Neutral Endopeptidase Is Activated in Cardiomyocytes in Human Aortic Valve Stenosis and Heart Failure

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Background—The regulation of the cardiac neutral endopeptidase (EC 24.10, NEP) that degrades bradykinin and natriuretic peptides has been investigated in human cardiac hypertrophy and heart failure.

Methods and Results—NEP mRNA was quantitated by real-time polymerase chain reaction (PCR) in left ventricular biopsies from patients with aortic valve stenosis (AS, n=19) and heart failure due to dilated cardiomyopathy (DCM, n=14), and control subjects with normal systolic function (CON, n=14). Left ventricular NEP mRNA content was increased 3-fold in AS (P<0.005) and 4.1-fold in DCM (P<0.002). The increase in NEP mRNA was related to the increase in end diastolic pressure in AS and DCM. In a second series, myocardial NEP enzymatic activity was determined. It increased 3.6-fold in AS (P<0.02) and 4-fold in DCM (P<0.002). NEP was localized in the myocardium by immunofluorescence microscopy and in situ PCR to myocytes and nonmyocyte areas and cells.

Conclusions—Elevated cardiac NEP activity in pressure loaded and failing human hearts may increase the local degradation of bradykinin and natriuretic peptides. (Circulation. 2002;105:286-289.)

Key Words: hypertrophy ■ cardiomyopathy ■ heart failure ■ metalloproteinases

Neutral endopeptidase (EC 24.10, NEP) catalyzes the degradation of vasodilator and growth-inhibitory peptides such as bradykinin and atrial and brain natriuretic peptide (ANP, BNP), as well as vasoconstrictor peptides like angiotensin and endothelin.1 In the human myocardial interstitium, NEP represents the major bradykinin degrading enzyme.2 Along with the renin-angiotensin system (RAS) and the sympathetic nervous system, the kallikrein-kinin system is involved in the onset and progression of heart failure and ventricular remodeling after myocardial infarction.3 Kinins reduce left ventricular hypertrophy and fibrosis and prevent progression of heart failure.3 Knockout of the predominant kinin receptor, the B2 receptor, in mice leads to cardiac hypertrophy and heart failure.4 In hypertensive rats, NEP inhibitors led to urinary kinin accumulation and natriuresis, and prevented cardiac hypertrophy and fibrosis.5 Therefore, in addition to the RAS, NEP may represent a determinant of cardiac hypertrophy and fibrosis.

We recently described an upregulation of myocardial angiotensin converting enzyme (ACE) mRNA and activity in patients with ventricular hypertrophy caused by AS and in heart failure.6 Much less is known about the effects of human cardiac diseases on the regulation of myocardial NEP. Therefore, we studied the mRNA expression, activity, and cellular localization of NEP in the myocardium of patients with chronic pressure overload due to aortic stenosis and in patients with heart failure due to dilated cardiomyopathy.

Methods

Patients

Left ventricular myocardial samples from 19 patients with aortic valve stenosis (AS-I, LVEF 52±4%, aortic gradient 76±7 mm Hg), 14 patients with heart failure due to dilated cardiomyopathy (DCM-I, EF 25±4%), and 14 control subjects were analyzed for NEP mRNA content. In a second series, 8 new comparable patients with AS, 8 patients with DCM, and 10 control subjects were analyzed for NEP activity. Small tissue samples from patients with AS were obtained by biopsy of the left ventricular septum at elective aortic valve replacement surgery. Tissue samples from patients with dilated cardiomyopathy were obtained as left ventricular septum biopsies at cardiac catheterization (n=10) or at orthotopic heart transplantation. The control group was composed of donor hearts that were not used for logistic reasons. They had normal systolic cardiac function; cardiac history was absent and postmortem histology was normal. Clinical features and medical therapy are presented in detail in the Table (available in the Appendix at http://www.circulationaha.org). Written, informed consent was obtained from all patients. The study followed the rules for investigation of human subjects, as defined in the Declaration of Helsinki.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

RNA was extracted and reverse transcribed after complete DNA digestion as previously described.6 Yields of RNA were comparable.
in all groups (data not shown). A “hot start” real-time polymerase chain reaction (PCR) procedure with SYBR Green that was validated with respect to reproducibility and linearity within the measuring range was performed in quadruplicate with the Light Cycler instrument (Roche). To correct for potential variances between samples in mRNA extraction or in RT-efficiency, the mRNA content of NEP was normalized to the expression of the stably expressed reference gene GAPDH in the same sample.

NEP Activity

The activity of myocardial NEP was determined with an enzymatic assay.7 Myocardial samples (8 to 24 mg) were homogenized in Tris/HCl buffer containing 1% Triton X-100. After removal of crude debris by centrifugation (1500g, 5 minutes), the supernatant was incubated with 0.6 mmol/L Suc-Ala-Ala-Phe-AMC (7-amino-4-methyl-coumarin) for 30 minutes at 37°C in the absence and presence of the NEP-inhibitor thiorphan (2.5 μmol/L). The reaction was stopped with Na-EDTA (2 mmol/L) and thiorphan (2.5 μmol/L), and the proteins were heat-precipitated. AMC was released from the reaction product Phe-AMC by incubation with aminopeptidase M, and was quantified by fluorometry. The thiorphan-sensitive reaction represented NEP activity.

Immunofluorescence Microscopy and In Situ PCR

Cryosections were permeabilized in 0.2% Triton X-100/PBS as described.8 Incubations with the primary antibodies, polyclonal rabbit anti NEP (Santa Cruz Biotech Inc, California, USA) and monoclonal mouse anti-myomesin were performed at 4°C overnight. Secondary antibodies, FITC-conjugated goat anti rabbit Igs (Cappel, via ICN, Germany) and Cy3-conjugated goat anti-mouse Igs (Jackson Immunochemicals, Switzerland) were incubated for 1 hour at room temperature, mounted as previously described,8 and viewed on a confocal microscope (Leica) equipped with argon/helium-neon lasers. The data sets were processed on a Silicon Graphics work station using the software Imaris (Bitplane, Switzerland).

In situ PCR on paraffin-embedded sections with hapten- (dinitrophenol)-labeled primers was used to localize NEP mRNA (detailed description Appendix, available at http://www.circulationaha.org). For each sample, 3 negative controls were performed by treatment of homologous sections with reaction mixtures without dNTP, taq-polymerase, or primers, respectively.

Statistics

Results are expressed as mean±SEM. The Mann-Whitney U test and ANOVA corrections for multiple testing were used. In the case of equal variances, the Tamhane-T2 procedure was used, as provided by the SPSS program. Pearson’s correlation coefficients were calculated by SPSS.

Results

Myocardial NEP mRNA and Activity

Left ventricular NEP mRNA content increased 3-fold in patients with AS (AS-I, Figure 1A). The increase was significantly greater in patients with elevated left ventricular
end-diastolic pressure (LVEDP) than in patients with normal LVEDP (Figure 1B).

In DCM, NEP mRNA increased 4.1-fold in comparison with controls (Figure 1A). NEP expression was higher in the 10 DCM patients with an ejection fraction <35% (431 ± 77% of controls) than in the 3 patients with ejection fractions >35% (207 ± 24%). A close correlation between NEP and LVEDP existed in the 10 patients in whom LVEDP was obtained at cardiac catheterization simultaneously with the biopsy (r = 0.70, P < 0.05, Figure 1C). Left ventricular NEP activity (in nmol · min⁻¹ · g⁻¹ protein) was increased 3.6-fold in AS (AS-II, P < 0.02) and 4-fold in DCM (DCM II, P < 0.002) compared with controls (Figure 1D).

**Myocardial Localization of NEP**

NEP protein was detected in nonmyocyte tissues as well as in ventricular cardiomyocytes (Figures 2A through 2L). In normal hearts, NEP was mainly associated with nonmyocyte areas or cells. Expression of NEP in the cardiomyocytes of the control hearts was low but detectable (Figures 2A through 2E). In contrast, in the failing hearts, there was pronounced binding of NEP antibodies to cardiomyocyte structures. In situ PCR confirmed that the expression of NEP mRNA was barely detectable in the ventricular myocytes of controls, whereas it was pronounced in myocytes from the failing hearts. NEP mRNA was also detected in nonmyocyte cells (Figure 2O).

**Effect of Medication**

No effect of medication on NEP could be detected. All patients with DCM and 5 with AS were treated with ACE inhibitors. These 5 patients tended to have a higher NEP mRNA expression than AS patients without ACE inhibitors, but the difference was not significant (486% versus 216% of controls, NS).

**Discussion**

We describe increased myocardial NEP mRNA expression and increased NEP activity in diseased human hearts. NEP mRNA was not only increased in patients with severe heart failure, but was also increased in patients with AS and increased filling pressures in the presence of a normal left ventricular ejection fraction and was related to filling pressures in AS and DCM. NEP was localized to myocytes and nonmyocyte areas or cells, the latter most likely representing fibroblasts.

It has been reported that NEP was the most important enzyme for bradykinin degradation in the extracellular space of human and rat hearts.²⁹ Our localization of NEP protein to nonmyocyte areas and cells, probably fibroblasts, supports this observation because the activity of the extracellularly orientated enzyme on these cells should at least in part be accessible from the interstitial space. The detection of NEP in nonmuscle cells probably corresponds to its already-reported expression by fibroblasts, neutrophils, or endothelial cells.¹⁰

In the normal hearts, the NEP protein expression in the myocytes was relatively weak, whereas a strong signal was detected in heart failure. In situ PCR confirmed the high expression of NEP mRNA in myocytes of failing hearts, suggesting NEP synthesis in myocytes. NEP mRNA seems also present in nonmyocytes.

The increase in NEP expression can already be detected in patients with hypertrophy and maintained systolic function.
Despite large interindividual differences in clinical parameters, the increase in NEP expression was higher in the patients with AS and elevated LVEDP than in patients with normal filling pressures. A positive correlation between LVEDP and NEP mRNA in DCM also suggests an association between NEP expression and filling pressures or wall stress. In contrast to NOS activity, the increase in NEP activity is not lost in endstage failure.

NEP contributes significantly to the degradation of the vasodilator and antifibrotic peptide bradykinin and to the degradation of the vasodilator and antihypertrophic peptides ANP and BNP. Most of the effects of NEP have been documented in plasma, but evidence exists that its local effects on bradykinin and ANP/BNP metabolism and on hypertrophy and fibrosis are also relevant at the cellular and tissue level. It may therefore be speculated that increased myocardial NEP activity leads to an increased degradation of local bradykinin and ANP/BNP, and thus reduced protection from the progression of hypertrophy and fibrosis. In the presence of ACE inhibitors, NEP may still continue to degrade bradykinin and counteract the beneficial myocardial effects of ACE inhibitors that are mediated via bradykinin. Thus, inhibition of NEP together with ACE may offer new aspects in heart failure therapy. Combined NEP and ACE inhibitors are now being developed for this purpose.

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