more than ACE inhibitors alone. These drugs are now being investigated in clinical trials for use in hypertension and congestive heart failure, and their use in the early and late postinfarction period is being considered. Given the indirect evidence for a role of BK in the cardioprotective effects of ACE inhibitors, it would appear essential to evaluate the effects of these new ACE/NEP inhibitors on the metabolism of BK in both healthy and pathological cardiac tissue. Omapatrilat (28) is a member of this new class of therapeutic agents (vasopeptidase inhibitors), and, in this study, its effects on BK metabolism in healthy and postinfarction (early and late) cardiac tissue were evaluated and compared with the effects of an ACE inhibitor. The experimental model used is clinically relevant, that of the postinfarction rat (26).

**MATERIALS AND METHODS**

**Drugs, Peptides, and Reagents**

BK and des-Arg²-BK were purchased from Peninsula Laboratories (Belmont, CA). The ACE inhibitor enalaprilat (IC₅₀ = 4 nM) was obtained from the pharmacy of the Institute of Cardiology of Montreal (Montreal, QC). The vasopeptidase inhibitor omapatrilat, which acts by combined inhibition of the ACE (IC₅₀ = 5 nM) and NEP (IC₅₀ = 9 nM), was provided for research purposes by Bristol-Myers Squibb (Princeton, NJ). Ketamine hydrochloride was obtained from Rogar/STB (Montreal); xylazine was from Bayer Canada (Etobicoke, ON); buprenorphine hydrochloride was from Reckitt Colman Pharmaceuticals (Richmond, VA); and heparin was from Leo Laboratories Canada (Ajax, ON). Halothane was manufactured by Halocarbon Laboratories (Riveredge, NJ). The 5'-nucleotidase 15.5 reagent, bovine serum albumin, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), hippuryl-l-histidyl-l-leucine, p-nitrophenyl phosphate, and phosphoramidon were from Sigma-Aldrich (Mississauga, ON). Aminopeptidase M and alkaline phosphatase-labeled anti-digoxigenin Fab fragments were from Boehringer Mannheim (Laval-des-Rapides, QC). Goat IgG and biotinylated goat anti-rabbit IgG were from Santa Cruz (Santa Cruz, CA), and the aminopeptidase complex was from Vector Laboratories (Burlingame, CA). Ethanol of HPLC grade was purchased from American Chemicals (Montreal). The biocinchonic acid protein assay procedure and heptafluorobutyric anhydride (HFB A) were from Pierce (Rockford, IL). 7-амино-4-methyl coumarin (AMC) and succinyl-alanyl-alanyl-phenyl-alanyl-AMC (Suc-Ala-Ala-Phe-AMC) were from Bachendorf (Switzerland), Acetonitrile (HPLC grade) and all other chemicals of analytic grade were obtained from Fisher Scientific (Montreal). All BK amino-truncated peptides were synthesized using solid-phase synthesis by Dr. G. Drapeau (Centre de Recherche, Hôtel-Dieu de Québec; see Ref. 10).

**Surgery and Animal Death**

All of the animal experiments followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Ethics Committee of the Institute of Cardiology of Montreal. Myocardial infarction (MI) was induced in 200- to 250-g male Wistar rats (Charles River, St. Constant, QC) through ligation of the left descending coronary artery as described earlier (3, 26). The rats were anesthetized with 3% halothane. During surgery, they were artificially ventilated with humidified room air supplied with oxygen, and the halothane concentration was gradually decreased to 1%. A Harvard Rodent ventilator (Harvard Apparatus, South Natick, MA) set at 2 ml, 70 strokes/min, and a Fluotec 3 halothane vaporizer (Cyrane) were used for this procedure. The heart was quickly exteriorized through a left-sided thoracotomy, and the left descending coronary artery was ligated ~2 mm from its origin. The heart was then replaced in its normal position in the thorax, and the incision was closed with a Mikron wound clip applicator (Clay Adams) after the crest was gently pressed to expel air from the cavity to avoid a pneumothorax. Once awakened after surgery, the rats were injected with 0.01–0.02 mg/kg buprenorphine to reduce the pain during recovery.

On days 1, 4, and 35 after the surgery, the surviving rats were killed to obtain the heart. The rats were anesthetized using an intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Before death, during the same anesthesia, an electrocardiogram (ECG) was performed, and the intraventricular pressures were measured by inserting a Millar Mikro-Tip Catheter Transducer (Millar Instruments, Houston, TX) with a pressure sensor at the tip in both the jugular vein and carotid artery and advancing it in the right and left ventricle, respectively. The ECG and pressures were recorded on a Gould 2,600S recorder (Gould, Cleveland, OH). The rats were classified as having shammed, small, medium, or large MIs according to the ECG readings taken before death. The presence of a Q wave in the lead I and lead aVL derivative signaled an MI. The heights of the R waves in the V1, V2, and V5 derivatives were summed, and their total value was used as a criterion for further classification. A value inferior to 0.6 mV indicated a large infarction, a value >1.0 mV indicated a small infarction, and a value between 0.6 and 0.85 mV indicated a medium infarction. Shamm hearts and hearts with small infarctions were pooled and compared with the second group consisting of medium and large infarctions. Before the heart was removed, 1,000 U/kg of heparin were injected in the jugular vein. The heart was then excised and carefully perfused with a 37°C saline solution to remove all blood. Once this was completed, the infarcted hearts were dissected into two pieces: the infarcted area of the left ventricular wall (scar) and the remainder of the heart (the noninfarcted area). In this model, the infarcted area is not clearly visible 24 h postinfarction and always involves the free wall of the left ventricle, and the septum is never implicated in the infarct heart. Hearts sampled 24 h postinfarction had the left ventricular wall dissected free of the septum. The left ventricular wall was then considered the infarcted area and the septum as viable myocardium. All
portions of the hearts were frozen at –80°C until used for biochemical investigations.

Preparation of the Total Heart Membrane Suspensions

To assess the metabolism of BK by enzymes located on cardiac cell membranes, membranes were extracted from the hearts following a procedure previously used by Kinoshiba et al. (15) to study the metabolism of angiotensin I and more recently by Blais et al. (5) to assess the metabolism of BK in the normal rat heart. The noninfarcted and infarcted portions of each heart were thawed, weighed, and then cut into 3- to 4-mm pieces. These pieces were placed in a 50 mM Tris·HCl buffer, pH 7.4, at 4°C (10 ml/g of tissue) and were homogenized with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON) at setting 8 for 15 s. The homogenate of each heart were thawed, weighed, and then cut into 3- to 4-mm pieces. These pieces were placed in a 50 mM Tris·HCl buffer, pH 7.4, at 4°C (10 ml/g of tissue) and were homogenized with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON) at setting 8 for 15 s. The homogenate was centrifuged at 40,000 g for 20 min at 4°C. After centrifugation, the tissue pellet consisting of membranes was separated from the cytosolic supernatant. The membranes were resuspended in a 50 mM Tris·HCl buffer, pH 7.4, containing 100 mM NaCl, at 4°C. A Wheaton Potter-Elvehjem tissue grinder (Fisher Scientific, Pittsburgh, PA), driven by a T-line motorized stirrer (Talboys Engineering, Emerson, NJ) turning at setting 8 for 60 s, was used for this procedure. During resuspension, the fibrous tissue was discarded from the infarcted pieces. The resulting membrane suspension was assayed for its 5'-nucleotidase activity (1). The protein concentration of the membrane suspensions was determined by the bicinchoninic acid method using bovine serum albumin as the standard.

BK Metabolism

Incubation of BK with the total heart membrane suspensions. The membrane suspensions were diluted with a 50 mM Tris·HCl buffer, pH 7.4, containing 100 mM NaCl to obtain a 5 mg/ml protein concentration. The membrane suspensions coming from the infarcted scar pieces were pooled two by two on the basis of an equal protein concentration to obtain a sufficient suspension volume for the incubation procedure. The metabolic profile of BK was measured at 37°C in the same conditions previously used for normal hearts (5). Briefly, 10 µl of saline containing 500 ng of synthetic BK were added to 990 µl of the heart membrane suspension. The final concentration of BK in this suspension was 471 nM. After various incubation periods at 37°C, ranging between 2 and 20 min, the reaction was stopped by precipitating the membrane proteins through the addition of cold (4°C) ethanol at a final concentration of 80% vol/vol. In two sets of parallel experiments, and before the synthetic BK was added, the membrane suspensions were preincubated for 15 min at 37°C either with enalaprilat or with omapatrilat. The inhibitor concentrations were, respectively, 130 nM for enalaprilat and 510 nM for omapatrilat. The precipitated samples were centrifuged 15 min at 4°C and 2,000 g. The clear supernatant containing BK and its metabolites was evaporated to dryness in a Speed Vac Concentrator (Savant, Farmingdale, NY). The residues were stored at –80°C until quantification of the residual BK was performed.

Quantification of BK. Immunoreactive BK was quantified in the residues of the evaporated ethanolic extracts using a highly specific enzyme immunoassay developed in our laboratory (8). This assay used highly specific polyclonal rabbit IgG raised against the carboxy-terminal end of BK, digoxigenin-labeled peptide as tracer, and alkaline phosphatase-labeled anti-digoxigenin Fab fragments with the substrate 5-nitrophenyl phosphate to detect and quantify the immune complexes. Each sample was measured in triplicate. Typical calibration curves were characterized by half-maximal saturation values of 0.78 pmol/ml. This method was precise and accurate.

Kinetic parameter analysis. BK hydrolysis rate constant (k) was evaluated with the first-order equation \[ [BK] = [BK]_0 \times e^{-kt} \] where [BK] is the concentration of BK at a given time and [BK]_0 is [BK] at time (t) = 0. The BK half-life (t_1/2) was represented as t_1/2 = ln(2)/k (20). The different t_1/2 values were expressed for 1 mg of protein.

ACE relative activity was estimated from k with (k^p) or without (k^p) enalaprilat using the equation %ACE = 100 × ((1 – k^-1/k^-1)). NEP relative activity was estimated by using k with (k^p) or without (k^p) omapatrilat and by subtracting the ACE relative activity, following the equation %NEP = [100 × ((1 – k^-1/k^-1))] – %ACE. This subtraction assumed that both enalaprilat and omapatrilat inhibit ACE to the same extent. In fact, the concentration chosen for each inhibitor was above its inhibitory constant so that both inhibitors fully inhibited ACE.

Separation and Identification of BK Amino-Truncated Metabolites

To assess if the immunoreactive BK measured at the t_1/2 corresponds to the native amino-terminal peptide, immunograms after HPLC were designed for each incubation condition (5). Briefly, in the conditions described above, BK was incubated with the heart membrane preparations for a period corresponding to the calculated t_1/2. Incubations were performed either in the presence of enalaprilat, in the presence of omapatrilat, or without inhibitor. After precipitation of proteins with cold ethanol and centrifugation, the ethanolic extracts were separated in two parts. After evaporation, the first part was used for the quantification of immunoreactive BK. The second part was dissolved in 0.025% HFBA (vol/vol) in distilled water before HPLC separation. An HPLC system (Waters Associates, Milford, MA) consisting of a model 600 Multisolvent Delivery System and a model 484 Tunable Absorbance Detector was employed for HPLC analysis. BK and four products of the amino-terminal enzymatic cleavage of BK were separated on a reverse-phase column (Vydac C18 5 µm, 4.6 × 250 mm; Hesperia, CA) at a constant flow rate of 0.7 ml/min using a 45-min linear gradient from 80% solvent A to 65% solvent A-35% solvent B. Solvent A was 0.025% HFBA (vol/vol) in distilled water, and solvent B was 0.025% HFBA (vol/vol) in 90% acetonitrile-10% distilled water. The column effluent was monitored continuously at 214 nm. Fractions of 0.7 ml were collected, evaporated to dryness in a Speed Vac Concentrator, and then frozen at –80°C until immunoreactivity profile determination. BK and metabolites were identified by comparing their retention times with those of reference peptides.

Measurement of ACE and NEP Activity

The membrane suspensions used for BK metabolism were solubilized in 8 mM CHAPS (6). ACE activity was measured using the method of Cushman and Cheung (7), and NEP activity was measured using the method of Nortier et al. (22). Each sample was quantified in duplicate for both assays. ACE activity was expressed as picomoles of hippuric acid per minute per milligram of protein, and NEP activity was expressed as picomoles of AMC per minute per milligram of protein.

Immunohistochemistry of NEP and ACE Expression

Expression of NEP and ACE was determined immunohistochemically. The hearts of the rats were fixed in a 10% Formalin-phosphate-buffered solution. The hearts were em-
bedded in paraffin, sectioned (6-µm width) with a microtome along the cross section of the specimen at the midpoint between the apex and the base of the heart, and applied on glass slides. The sections were deparaffinized in xylene and ethanol baths, and endogenous peroxidase activity was quenched in a methanol-hydrogen peroxide solution. A nonspecific antibody binding was prevented by preincubating the tissues with a 5% goat serum treatment. Sections were exposed to the primary antibodies of rabbit polyclonal anti-rat NEP IgG (1:500 dilution) raised and purified in our laboratory (P. Crine, unpublished observations) and monoclonal anti-rat ACE IgM (1:500 dilution) kindly provided by Dr. R. Auerbach (University of Wisconsin, Madison, WI) and were used according to Dr. Auerbach’s recommendations (2). A purified nonspecific goat IgG (1:500 dilution; Santa Cruz) was used as a primary negative control. The secondary antibodies were biotinylated goat anti-rabbit IgG (1:400 dilution; Vector Laboratories) and goat anti-mouse IgM (1:100 dilution; Vector Laboratories). Revelation of bound antibodies was achieved with an avidin-peroxidase complex (Vector Laboratories), and antibodies were counterstained in Gill’s hematoxylin solution. NEP and ACE expression (brown staining) was evaluated for each segment by using a dedicated 3CCD video microscope adapted to a customized software.

Statistical Analysis

All data are expressed as means ± SE. Different models of ANOVA were used to analyze the data. Two-way factorial analysis with days and tissues, sham and noninfarcted, or infarcted and noninfarcted were used with Scheffé’s contrasts. A two-way analysis with the factor days and a repeated factor, tissues (infarcted and noninfarcted), was also used with paired t-tests using Bonferroni inequality. Finally, in some cases, a one-way analysis with appropriate contrasts was used. In view of the multiple analysis performed on the data, the significance level was fixed at 1%. Because most of the ANOVA showed interaction, only the results of the analysis of contrasts are reported.

RESULTS

Hemodynamic Characteristics

Hearts with an MI had a significant increase in left ventricular end-diastolic pressure (LVEDP). On day 1, MI hearts had an LVEDP of 9 ± 2 mmHg, on day 4 an LVEDP of 9 ± 1 mmHg, and on day 35 an LVEDP of 7 ± 2 mmHg; all of these pressures were significant (P < 0.01) compared with sham-operated controls (day 1: 1 ± 2 mmHg; day 4: 3 ± 1 mmHg; day 35: 1 ± 2 mmHg, respectively).

Effect of MI on the Metabolism of BK

The t₁/₂ of exogenous BK measured in cardiac membranes from the sham hearts was consistent throughout the 5-wk study period, at between 114 ± 7 to 143 ± 6 s (Fig. 1). The t₁/₂ in the viable portion of the infarcted hearts was similar to that of sham at 1 day postinfarction (107 ± 8 s, n = 21; and 123 ± 6 s, n = 10, respectively) but was significantly decreased by 4 days (85 ± 4 s, n = 20 in MI vs. 114 ± 7 s, n = 11 in sham; P < 0.01). BK was thus metabolized 1.3 times faster in the viable portion of the MI hearts compared with sham hearts. However, by 35 days postinfarction, the t₁/₂ had returned to levels similar to those of the sham group (137 ± 9 s, n = 14 and 143 ± 6 s, n = 10, respectively). The infarcted portion of the heart had no difference in BK t₁/₂ compared with sham or viable myocardium of MI hearts 1 day postinfarction. By 4 days postinfarction, BK t₁/₂ was decreased markedly to 28 ± 2 s (n = 10), levels that were ∼25% of those of sham and 33% of those of the noninfarcted portion of the same hearts (P < 0.01). By 35 days postinfarction, BK t₁/₂ in the infarcted region had nearly doubled (55 ± 4 s; n = 7) compared with 4 days postinfarction (28 ± 2 s, n = 10; P < 0.01) but remained less than one-half of that of the noninfarcted portion of the same hearts and of sham hearts (P < 0.01). BK was thus metabolized 2.5 times faster in the infarcted region at 35 days compared with sham or the viable myocardium of these hearts.

Effect of Enalaprilat and Omapatrilat on BK t₁/₂

The preincubation of the membrane preparations with enalaprilat increased BK t₁/₂ significantly (P < 0.01) in every tissue type and at every sampling time after surgery (Fig. 2). Omapatrilat increased BK t₁/₂ significantly even more than did enalaprilat (P < 0.01; Fig. 2). In sham hearts, the effect of both inhibitors remained unchanged over time (Fig. 3). However, in the infarcted and noninfarcted portions of the MI hearts, the effects of both inhibitors increased over time (P < 0.01; Fig. 3). In infarcted pieces, the effect of both inhibitors on BK t₁/₂ was the greatest at 35 days postinfarction (163 ± 8 s for enalaprilat and 199 ± 14 s for omapatrilat; n = 7) compared with day 1 (36 ± 8 and 68 ± 7 s, respectively; n = 11; P < 0.01) or day 4 (35 ± 4 and 43 ± 4 s, respectively; n = 10; P < 0.01) after infarction. In the noninfarcted pieces, an increase in effect of both inhibitors on BK t₁/₂ occurred earlier, at 4 days postinfarction (126 ± 7 s for enalaprilat and 185 ± 12 s for omapatrilat; n = 20) vs. 1 day postinfarction (63 ± 10 and 97 ± 14 s, respectively; n = 21; P < 0.01). By 35 days postinfarction, the increase in effect of
enalaprilat on BK $t_{1/2}$ in noninfarcted portions of MI hearts was no longer significant compared with day 1. However, the increase in effect of omapatrilat on BK $t_{1/2}$ compared with day 1 ($97 \pm 14$ s, $n = 21$) was maintained up to 35 days ($168 \pm 14$ s, $n = 14$; $P < 0.01$).

Relative Contribution of ACE and NEP on BK Metabolism

The effect of enalaprilat on the BK $t_{1/2}$ was used as a measure of the involvement of ACE in the metabolism of BK. The difference between the effects of omapatrilat and enalaprilat was used as a reflection of the involvement of NEP in this metabolism (Fig. 4). In sham hearts, the additive effect of NEP on BK metabolism remained unchanged throughout the study period. In the noninfarcted pieces of MI hearts, the involvement of NEP increased gradually over time such that it was significantly higher ($P < 0.01$) by 35 days postinfarction ($69 \pm 7$ s, $n = 14$) compared with 1 day postinfarction ($34 \pm 6$ s, $n = 21$). In infarcted pieces, NEP inhibition significantly prolonged the $t_{1/2}$ of BK 1 day postinfarction ($32 \pm 5$ s, $n = 11$); however, by 4 days postinfarction, this was no longer true ($8 \pm 1$ s, $n = 10$). The effect of NEP inhibition on BK $t_{1/2}$ was reestablished by 35 days postinfarction ($37 \pm 9$ s, $n = 7$).

If one adjusts the additive effects of ACE and NEP inhibition to BK $t_{1/2}$ without drugs, the relative contribution of ACE and NEP on BK metabolism in the various tissues at any given point in time can be calculated (Table 1). The relative importance of ACE and NEP on the metabolism of BK varies according to the nature of the tissue and over time. When compared with NEP, ACE played a greater role in the metabolism of BK in every tissue and at every time point evaluated. In the sham hearts, the relative participation of ACE remained unchanged over time. In the infarcted pieces, the relative participation of ACE to the metabolism of BK was similar at day 1 postinfarction compared with sham; however, it rose steadily over time, more than tripling between days 1 and 35 ($P < 0.01$) such that, at day 35, it was two times that of sham ($P < 0.01$). In the noninfarcted pieces, the relative participation of ACE was similar to that of sham on day 1 postinfarction. It then rose from day 1 to day 4 postinfarction ($P < 0.01$) and returned to basal levels by day 35. The relative importance of NEP in sham or noninfarcted pieces of myocardium was similar at day 1 postinfarction and did not vary over time. In the infarcted pieces, the relative participation of NEP was similar at day 1 postinfarction compared with the other two tissues, but it decreased over time ($P < 0.01$) such that it was lower than the sham hearts by days 4 and 35 postinfarction ($P < 0.01$).

Evolution of ACE and NEP Enzymatic Activities Over Time

Both ACE and NEP enzymatic activity exhibited important variations according to the nature of the tissue and its sampling time (Fig. 5). In the sham hearts, ACE activity remained unchanged over time from $164 \pm 8$ pmol · min$^{-1}$ · mg protein$^{-1}$ ($n = 9$) on day 1 to...
to 156 ± 6 pmol min⁻¹·mg protein⁻¹ (n = 9) on day 35. NEP activity was similar at day 1 and day 4 (24.9 ± 1.9 pmol min⁻¹·mg protein⁻¹, n = 10 and 25.4 ± 1.9 pmol min⁻¹·mg protein⁻¹, n = 11, respectively), and it decreased to 17.1 ± 1.8 pmol min⁻¹·mg protein⁻¹ (n = 9) on day 35, but it did not reach statistical significance (P > 0.01).

In the infarcted pieces from MI hearts, the ACE activity was similar on day 1 (120 ± 11 pmol min⁻¹·mg protein⁻¹, n = 15) compared with sham. It then increased progressively over time such that by 4 days postinfarction the highest values for any tissue were recorded (357 ± 19 pmol min⁻¹·mg protein⁻¹, n = 8; P < 0.01). By 35 days postinfarction, ACE activity (338 ± 15 pmol min⁻¹·mg protein⁻¹, n = 8) remained similar to day 4. The activity of NEP was higher in the infarcted pieces compared with sham on the three time points (P < 0.01). Moreover, NEP activity was similar after 1 and 4 days, but it decreased at day 35 (41.7 ± 3.7 pmol min⁻¹·mg protein⁻¹, n = 7) compared with day 4 (70.7 ± 5.7 pmol min⁻¹·mg protein⁻¹, n = 4; P < 0.01).

In the noninfarcted pieces from MI hearts, ACE activity was higher on day 1 than in sham (P < 0.01), but it remained unchanged over time from 205 ± 17 pmol min⁻¹·mg protein⁻¹ (n = 20) on day 1 to 143 ± 15 pmol min⁻¹·mg protein⁻¹ (n = 14) on day 35. NEP activity was lower at day 4 (31.2 ± 1.9 pmol min⁻¹·mg protein⁻¹, n = 17) and day 35 (30.8 ± 4.2 pmol min⁻¹·mg protein⁻¹, n = 10) compared with day 1 (45.4 ± 2.7 pmol min⁻¹·mg protein⁻¹, n = 17; P < 0.01).

Amino-Terminal Metabolism of BK in the Heart Membrane Preparations

HPLC analysis performed with residual BK after incubation with membranes coming from 4-day infarcted pieces indicated that the immunoreactive BK measured at the t½, in the absence and in the presence of enalaprilat or omapatrilat (Fig. 6), corresponds to the native peptide. In fact, >95% of the detected immunoreactive BK was native BK.

Table 1. Relative participation of ACE and NEP in the hydrolysis of exogenous BK incubated with different heart pieces

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<th>Day 1</th>
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<td>Sham</td>
<td>42.1 ± 3.4</td>
<td>49.2 ± 3.0</td>
<td>34.0 ± 3.5</td>
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<td>9.2 ± 2.8</td>
<td>12.5 ± 1.6</td>
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<td>Infarcted</td>
<td>22.4 ± 4.9</td>
<td>54.3 ± 3.1*</td>
<td>74.8 ± 1.7*</td>
<td>Day 1</td>
<td>15.1 ± 3.0</td>
<td>5.3 ± 1.1*</td>
<td>3.5 ± 0.8*</td>
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<td>Noninfarcted</td>
<td>39.9 ± 4.2</td>
<td>59.4 ± 1.8*</td>
<td>41.5 ± 3.0†</td>
<td></td>
<td>9.0 ± 1.6</td>
<td>8.4 ± 1.0</td>
<td>12.5 ± 1.3</td>
</tr>
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Values are means ± SE. ACE, angiotensin-converting enzyme; NEP, neutral endopeptidase; BK, bradykinin. *P < 0.01 vs. day 1; †P < 0.01 vs. day 4.
active BK corresponds to the non-amino-truncated B₂ agonist. Similar recovery values were measured for sham hearts and the noninfarcted parts of the infarcted hearts.

Protein Expression of NEP and ACE in Normal and Hypertrophied Hearts

In the absence of cardiac injury in normal hearts, we could not detect the expression of NEP immunohistochemically with an antibody that specifically recognizes the expression of this protein (Fig. 7D). We also analyzed the expression of NEP on the hearts of rats with an MI at 35 days postprocedure. In the noninfarcted region of the heart, we could not detect the expression of NEP (Fig. 7E); however, in the infarcted area of the heart, we observed a clear and localized expression of NEP in some of the cardiomyocytes (Fig. 7F). In each study, a purified nonspecific goat IgG was used as a primary negative control, and in each case we could not detect any positive staining (Fig. 7, A–C). In a similar manner, we also evaluated the expression of ACE. In all hearts (normal and infarcted), we observed a ubiquitous expression of ACE on cardiomyocytes and endothelial cells (Fig. 8, D–F). In each study, a purified nonspecific goat IgG was used as a primary negative control, and in each case we could not detect any positive staining (Fig. 8, A–C).

DISCUSSION

In this study, we demonstrated for the first time an alteration in the metabolism of BK by cardiac membranes during the acute, subacute, and chronic postinfarction periods. These alterations varied according to the time postinfarction and the tissue (infarcted or noninfarcted) being studied. Moreover, we showed clearly that an ACE inhibitor, and even more so the new vasopeptidase inhibitor omapatrilat, a dual ACE/NEP inhibitor, prevents the degradation of this vasodilatory and antiproliferative peptide at each time point and in each tissue. Again, these effects varied according to the tissue and the time point postinfarction, and, at each time of our experimental protocol, the effect of omapatrilat was significantly more important than that of enalaprilat on the degradation of BK not only by sham and noninfarcted tissues but also by the scar.

The rat postinfarction model has been used widely to study the pathophysiological processes involved in acute and chronic postinfarction left ventricular remodeling (23, 24). In the early postinfarction, there is an acute inflammatory process in the area of the scar that involves cardiac necrosis and infiltration by numerous inflammatory cell types. This inflammatory process intensifies within the next few days and then gradually disappears as a fibrotic scar develops. The noninfarcted area is under significant hemodynamic stress and intense neurohumoral stimulation during the acute and subacute postinfarction periods (23, 24, 29). Later in postinfarction, hemodynamic stress and neurohumoral activation abate in those tissues in which beneficial compensatory ventricular remodeling occurs. A number of studies suggest that BK may play a critical role in promoting beneficial remodeling and that the cardioprotective effects of ACE inhibitors in this setting are largely due to their prolongation of BK $t_{1/2}$ (17, 19).

In those tissues where adverse ventricular remodeling occurs or MI size is very large, hemodynamic stress and neurohumoral activation abate in those tissues in which beneficial compensatory ventricular remodeling occurs. A number of studies suggest that BK may play a critical role in promoting beneficial remodeling and that the cardioprotective effects of ACE inhibitors in this setting are largely due to their prolongation of BK $t_{1/2}$ (17, 19).

In the heart, endogenous kinins can be produced locally during an MI from two mechanisms. First, because heart possesses an independent kallikrein-kinin system (21), BK can originate from the tissue itself. Second, BK can also originate from plasma. Indeed, in a previous clinical study, we have shown a decrease of prekallikrein and plasma kininogens in the context of MI.
postinfarction period in humans (A. Adam, unpublished observation). In this paper, we applied to the postinfarction period the same experimental in vitro metabolic approach that we had used to define the metabolism of BK in the normal heart of different animal species (5). We clearly showed that MI significantly shortens the $t_{1/2}$ of BK. In the infarcted zone, this decrease only becomes evident 4 days postinfarction, a time at which the infarcted zone is scarring and the acute inflammatory response is still important. It also persists until at least 35 days postinfarction, a time at which the inflammatory response has largely abated. In the noninfarcted portions of the MI hearts, BK $t_{1/2}$ also only decreases by 4 days postinfarction and returns to the level of sham by 35 days postinfarction, a period during which hypertrophy of the remaining viable heart is well established.

Because BK was incubated with membrane preparations and $t_{1/2}$ was expressed per milligram of total membrane proteins, the metabolic changes documented cannot be attributed to an artefactual dilution factor or to soluble intracellular or extracellular enzymes modified during and after MI but rather to enzyme activities modified at the plasmatic cell membrane level. Membrane preparations used in this protocol are representative of normal cardiac muscle because they are composed of at least 75% cardiomyocyte membranes (36). However, we cannot exclude that membranes prepared from infarcted and hypertrophied samples contain membranes of cells other than cardiomyocytes. That is particularly true 4 days post-MI when infiltrating inflammatory cells (neutrophils, macrophages, and fibroblasts) are at their peak in the necrotic zone (33) and could release cytokines that could upregulate the enzyme activity in an autocrine or paracrine way (16).

Preincubation of membranes with an ACE inhibitor significantly increased the $t_{1/2}$ of BK in the different tissue samples evaluated. The potentiating effect of an ACE inhibitor was similar in sham and noninfarcted tissues. In the infarcted portion of the heart, however, the effect of ACE inhibitor on the BK $t_{1/2}$ was less important 1 and 4 days post-MI. Four days post-MI, BK $t_{1/2}$ was markedly decreased in these membranes, and preincubation with enalaprilat did not succeed in normalizing $t_{1/2}$. In these infarcted pieces, the effect of the ACE inhibitor was mainly evident 35 days postinfarction, a time at which hypertrophy of the remaining viable myocardium has developed. At that time, ACE inhibitor increased BK $t_{1/2}$ fourfold. These results are consistent with those of Johnston et al. (14), who, using a quantitative autoradiographic method, showed an increased ACE expression in rat heart 4 wk postinfarction. This increase was particularly important in the fibrous scar tissue of the infarcted area. However, when the relative participation of ACE in the metabolism of BK is calculated from the $t_{1/2}$ values in the presence and in the absence of ACE inhibitor, the values of both
kinetic parameters show clearly that, although important in the metabolism of BK, ACE is not the unique enzyme responsible for the inactivation of BK, and the relative participation of these other enzymes varies according to the tissue and timing postinfarction. Differences in the activation of other enzymes (5, 15) would thus explain the only incomplete correlation between ACE activity and the relative contribution of ACE inhibitor on BK metabolism.

Among the other enzymes potentially responsible for the degradation of BK, NEP must be considered as a serious candidate. Recently, in defining the metabolism of BK by the coronary vascular bed of normal rat heart, we have shown that coperfusion of retrothiorphan, a highly specific NEP inhibitor, with BK did not modify its metabolism. However, when perfused in the presence of enalaprilat, retrothiorphan significantly increased the recovery of BK by 36% when compared with enalaprilat alone (11). These in vitro results with normal coronary endothelium show clearly that, when ACE is inhibited, NEP takes over and plays an important role in the metabolism of BK. The behavior of both enzymes can be explained by their respective affinity for BK. These results constitute the experimental basis

Fig. 8. ACE expression on representative cross sections of control and infarcted hearts (35 days postinfarction) in absence (A–C) and presence (D–F) of primary antibodies in control heart (A and D), infarcted heart (noninfarcted region; B and E), and infarcted region (scarred; C and F). Positive ACE expression is revealed by brown staining on cardiomyocytes (filled arrow) and endothelial cells (open arrow). Magnification, ×600. Immunohistochemistry protocol is described in MATERIALS AND METHODS.

Fig. 9. Synoptic representation of relative contribution of ACE + NEP to the metabolism of BK in heart membranes from sham hearts (A) and infarcted zone (B) and noninfarcted zone (C) of infarcted hearts.
for the simultaneous inhibition of both enzymes by a vasopeptidase inhibitor that exhibits similar inhibitory potency for both ACE (IC50 = 5 nM) and NEP (IC50 = 9 nM). Our results obtained using cardiac membranes in normal and pathological hearts not only confirms but also extends these previous observations at the normal coronary endothelium level.

When heart membranes were preincubated with omapatrilat, an increase in BK t½ occurred that was greater than that measured in the presence of the ACE inhibitor. Although omapatrilat reduced the metabolism of BK in all tissues evaluated, the simultaneous inhibition of NEP and ACE was particularly effective in prolonging BK t½ in the infarcted portion of the heart 1 day post-MI. At that time, the relative participation of NEP in the degradation of BK averaged that of ACE. This observation suggests that the use of omapatrilat may be particularly beneficial early in the postinfarction period, when BK appears to be involved in the regulation of the acute inflammatory reaction that stabilizes the scar. Although still significant, the protective effect of omapatrilat on BK was less important in the injured zone at 4 and 35 days postinfarction and in the noninjured zone, as well as in sham hearts, at all time points measured. The superiority of omapatrilat over a simple ACE inhibitor in reducing the degradation of BK could explain some of the experimental data recently obtained in vivo, which could involve the effects of endogenous BK.

In the rat, inhibition of NEP protected the heart against ischemia-reperfusion injury, as evidenced by a significant reduction of MI size and a tendency toward a reduction of reperfusion arrhythmias (38). These effects of NEP inhibitor were blocked by the B2-receptor-activator (21), our observations for exogenous BK may be transposed to endogenous BK. However, in that case, membrane enzymes and also circulating enzymes released from the membranes and cytosol during ischemia must be considered.

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