Neutral Endopeptidase in the Heart
Neutral Endopeptidase Inhibition Prevents Isoproterenol-Induced Myocardial Hypoperfusion in Rats by Reducing Bradykinin Degradation

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Abstract Peptide mediators may play a role in the control of myocardial perfusion. We found immunohistochemical evidence of the peptide-degrading enzyme neutral endopeptidase (NEP) in cultured rat myocytes. Therefore, we examined the effect of an NEP inhibitor, phosphoramidon, on myocardial perfusion in rats after (1) stimulating sensory nerves with capsaicin and (2) inducing myocardial hypoperfusion with isoproterenol, with or without pretreatment with selective antagonists of the substance P (NK1) and bradykinin (B2) receptors. Three to five sequential determinations of myocardial blood flow were made in anesthetized rats by injecting 100,000 radionuclide-labeled microspheres suspended in 70% dextrose into the left ventricle. Phosphoramidon doubled coronary blood flow in response to a dose of capsaicin that was ineffective in the absence of the inhibitor. Isoproterenol (50 mg/kg IP) caused an immediate fall in blood pressure and coronary blood flow; after 20 minutes, flow had returned to normal but pressure was still subnormal. Administration of phosphoramidon reduced the recovery of blood pressure but greatly increased coronary blood flow. These changes were not altered by a substance P NK1 receptor blocker but were completely abolished by a selective bradykinin B2 receptor blocker. Our data indicate that (1) NEP is present in the rat myocardium, (2) sensory nerve-induced coronary vasodilation is markedly potentiated by NEP inhibition, (3) isoproterenol-induced myocardial hypoperfusion is prevented by NEP inhibition, and (4) this effect of NEP inhibition is due to reduced degradation of bradykinin. (Circ Res. 1994;75:770-779.)

Key Words • myocardial blood flow • myocardial ischemia • enkephalinase • EC 3.4.24.11 • substance P

Peptide mediators play a role in the complex neurohumoral control of myocardial perfusion. Among vasodilator peptides, substance P and bradykinin have been studied extensively. Substance P coexists in the terminal varicosities of unmyelinated and small myelinated sensory nerves with other neuropeptides (neurokinin A [NKA] and calcitonin gene-related peptide [CGRP]) that are coreleased after nerve stimulation by a variety of physical and chemical stimuli. Bradykinin is a nonapeptide derived by enzymatic action of plasma and tissue kallikreins on inactive precursors (kininogens) present in most tissues.

The biological effects of peptides are modulated by degradative cleavage by enzymes expressed on the cell surface. One of these peptides, angiotensin-converting enzyme (ACE; also called kininase II, dipeptidyl carboxypeptidase I, peptidyl dipeptidase A, EC 3.4.15.1), has been shown to exist in the heart and has been studied extensively. Another membrane-bound enzyme, neutral endopeptidase (NEP; also called enkephalinase, CALLA/CD10, EC 3.4.24.11), plays important roles in the modulation of peptide actions in various organs, including the respiratory tract, kidney, central nervous system, and gastrointestinal tract. However, at present no data are available concerning the expression and physiological significance of NEP in the heart.

In the present study, we present evidence of NEP-like immunoreactivity in rat myocardial cells. In vivo, we show that NEP inhibition potentiates the increase in coronary flow evoked by stimulation of sensory nerves in rats. Furthermore, we show that the acute myocardial hypoperfusion caused by isoproterenol overdosage is prevented by NEP inhibition and is converted to a markedly, long-lasting increase in myocardial blood flow. Finally, we provide evidence that the effect of NEP inhibition in isoproterenol-induced ischemia is due to decreased degradation of bradykinin. Thus, bradykinin may contribute significantly to the regulation of myocardial perfusion.

Materials and Methods

Animals

Heart cells were harvested for culture from the ventricles of 1-day-old Sprague-Dawley rats. For the experiments on myocardial blood flow, we used a total of 38 adult male Fischer F344 rats, 12 to 14 weeks of age, with body weight ranging from 277 to 326 g (301 ± 1 g) from Simonsen Laboratories, Gilroy, Calif. Experimental procedures followed in the present study were approved by the Committee on Animal Research of the University of California, San Francisco.

NEP Immunohistochemistry

NEP immunohistochemistry was performed on primary cultures of neonatal rat heart muscle and nonmuscle cells prepared as described previously. In brief, cells obtained by
trypsinization from the ventricles of 1-day-old rats were plated in MEM (Hanks’ salts) with 5% calf serum. After 12 hours in culture, these cells were transferred to a serum-free medium supplemented with transferrin and insulin (each 10 µg/mL) and maintained in 100-mm culture dishes (Falcon) at a density of ≈150 cells per square millimeter. Contaminating nonmuscle cells were kept at <10% by preplating and by addition of bromodeoxyuridine (0.1 mmol/L) through day 2 of culture. Under these control conditions, there is no change in cell size or number and no beating of the myocytes.13 Nonmuscle cells separated during preplating were subcultured and grown to confluence in MEM with 5% calf serum. Previous studies have shown that these cardiac nonmuscle cells consist predominantly of fibroblast/mesenchymal cells.12

The cell cultures were washed with phosphate-buffered saline (PBS) and fixed with a 1:1 mixture of methanol and acetone for 15 minutes at 4°C. After fixation, cells were incubated in normal goat serum diluted 1:10 for 30 minutes in a humid chamber to minimize nonspecific staining. Cell nuclei were stained with DAPI, a fluorescent dye with high affinity for DNA and widely used as a counterstain for immunohistochemistry.13 As primary antibodies, we used: (1) a previously characterized antisera10 raised in rabbits to recombiant human NEP (antisera No. 20, 1:1000 dilution) and (2) a mouse anti-myosin monoclonal antibody14 specific for myosin of striated muscle (MF-20, 1:2 dilution). Both antibodies (60 µL of each antibody) were added to areas previously circled on the dish. These areas were covered with coverslips, and the cells were incubated in a humid chamber for 90 minutes at 37°C. Then cells were washed twice with ice-cold PBS, blotted, and incubated in a humid chamber for 30 minutes at 37°C with the secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG (60 µL of each antibody, 1:100 dilution). As a control for the specificity of the antisera for NEP, we repeated the staining procedure by using as a primary antibody diluted antisera preincubated with recombiant human NEP (rhNEP, 10 µg/mL) at 4°C for 24 hours or normal rabbit serum.

**Blood Flow Technique**

Except for minor modifications, the procedure used in the present study to measure regional blood flow has been described in detail previously.15 In brief, rats were anesthetized with sodium pentobarbital (65 mg/kg IP). A Teflon catheter (22 gauge, 2.54 cm, Becton-Dickinson) was inserted into the right femoral artery and advanced to the abdominal aorta for the withdrawal of reference blood samples. A similar catheter was inserted into the left femoral artery for arterial pressure monitoring. The catheters were secured with silk suture and connected to 15-ml-long Silastic tubing (0.64-mm internal diameter, Dow Corning) filled with heparinized (100 U/mL) 0.9% NaCl. The right femoral artery cannula was connected to the withdrawal channel of a reciprocal infusion/withdrawal pump (Harvard). The left femoral artery cannula was connected to a pressure transducer (Hewlett-Packard). The signal from the transducer was amplified (Lectromed MT8P), displayed on a video monitor, and recorded (Astromed MTI-9500). The right carotid artery was dissected carefully from the vagus nerve, and a Teflon catheter (22 gauge, 4.45 cm, Arrow) was inserted by using an arterial catheterization set. The catheter was connected to a pressure transducer by using 15-ml-long Silastic tubing filled with heparinized 0.9% NaCl and advanced until the pressure tracing showed that the catheter tip had reached the left ventricular cavity. The catheter was then secured with silk sutures, disconnected from the pressure transducer, and connected to the infusion channel of the reciprocal pump.

Blood flow was measured with radioactive microspheres 15.5±0.1 µm in diameter (mean±SD) labeled with either 51Cr, 55Co, 123I, 125I, 113In, 99mTc, or 111Sn (New England Nuclear). The medium of the stock suspensions (10% dextan-saline suspension containing 0.05% Tween 80) was replaced with 70% dextrose solution (Baxter), following a procedure described previously.15 Aliquots of the final suspension were counted for radioactivity to determine the number of microspheres, and the final volume was adjusted with 70% dextrose to contain ≈100 000 microspheres in 0.25 mL.

For each determination of blood flow, a volume of 0.25 mL of the microsphere suspension, corresponding to 100 000 microspheres, was injected into the left ventricle. Different isotopes were used in rotating sequence to avoid systematic errors. Before each injection, the multi-injection vials containing the microspheres were vortexed for 5 seconds and then sonicated in water at 37°C for at least 5 minutes by using an ultrasonic bath (Branson) to obtain a homogeneous suspension.16 Two 0.25-mL aliquots of microsphere suspension were withdrawn in a disposable tuberculin syringe. One aliquot was transferred to a counting vial to assess the total injected radioactivity; the calculated number of injected microspheres was within 5% of the estimated value (97 541±4004). The other aliquot was injected into the ventricular catheter. The catheter was then connected to the infusion port of the reciprocal pump, which was set at a rate of 0.68 mL/min and activated for 1 minute. During this time, sterile 0.9% NaCl at 37°C was infused via the ventricular catheter, and the reference blood sample was withdrawn simultaneously from the right femoral catheter at the same rate.15

The rats were killed by exsanguination, and the heart was removed and cleaned of connective tissue. The heart was first cut into three parts: atria, right ventricular free wall (RV), and left ventricle (LV). Then, the interventricular septum was separated from the LV free wall, and the latter was cut into the subendocardial half (LVsub endo; wet weight, 0.197±0.004 g) and the subepicardial half (LVsub epi; wet weight, 0.220±0.004 g). Each sample was blotted, weighed, and placed in counting vials containing phosphate-buffered 2% formalin solution (pH 7.4). All blood, tissue, and total count samples were counted for 3 minutes for radionuclide emissions with a NaI (Tl) detector (Tracor Analytic), a multichannel pulse-height analyzer (Inotech), and a NOVA-3 minicomputer (Data General). Nuclide activities were determined by least-squares radionuclide separation technique, as described previously.16

**Blood Flow: Experimental Protocols**

To determine whether NEP inhibition potentiates the changes in myocardial blood flow produced by cardiac sensory nerves, we stimulated these nerves with capsaicin17 and blocked NEP activity with the inhibitor phosphoramidon.18

The dose of this inhibitor (2.5 mg/kg) was chosen because it was found in a previous study to produce in vivo maximal inhibition of NEP activity in the rat trachea, as shown by the potentiation of substance P-induced plasma extravasation.19,20 Phosphoramidon was injected into the ventricular cannula and flushed with 0.9% NaCl 5 minutes before the first (baseline) measurement of myocardial blood flow (n=5 rats). A control group of rats received an equivalent volume of 0.9% NaCl (n=5 rats) 5 minutes before the baseline measurement. Myocardial blood flow was measured at baseline, at the end of a left ventricular injection of a subthreshold dose of capsaicin (3 µg/kg), which was based on exploratory dose-response curves,15 and 5 minutes after capsaicin injection.

To investigate the influence of NEP on myocardial blood flow during and after myocardial hypoperfusion, we used a rat model of myocardial ischemia in which a large dose of the β-adrenergic agonist isoproterenol (50 mg/kg) is given intraperitoneally.21 This dose of isoproterenol produces a transient reproducible fall in myocardial blood flow (myocardial hypoperfusion), followed within 2.5 minutes by increased myocardial lactate content and 24 hours later by infarctlike necrosis. In the present study, the same dose of phosphoramidon that
effectively potentiates capsaicin-induced coronary vasodilation (2.5 mg/kg) was injected into the ventricular canula and flushed with 0.9% NaCl 5 minutes before the first (baseline) measurement of myocardial blood flow (n=7 rats). A control group of rats (n=8 rats) received an equivalent volume of 0.9% NaCl 5 minutes before the baseline measurement. In each of the rats, isoproterenol was injected rapidly into the peritoneal space 2.5 minutes after the baseline measurement, and myocardial blood flow was measured again at 0, 2.5, 10, and 20 minutes after the injection of isoproterenol.

After detecting that phosphoramidon prevents isoproterenol-induced myocardial hyperperfusion, we studied the roles of substance P and bradykinin, two coronary vasodilators that are inactivated by NEP. Because their cardiovascular effects are mediated by NK1, receptors22-23 and B1 receptors,24 respectively, we tried to abolish the effect of phosphoramidon on perfusion by using the selective antagonists CP-99,994 (4 mg/kg)26 and Hoe-140 (130 μg/kg).27,28 Phosphoramidon and CP-99,994 (n=8 rats) or phosphoramidon and Hoe-140 (n=5 rats) were injected into the left ventricular canula and flushed with 0.9% NaCl 5 minutes before the measurement of myocardial blood flow. Myocardial blood flow was measured again at 0, 2.5, 10, and 20 minutes after the injection of isoproterenol.

Drugs and Chemicals

Antiserum No. 20 and hrNEP were kindly provided by Dr N.W. Bunnell. The anti-myosin monoclonal antibody MF-20 was purchased from the Developmental Studies Hybridoma Bank. FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG were purchased from Cappel. DAPI (4’,6-diamidino-2-phenylindole hydrochloride) was purchased from Sigma Chemical Co.

All drugs used in the present study were delivered in a volume of 1 mL/kg body wt. Capsaicin (8-methyl-N-vanillyl-6-nonanamide, Sigma) was dissolved in 70% ethanol (10-2 mol/L stock) and diluted to final concentration with 0.9% NaCl. (±)-Isoproterenol [1-(3’-4’-diisopropylamino)-2(isopropylamino)ethanol hydrochloride, Sigma] and phosphoramidon [N-(α-homophenylaminoxyhydroxyphosphinyl)-L-leucyl-L-tryptophan, Peninsula Laboratories] were dissolved in 0.9% NaCl immediately before administration. CP-99,994 [(-)-25-35)-3-(2-methoxyphenylamino)-2-phenylpyrrolidine, kindly provided by Dr R.M. Snider (Pfizer Central Research Division), was dissolved in 0.9% NaCl, Hoe-140 [(3’-Arg’-Hyp’-Thi’-D-Tic’-Oic’)-bradykinin], kindly provided by Dr F.J. Hack (Hoecht AG), was dissolved in dimethyl sulfoxide (10-3 mol/L stock) and diluted to final concentration with 0.9% NaCl.

Calculations and Statistical Analysis

Regional blood flow was calculated by the following equation:

\[ Q = (Q_0 \times A_0) / (A_0 \times W_0) \]

where \( Q \) is blood flow in the tissue sample (mL/min/g), \( Q_0 \) is reference withdrawal sampling rate (mL/min), \( A_0 \) is radioactivity in the tissue sample (cpm), \( A_0 \) is radioactivity in the reference blood sample (cpm), and \( W_0 \) is wet weight of the tissue sample (g). Vascular conductance was calculated by dividing the measurement of regional blood flow by the mean arterial pressure measured at the time of the corresponding microsphere injection.

Data are expressed as mean±SEM. The effects of different drugs on mean values of mean arterial pressure, heart rate, myocardial blood flow, and conductance were analyzed by two-factor ANOVA.29 Differences in myocardial perfusion at different time points and in different myocardial regions were analyzed by one-factor ANOVA.29 One-factor ANOVA was also used to analyze the effect of capsaicin on myocardial blood flow and mean arterial pressure. Multiple comparisons between means were performed with Fisher’s protected least significant difference test.30 Differences having a value of \( P<.05 \) were considered significant.

Results

Immunohistochemistry

Cultured rat myocytes, identified by positive myosin staining, revealed intense NEP immunoreactivity (Fig 1A through 1C). Contaminating nonmyocytes (mainly fibroblasts) negative for myosin staining were also negative for NEP staining, and no staining was observed in confluent nonmyocyte cultures (Fig 1D through 1F). Myocytes contaminating nonmyocyte cultures underwent hypertrophic growth, as seen in previous studies,12 but still expressed NEP immunoreactivity. NEP immunoreactivity was abolished by preincubation of the primary antisera with rhNEP. There was no staining when the primary antisera was replaced with normal rabbit serum.

Sensory Nerve Stimulation

In control rats injected with a subthreshold dose of capsaicin, myocardial flows measured immediately after (2.5±0.3 mL·min⁻¹·g⁻¹ wet tissue wt) and 5 minutes after (2.8±0.5 mL·min⁻¹·g⁻¹) capsaicin were not significantly different from baseline (3.0±0.4 mL·min⁻¹·g⁻¹, \( P>.5 \)); this dose of capsaicin had no effect on mean arterial pressure (104±7 versus 102±6 mm Hg, \( P=.86 \)). Rats pretreated with the NEP inhibitor phosphoramidon had only minimal changes in baseline myocardial blood flow (3.4±0.5 mL·min⁻¹·g⁻¹, \( P=.75 \)) and mean arterial pressure (100±4 mm Hg, \( P=.79 \)) compared with control rats. In these rats, the same dose of capsaicin caused myocardial blood flow to increase by 97% (6.6±1.3 versus 3.4±0.5 mL·min⁻¹·g⁻¹, \( P<.01 \)) without significant change in mean arterial pressure (106±6 mm Hg, \( P=.48 \)). Blood flow was still 61% higher than baseline 5 minutes after capsaicin (5.4±1.2 mL·min⁻¹·g⁻¹, \( P=.08 \)), again without change in mean arterial pressure (103±5±5 mm Hg, \( P=.73 \)). Although dose-response curves have not been done for the rat myocardial circulation, the dose of phosphoramidon chosen on the basis of its effect on the rat trachea19,20 had a profound effect on the myocardial circulation and clearly exceeded the threshold needed for response.

Effect of Isoproterenol Overdose

Before the isoproterenol was given, mean arterial blood pressures and coronary conductances were almost identical in all four groups, and heart rates were similar, although slightly lower after phosphoramidon alone. The systemic hemodynamic changes produced by isoproterenol overdosage were qualitatively similar in all four groups of rats. Isoproterenol produced marked and prolonged hypotension and tachycardia (Fig 2). The magnitude of the tachycardia induced by isoproterenol was greater in rats treated with phosphoramidon alone than in the other treatment groups (\( P<.05 \)). Furthermore, in rats treated with phosphoramidon alone, arterial pressure failed to recover after the initial fall and was significantly different from the other groups at 10 and 20 minutes after the injection of isoproterenol (\( P<.05 \)). As shown in the Table, the pretreatment with phosphoramidon, Hoe-140, and CP-99,994 produced...
only minor changes in the absolute hemodynamic data measured at baseline.

We also found important differences in myocardial perfusion after isoproterenol: in control rats, isoproterenol overdosage caused a marked fall in perfusion pressure but a relatively modest coronary vasodilation (Fig 3). This resulted in a 31% decrease in myocardial blood flow, followed by a rapid return to baseline values. In contrast, isoproterenol-induced hypoperfusion did not occur in phosphoramidon-treated rats: myocardial blood flow increased by 22% because vascular conduc-
tance increased by 98% (different from control rats, \( P<.05 \)). Moreover, in phosphoramidon-treated rats cor-
ony conductance continued to increase during reper-
fusion up to 218% 20 minutes after isoproterenol, with a 99% increase in coronary flow from baseline.

The substance \( P \) receptor antagonist CP-99,994 did not reduce the effect of phosphoramidon on myocardial perfusion (not different from phosphoramidon alone, \( P=.85 \)). In contrast, the effect of phosphoramidon was abolished by the bradykinin receptor antagonist Hoe-140 (different from phosphoramidon alone, \( P<.05 \)). After the administration of Hoe-140, the changes in coronary flow caused by isoproterenol in phosphoramidon-treated rats were the same as the changes seen in control rats (\( P=.99 \)), suggesting that the increase in coronary conductance after iso-
proterenol overdosage in rats pretreated with the NEP inhibitor is due to reduced degradation of bradykinin.

**Regional Distribution of Changes in Myocardial Blood Flow**

The effect of phosphoramidon on myocardial flow after isoproterenol overdosage was found in all myocardial regions (Fig 4): a significant increase in vascular conductance and in blood flow compared with non-
treated control rats was found in the atria (\( P<.01 \)), in the RV (\( P<.01 \)), and in the LV including the inter-
ventricular septum (\( P<.05 \)). Within the LV (Fig 5), phosphoramidon increased conductance and blood flow in both \( LV_{end} \) (\( P<.05 \)) and \( LV_{epi} \) (\( P<.01 \)). The effect of phosphoramidon was abolished by Hoe-140 in the RV (\( P<.05 \)), \( LV_{end} \) (\( P<.01 \)), and \( LV_{epi} \) (\( P<.01 \)). However, Hoe-140 did not reverse the effect of phosphoramidon on atrial perfusion (\( P=.53 \)), which remained signi-
cantly different from the control value (\( P<.05 \)). CP-
99,994 did not affect perfusion in any myocardial region (\( P>.5 \)).

The magnitude of the changes produced by isoproterenol varied in different myocardial regions, both with or without phosphoramidon pretreatment. In control rats, isoproterenol-induced myocardial hypoperfusion was significantly greater in the RV than in the LV at

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**Fig 1.** Photomicrographs showing immunofluorescent staining for neutral endopeptidase (NEP) in cultured rat myocytes. A, B, and C show the same field in a myocyte culture with \(<10\% \) nonmyocytes. D, E, and F show the same field in a culture of confluent nonmyocardial cells with two hypertrophic myocytes. Both cultures were stained with (1) nuclear stain (DAPI) to identify individual cells (A and D), (2) mouse anti-myosin monoclonal antibody detected by rhodamine-conjugated goat anti-mouse IgG to identify myocytes (B and E), and (3) rabbit anti-recombinant human NEP polyclonal antibody detected by fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (C and F). Original magnification \( \times 100 \).
Fig 2. Graphs showing changes in mean arterial pressure and heart rate (percent change from baseline) after injection of isoproterenol in control rats (solid circles) and in rats pretreated with the neutral endopeptidase inhibitor phosphoramidon (open symbols). Phosphoramidon-treated rats also received either saline (circles), the B2 (bradykinin) receptor antagonist Hoe-140 (squares), or the NK1 (substance P) receptor antagonist CP-99,994 (triangles). The effect of isoproterenol was more pronounced in rats treated with phosphoramidon alone than in the other groups (P<.05). Values are mean±SEM (n=5 to 8 rats per group).

every time point (P<.05, Fig 4). In phosphoramidon-treated rats, the increase in myocardial perfusion after isoproterenol was more pronounced in the LV than in the RV, but this difference was not statistically significant. Only minor differences were found between different layers of the LV free wall (Fig 5). In phosphoramidon-treated rats, the increase in subepicardial flow after isoproterenol overdose appeared to be more pronounced than the corresponding increase in subendocardial flow, but this trend was not statistically significant.

Baseline Hemodynamic Measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Arterial Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>Coronary Conductance, mL·min⁻¹·mm Hg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>126±3</td>
<td>422±16</td>
<td>0.05±0.003</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>122±4</td>
<td>359±11</td>
<td>0.04±0.010</td>
</tr>
<tr>
<td>Phosphoramidon + Hoe-140</td>
<td>126±5</td>
<td>418±17</td>
<td>0.05±0.002</td>
</tr>
<tr>
<td>Phosphoramidon + CP-99,994</td>
<td>123±3</td>
<td>392±14</td>
<td>0.03±0.004</td>
</tr>
</tbody>
</table>

bpm indicates beats per minute. Values are mean±SEM.

Discussion

NEP in the Heart

We found evidence of NEP immunoreactivity in cultured rat myocytes but not in nonmyocytes from the same source. Nonmyocytes did not react with antibodies to myosin heavy chain. Furthermore, previous work showed that these cells do not react with antibodies to factor VIII, nor do they take up acetylated LDL, confirming their nonendothelial origin.12 These observations indicate that nonmyocytes in primary cultures from neonatal rat heart are predominantly fibroblasts. Cardiac nonmyocytes stimulate hypertrophic growth of cocultured cardiac myocytes, probably by release of a paracrine growth factor.12 Hypertrophic myocytes were easily identified among surrounding nonmyocytes because of their immunofluorescent staining for NEP. However, this staining was less intense than the staining seen in nonhypertrophic myocytes. This difference could be explained by the disproportion between myocyte growth and NEP expression, suggesting that growth may contribute to the modulation of NEP activity in myocytes.

We used cultured neonatal myocytes as being an appropriate system for demonstrating an exoenzyme.
Many proteins show developmental changes, and we cannot be sure that the amount of enzyme disclosed in these cultures is the same as that present in the adult rats studied, nor do we know whether there are differences between different strains of rats. Nevertheless, the results of this and other studies attest to the presence of NEP in adult rat hearts in sufficient amounts to have a major effect on peptides.

**NEP and Sensory Nerves**

We tried to understand the physiological significance for the presence of this enzyme in the heart. Inasmuch
as previous studies in other organs consistently indicate that NEP plays a pivotal role in modulating biological responses to tachykinins released from sensory nerves, we measured the changes in myocardial blood flow in rats injected with a subthreshold dose of capsaicin, a drug known to stimulate sensory afferents selectively.17 A dose of capsaicin causing no change in myocardial blood flow or vascular resistance in control rats caused a marked increase in myocardial blood flow and decrease in myocardial vascular resistance in rats pretreated with the NEP inhibitor phosphoramidon; phosphoramidon by itself had only minor effects on the heart rate–blood pressure product, myocardial blood flow, and vascular conductance and so probably did not alter myocardial oxygen consumption in any major way. This effect of phosphoramidon is probably due to reduced inactivation of substance P, a peptide that is released from sensory nerves3 and that is rapidly inactivated by NEP.31 Of the other peptides released from sensory nerves, CGRP is a poor substrate for NEP,32 and NKA does not have a significant vasodilator effect in the coronary bed (authors’ unpublished observations). Capsaicin stimulates unmyelinated sensory nerves in many organs, so that substance P release may be local in the heart or remote in other organs and then travel to the heart.

Membrane-bound peptidases can act only in the proximity of the cell surfaces where they are expressed.5 Thus, their selectivity and efficiency in vivo are restricted by the geometrical factors that determine the “accessibility” of the enzyme to potential substrates. Because of its topology, NEP seems to play a particularly relevant role in the extravascular compartments: eg, in the airway, epithelium NEP is concentrated in the basal cells7 and thus is in a strategic position to cleave and inactivate the neuropeptides near their sites of release from the network of sensory nerve terminations concentrated in close association with the basal cells.33 NEP localized in myocardial cells may have a similar role: in the normal state, as the peptides released from cardiac nerves or locally produced from tissue kininogens diffuse toward their receptors on coronary vessels, the enzymatic cleavage by NEP present in the surrounding myocardium reduces the concentration of active peptide. However, physical or metabolic changes may upregulate or downregulate myocardial NEP activity, thus modulating the potent vasoactive effects of the peptides cleaved by this enzyme.

**NEP and Myocardial Ischemia**

Because NEP effectively cleaves and inactivates potent vasodilators, we hypothesized that this enzyme may contribute to the control of myocardial perfusion. We induced acute myocardial hypoperfusion in rats by injecting a large dose of isoproterenol23 and compared the changes in myocardial perfusion observed in control rats with those observed in rats pretreated with phosphoramidon, an inhibitor of NEP activity. In all rats, administration of the β-adrenergic agonist isoproterenol was followed by marked and persistent tachycardia and hypotension caused by β1-mediated myocardial stimulation and β2-mediated systemic vasodilation, respectively.34 In our control rats, these systemic hemodynamic effects of isoproterenol led to a transient fall in myocardial perfusion, followed by return only to base-line values despite the increased metabolic demand. In contrast, rats treated with the NEP inhibitor did not experience the initial fall in myocardial perfusion; in the rats treated with phosphoramidon, the mean arterial pressure decreased by 37% compared with 43% in the control rats, so that they had a much greater increase in conductance when isoproterenol was given (Fig 3). In addition, they were able to respond to the vasodilator and metabolic effects of isoproterenol with a secondary increase in coronary conductance, leading to a marked increase in myocardial perfusion over the 20 minutes following the isoproterenol injection. The effect on myocardial blood flow of phosphoramidon was qualitatively similar in different myocardial regions but was more pronounced in the LV than in the RV or in the atria. These observations suggest that one or more vasodilator agent(s), spared from inactivation when NEP is inhibited, can alter the regulation of myocardial perfusion.

Another peptidase, ACE, has been proven to exist in the heart2 and has been shown to play a role in the intracardiac generation of angiotensin II35 and in the control of kinin degradation.36 Some studies in animals and in humans have shown significant beneficial effects of ACE inhibitors on myocardial ischemia, congestive heart failure, and left ventricular hypertrophy.37,38 In particular, captopril given during acute myocardial ischemia in dogs reduces myocardial necrosis and increases regional blood flow,39,40 effects that are abolished by the bradykinin antagonist Hoe-140.41 However, other studies have failed to confirm this effect of ACE inhibitors.42 Similarly, controversial results were recently obtained in large clinical trials exploring the effects of ACE inhibitors after myocardial infarction: one trial found a significantly lower mortality rate in captopril-treated patients,43 but other trials did not confirm the cardio-protective effect of ACE inhibitors.44,45

NEP and ACE share several structural characteristics. They both are zinc metalloproteins with variable degrees of glycosylation, anchored to different cell types by a single hydrophobic membrane-spanning domain, with their catalytic site facing the extracellular milieu. Substance P and bradykinin are effectively degraded in vivo by both enzymes.36,47 In preliminary experiments done in the same rat model described in the present study, we found that the effect of phosphoramidon against isoproterenol-induced myocardial hypoperfusion was not enhanced by concurrent administration of the ACE inhibitor captopril (A.J. Maxwell, W.K. Hussein, G. Piedimonte, J.I.E. Hoffman, unpublished observations). Furthermore, in open-chest dogs we have found that the increase in blood flow measured in the left circumflex artery after local injections of substance P or bradykinin was maximally potentiated by local injection of phosphoramidon, and no further increase was measured after addition of captopril (authors’ unpublished observations). These findings indicate that ACE is not the only, and perhaps not even the most important, enzyme controlling kinin levels in the heart. In fact, NEP may play a role in the regulation of myocardial perfusion. The existence of multiple myocardial peptidases may explain, at least in part, the variability found in the cardioprotective effect of ACE inhibitors. Additional studies are necessary to explore
further the relative importance and the possible interactions between the two peptidases.

**NEP Substrates: Substance P Versus Bradykinin**

After discovering that phosphoramidon prevents isoproterenol-induced myocardial hypoperfusion, we tried to determine which of the potential substrates of NEP was responsible for the marked increase in coronary conductance seen after NEP inhibition. NEP degrades a variety of small peptides by cleaving the internal peptide bonds at the amino side of hydrophobic amino acids.\(^{47}\) We focused our study on bradykinin and substance P because it is known that the ischemic myocardium releases bradykinin,\(^{48}\) which is believed to evoke cardiac pain by stimulating chemosensitive unmyelinated sensory fibers\(^ {49}\) containing substance P.\(^ {50}\) Also, we studied substance P because of the potentiating effect of phosphoramidon on sensory nerve–induced coronary vasodilation.

The potentiating effect of phosphoramidon on sensory nerve–induced coronary vasodilation suggested that the sensory neuropeptide substance P may be responsible for the effect on myocardial blood flow of phosphoramidon during myocardial ischemia. However, the selective NK\(_1\) receptor antagonist CP-99,994\(^ {25,26}\) did not affect myocardial perfusion after isoproterenol in rats pretreated with phosphoramidon, and we actually observed a trend for increased blood flow during reperfusion in rats receiving both phosphoramidon and CP-99,994. In contrast, CP-99,994 reversed the effect of phosphoramidon on mean arterial pressure and heart rate. We have previously described profound differences in the vasodilator potency of substance P in different organs;\(^ {51}\) a possible explanation for these findings is that the systemic effects of the substance P antagonist are dependent on its effect on the regional circulation of other organs in which substance P plays a hemodynamic role more important than that found in the heart.

Although substance P can activate all types of tachykinin receptors,\(^ {51}\) previous studies with selective receptor agonists and antagonists have shown that the vasodilator effect of substance P is mediated by activation of the NK\(_1\) receptor subtype.\(^ {22,23}\) Furthermore, in studies of dog myocardial blood flow in vivo, we found that substance P has a marked vasodilator effect that is abolished by an NK\(_1\) receptor antagonist, whereas the NK\(_2\) agonist NKA did not have any significant vasoactive effect (authors’ unpublished observations). Among the many compounds recently found to produce selective NK\(_1\) receptor antagonism, CP-99,994 is suitable for studies of vascular physiology in vivo because of its lack of activity on verapamil-sensitive calcium channels and thus its lack of a significant, long-lasting hypotensive effect.\(^ {26}\)

Previous studies have shown that the cardiovascular effects of bradykinin are mediated by activation of the B\(_2\) receptor subtype.\(^ {24}\) Hoe-140, a potent and selective antagonist of B\(_2\) bradykinin receptors\(^ {27,28}\) abolished the effect of phosphoramidon on myocardial perfusion after isoproterenol. Rats pretreated with both Hoe-140 and phosphoramidon experienced the same degree of isoproterenol-induced hypoperfusion as control rats that did not receive phosphoramidon. Hoe-140 reversed the effect of phosphoramidon in both ventricles but not in the atria. These observations suggest that the difference in ventricular perfusion after isoproterenol observed in phosphoramidon-treated rats is due to reduced NEP degradation of bradykinin. Atrial perfusion appears to depend on a peptide different from bradykinin and substance P but still inactivated by NEP.

Another excellent substrate for NEP is the atrial natriuretic peptide (ANP).\(^ {52}\) This is released mainly from the atrial myocytes, the principal release mechanism being atrial stretch.\(^ {53}\) Much of the ANP released is broken down by NEP activity in the lungs.\(^ {54}\) Reversal of this degradation by inhibiting NEP might then increase the circulating concentration of ANP, which is known to be a vasodilator.\(^ {55}\) There are, however, several arguments that negate the role of ANP in our studies. First, ANP has little vasodilator activity, less than C-type natriuretic peptide (CNP).\(^ {56}\) Second, in our hands, physiological concentrations of ANP and CNP (10\(^ {-9}\) and 10\(^ {-8}\) mol/L) given by intracoronary arterial injection in the dog do not cause coronary vasodilation (authors’ unpublished observations). Finally, the results of the selective B\(_2\) receptor blocker exclude the role of these peptides in explaining our results.

Phosphoramidon has been shown to inhibit another enzyme distinct from NEP and responsible for the conversion of big endothelin 1 to endothelin 1 (ET-1),\(^ {57}\) which is a potent coronary vasoconstrictor.\(^ {58}\) If isoproterenol caused secretion of big endothelin 1, conversion of this to ET-1 might explain the relatively minor increase in coronary conductance after the isoproterenol dose. Inhibiting the conversion of big endothelin 1 to ET-1 by giving phosphoramidon would then allow for greater myocardial flows and coronary conductances. Again, the observation that a highly selective antagonist of the B\(_3\) receptor abolishes the effect of phosphoramidon completely provides strong evidence that this effect is mediated by bradykinin. Thus, it is unlikely that ET-1 is involved in this phenomenon. The same argument probably applies to other peptides that may be present.

We speculate that bradykinin is produced in the ventricular myocardium as a response to the increased oxygen demand created by \(\beta\)-adrenergic stimulation; however, the vasodilator effect of bradykinin is negligible in the presence of the degradative activity of NEP. When NEP activity is inhibited, then the increase in heart rate is associated with an immediate and persistent increase in myocardial perfusion because of the higher levels of active bradykinin. Thus, at least when NEP activity is reduced, bradykinin may contribute to the regulation of myocardial perfusion. Further studies will be needed to explore possible indirect mechanisms involved in this effect of bradykinin. Sensory nerve stimulation and consequent local release of substance P do not appear to play an important hemodynamic role in this experimental model.

**Conclusions**

In summary, we found evidence of NEP-like immunoactivity and biological activity in the rat myocardium. This enzyme exerts an inhibitory control on the activity of vasodilator peptides in the coronary circulation, as shown by the exaggerated vasodilator response to endogenously released neuropeptides in rats pretreated with an NEP inhibitor. After NEP inhibition, we observed marked coronary vasodilation in response to
isoproterenol overdosage, whereas in the absence of NEP inhibition isoproterenol produced the expected acute myocardial hyperperfusion. Studies with selective receptor antagonists revealed that this modified response to isoproterenol is due to reduced inactivation of bradykinin, suggesting that this peptide may play an important role in the regulation of myocardial perfusion. If confirmed in humans, these studies may provide a new tool to modulate myocardial perfusion by using inhibitors of the enzymes responsible for the degradation of endogenous coronary vasodilators.

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References
Neutral endopeptidase in the heart. Neutral endopeptidase inhibition prevents isoproterenol-induced myocardial hypoperfusion in rats by reducing bradykinin degradation.
G Piedimonte, J A Nadel, C S Long and J I Hoffman

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