Degradation of Bradykinin by Neutral Endopeptidase (EC 3.4.24.11) in Cultured Human Endothelial Cells

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Summary: The presence of neutral endopeptidase 24.11 was demonstrated in human umbilical vein endothelial cells by immunostaining. Enzymatic activity of neutral endopeptidase was determined as 0.167 ± 0.02 mU/mg protein in the membrane fraction of human umbilical vein endothelial cells, using the fluorogenic peptide substrate, dansyl-D-Ala-Gly-Phe(pNO$_2$)-Gly. No activity was found in the cytosolic fraction of endothelial cells. The role of this peptidase in the degradation of the endogenous vasodilator bradykinin was investigated by incubating human umbilical vein endothelial cell monolayers with bradykinin (10^{-8} mol/l). The inhibitor of neutral endopeptidase, phosphoramidon (10^{-8} mol/l), decreased the degradation of bradykinin in the supernatant of endothelial cells; the half-life of bradykinin was then increased from 29 ± 1 to 46 ± 2 minutes. The angiotensin-converting enzyme inhibitor, lisinopril (10^{-8} mol/l), increased the half-life of bradykinin to 244 ± 20 minutes; the combination of both inhibitors increased the half-life of bradykinin to 381 ± 51 minutes. Inhibitors of aminopeptidase (amastatin) and carboxypeptidase (2-mercaptomethyl-3-guanidinoethyl-thiopropionic acid) caused no significant effect. The effect of phosphoramidon was small in comparison with that of lisinopril, but was pronounced in combination with lisinopril. Neutral endopeptidase activity is localized in the membranes of human endothelial cells and seems to be involved in the degradation of bradykinin by the vascular endothelium, particularly during angiotensin converting enzyme inhibition.

Introduction

The emerging importance of vasoactive peptides in the regulation of vascular tone has directed interest to enzymes which could be involved in their local generation and degradation. Bradykinin acts as a potent vasodilator by inducing the rapid release of endothelium-derived relaxing factor, nitric oxide, and prostacyclin from vascular endothelial cells (1). Angiotensin converting enzyme[1]) accounts for the major part of kininase activity in human endothelial cells (2, 3). However, the role of other enzymes in the endothelial degradation of kinins is not precisely known. Other pathways of vasoactive peptide degradation in the vascular endothelium may be of particular interest when angiotensin converting enzyme is inhibited. A second kininase II[1]), which is identical to neutral endopeptidase[1]), has been described in the brush border of the kidney (4) and appears to be involved in the renal degradation of kinins (5). We therefore investigated the presence of neutral endopeptidase and its role in the degradation of bradykinin in cultured human endothelial cells.

[1]) Enzymes
    Neutral endopeptidase = Membrane metallo-endopeptidase, EC 3.4.24.11
    Angiotensin converting enzyme = Kininase II = Dipeptidyl carboxypeptidase I, EC 3.4.15.1
    Aminopeptidase, EC 3.4.11.11
    Carboxypeptidase = Serine carboxypeptidase, EC 3.4.16.1

Materials and Methods

Cell culture

Human umbilical vein endothelial cells were prepared according to the method of *Jaffe* (6) with minor modifications (7). Cells of the second passage were used throughout all experiments. The purity and identity of the endothelial cell cultures was verified by the presence of factor VIII related antigen (Dako, Hamburg, Germany) and the uptake of fluorescence conjugated-low density lipoprotein (Paesel & Lorei, Frankfurt, Germany).

Membrane preparation of human umbilical vein endothelial cells

Confluent monolayers were washed with ice-cold 50 mmol/l Tris-HCl buffer, scraped off the dish with a rubber policeman and centrifuged at 400 g for 4 minutes. The cell pellet was lysed with ice-cold distilled water and centrifuged at 56 000 g for 35 minutes. The 56 000 g membrane fraction and the supernatant were aliquotted and frozen immediately. Protein concentration was determined by the method of *Lowry* et al. (8).

Immunostaining

Cytocentrifuge preparations of human umbilical vein endothelial cells were stained with an anti-human neutral endopeptidase antibody ALB1 (Dianova, Hamburg, Germany), using the alkaline phosphatase anti-alkaline phosphatase method (9).

Flow cytometry

The endothelial cells were labelled with fluorescence-conjugated ALB1 antibody. Cell counting and quantitation of fluorescence activity of endothelial cells were performed with a flow cytometry analyser (Becton-Dickinson, Heidelberg, Germany).

Assay for neutral endopeptidase activity in endothelial cell fractions

The assay, which has been described in detail by *Florentin* & *Roques* (10), employs dansyl-D-Ala-Gly-Phe(pNO$_2$)-Gly as the substrate for neutral endopeptidase (K_m = 45 μmol/l, V = 0.65 μmol/min). Measurements were performed with 50 μmol/l dansyl-D-Ala-Gly-Phe(pNO$_2$)-Gly in 50 mmol/l Tris-HCl buffer in the presence of phosphoramidon (10^{-8} mol/l, Sigma, München, Germany) or diluent (for total activity) at a pH of 7.4 and 37 °C over a period of 24 hours. The increase of fluorogenic activity was measured at 562 nm with a fluorescence spectrometer (Pharmacia, Freiburg, Germany). Enzymatic activity was defined as the amount of substrate (dansyl-D-Ala-Gly-Phe(pNO$_2$)-Gly) cleaved at 37 °C per minute (nmol/min · mg protein = mU/mg protein) and was calculated from the maximal slope of the degradation curve. The activity inhibited by phosphoramidon was assigned to neutral endopeptidase activity.

Degradation of bradykinin

The degradation of bradykinin was measured using intact endothelial cell monolayers, seeded into 35 mm dishes (Falcon, Heidelberg, Germany), and grown to confluency. Phosphoramidon (10^{-8} mol/l), lisinopril, (10^{-6}, ICI, Plankstadt, Germany) amastatin (10^{-5} mol/l, Sigma, München, Germany) and DL-2-mercaptomethyl-3-guanidinoethyl-thiopropionic acid (10^{-4} mol/l, Calbiochem, Frankfurt/Main, Germany) were added to the incubation medium (RPMI, 10 g/l bovine serum albumin, 10 mmol/l HEPES) 15 min before the addition of bradykinin stock solution (Sigma, München, Germany) in peptide free medium to a final concentration of 10 μg/l bradykinin.

Samples of 0.3 ml were taken at 15, 30 and 60 minutes, mixed with 0.3 ml 0.2 mol/l Tris + 0.01 mol/l EDTA buffer (pH 6.4), frozen immediately in liquid nitrogen, and stored at −70 °C until further use. In experiments with lisinopril, the bradykinin concentration was determined over an incubation period of 240 minutes. No bradykinin immunoreactivity was detected in the incubation medium, the cell supernatant and the stock solution of the used peptidase inhibitors. No bradykinin degradation was observed in the 4 hour incubation period in cell free incubation medium at 37 °C.

Bradykinin radioimmunoassay

Immunoreactive kinins were measured by a radioimmunoassay (RIA) as described previously (10, 11). The minimal amount of bradykinin distinguishable from zero was 2 pg and thus the detection limit of the RIA was 20 ng per litre of cell supernatant medium. The inter- and intra-assay coefficients of variation were 4% and 5%, respectively. The cross reactivity was less than 1% to des-9arg-bradykinin. The peptidase inhibitors added to the incubation medium did not interfere with the RIA at the concentrations used in the experiments.

Calculations and statistics

Experiments were performed in triplicate. Results are shown as means ± SEM (n = number of dishes or experiments). The bradykinin half-life was calculated by linear regression analysis in each experiment from the degradation of bradykinin during the first 60 minutes. In experiments with lisinopril, the half-life was calculated from the bradykinin degradation during the first 240 minutes. Statistical significance was analysed by the *Mann-Whitney*-U Test for unpaired samples. P values less than 0.05 were accepted as statistically significant.

Results

Immunohistochemistry

Staining with the anti-human neutral endopeptidase antibody demonstrated the presence of neutral endopeptidase antigen (fig. 1). Control staining without the anti-neutral endopeptidase antibody resulted in low unspecific binding of the alkaline-phosphatase complex. Flow cytometry analysis showed that 67 ± 6% (n = 4) of endothelial cells express neutral endopeptidase antigen on their cell membrane.

Neutral endopeptidase activity

The activity of endothelial neutral endopeptidase was measured in the 56 000 g crude membrane preparation of human umbilical vein endothelial cells. In the membrane preparation, a neutral endopeptidase activity of 167 ± 1.7 μU/mg protein (mean values ± SEM; n = 6) was measured. No neutral endopeptidase activity was detected in the supernatant of the 56 000 g fraction, which mainly consists of cytosolic particles (fig. 2).
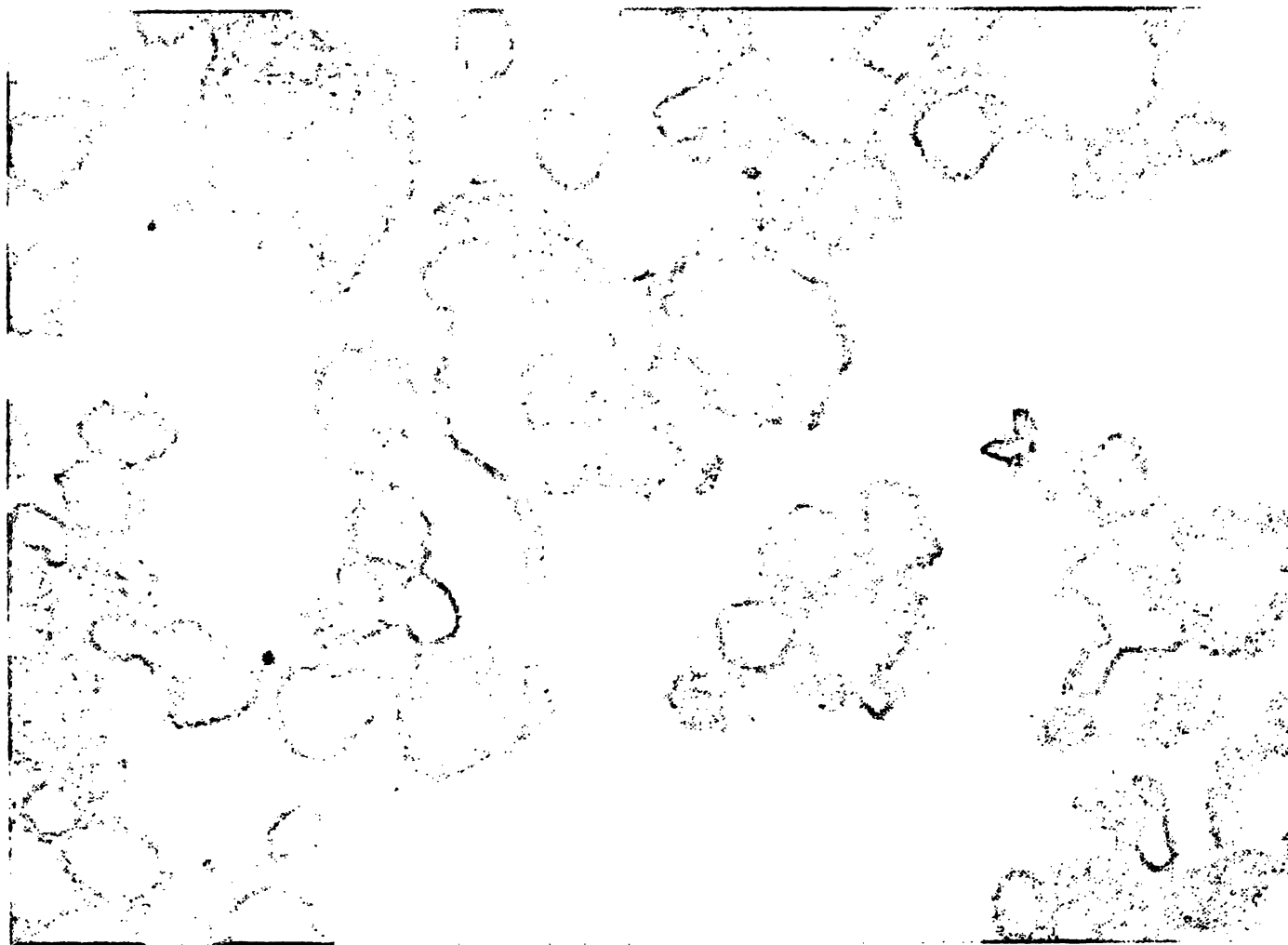
Fig. 1. Light micrograph of human umbilical vein endothelial cells stained with an antibody directed against neutral endopeptidase (haematoxylin stain, magnification × 200).
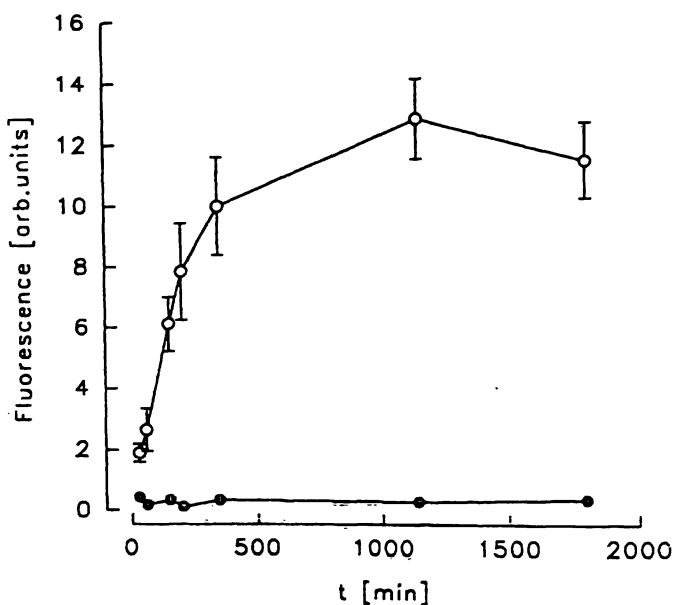

Fig. 2. Time course of dansyl-D-Ala-Gly-Phe(pNO$_2$)-Gly (50 µmol/l) degradation by crude membrane preparation of human umbilical vein endothelial cells (56 000 g fraction) (o) and the cytosol fraction (●) (n = 6 each) in 50 mmol/l Tris-HCl buffer, pH 7.4, at 37 °C. Data represent the specific neutral endopeptidase activity, which was inhibited by phosphoramidon (10^{-8} mol/l).

Endothelial degradation of bradykinin

In the supernatant of confluent endothelial monolayers, the decrease of 10^{-8} mol/l exogeneous bradykinin shows a time dependent kinetic (fig. 3) in the absence of inhibitors. The rapid degradation during the first 15 minutes is followed by a slow degradation during the following time period. In the presence of phosphoramidon (10^{-8} mol/l) significantly higher bradykinin concentrations were measured in the supernatant of human umbilical vein endothelial cells compared with the control (p < 0.05, n = 9). The angiotensin converting enzyme inhibitor, lisinopril, inhibited most of the bradykinin degradation by intact endothelial monolayers. After 240 min incubation with phosphoramidon in the presence of lisinopril, the bradykinin concentration was significantly higher than after incubation with lisinopril alone (lisinopril: 3680 ± 458 ng/l, n = 12); lisinopril + phosphoramidon: 5060 ± 568 ng/l, n = 9; p < 0.03 vs. lisinopril).
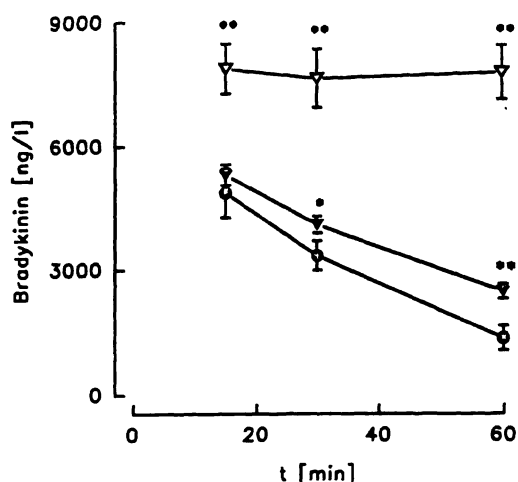
Fig. 3. Time course of degradation of exogeneous bradykinin in the absence (control, circle) and presence of lisinopril (10^{-6} mol/l, filled triangle), phosphoramidon (10^{-8} mol/l, open triangle) and the combination of phosphoramidon and lisinopril in the supernatant of a confluent monolayer of endothelial cells (human umbilical vein endothelial cells; n = 9—16, * = $P < 0.05$ vs. control, ** = $P < 0.01$ vs. control).
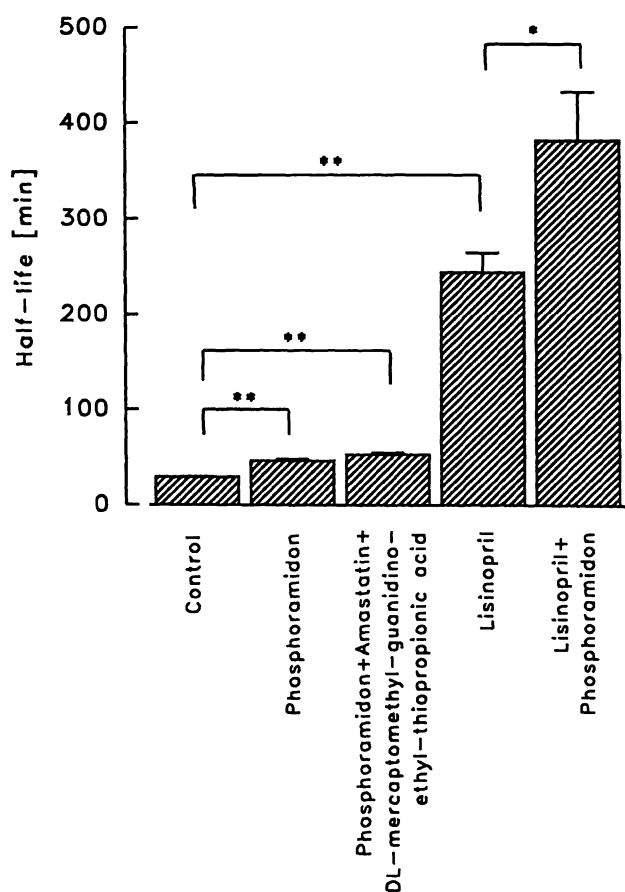

Fig. 4. Half-lives of bradykinin in the presence or absence of various peptidase inhibitors phosphoramidon, 10^{-8} mol/l; lisinopril, 10^{-6} mol/l; amastatin, 10^{-6} mol/l; DL-mercaptomethyl-guanidinoethyl-thiopropionic acid, 10^{-4} mol/l; mean ± SEM minutes; * = $P < 0.05$ vs. control, ** = $P < 0.01$ vs. control.

The half-life of bradykinin in the absence of inhibitors was 29 ± 1 minutes (n = 18) and it was prolonged up to 244 ± 20 minutes (n = 15, p < 0.005) in the presence of the angiotensin converting enzyme inhibitor, lisinopril. Phosphoramidon alone significantly prolonged the half-life of bradykinin up to 46 ± 2 minutes (n = 15, p < 0.01; fig.4). The addition of phosphoramidon to lisinopril resulted in a significant prolongation of the half-life up to 381 ± 51 minutes (n = 12, L vs. L + P p < 0.03). The half-life of bradykinin in the presence of all inhibitors (lisinopril, amastatin, phosphoramidon, DL-2-mercaptomethyl-3-guanidinoethyl-thiopropionic acid) did not exceed the half-life of bradykinin in the presence of the combination of lisinopril and phosphoramidon (L + P + A: 386 ± 52 minutes, n = 12; L + P + A + M: 275 ± 22 minutes, n = 9). The combination of both inhibitors with amastatin and DL-2-mercaptomethyl-3-guanidinoethyl-thiopropionic acid affected the half-life of bradykinin.

It is estimated that the degradation of bradykinin by neutral endopeptidase, as judged from the prolongation of the bradykinin half-life, is equivalent to about 9% of the degradation due to angiotensin converting enzyme activity.

Discussion

Our study shows that two kininases are largely responsible for the degradation of the vasoactive peptide, bradykinin, by intact endothelial cells in culture, i.e. angiotensin converting enzyme and neutral endopeptidase. The decay of the bradykinin concentration indicated that about 90% of bradykinin is degraded by angiotensin converting enzyme, whereas the minor part is cleaved by neutral endopeptidase. Aminopeptidase[1]) or carboxypeptidase[1]) activity seems not to play a role in bradykinin degradation by intact endothelial cells.

In order to verify the presence of neutral endopeptidase we performed alkaline phosphatase staining of human endothelial cells with a monoclonal mouse antibody. Quantification by flow cytometry analysis performed with the same antibody detected 67% antigen-positive cells. Since the antibody was incubated with intact living cells and could not penetrate the cellular membrane this result indicates the presence of neutral endopeptidase on the surface of endothelial cells.

This corresponds to the results of the functional assay with the artificial substrate, dansyl-D-Ala-Gly-Phe(pNO$_2$)-Gly, which show considerable neutral endopeptidase-activity in the membrane preparations of

human endothelial cells. Neutral endopeptidase activity was not, however, detected in the cytosolic fraction. The lack of detectable neutral endopeptidase activity in the soluble fraction indicates that this peptidase is mainly located in the cellular membranes of the endothelial cell, where it is in close contact with circulating peptides. The enzymatic activity measured in the membrane preparation corresponds to previously published data of *Florentin* and coworkers for various rat tissues (10).

The vascular endothelium plays an important role in the regulation of the vascular tone. We used the confluent living endothelial monolayer to study the degradation of physiologically effective concentrations of bradykinin, a potent vasodilator. Our data show that living endothelium degrades bradykinin by enzymatic cleavage, and that this was prevented by two specific inhibitors. The neutral endopeptidase inhibitor, phosphoramidon, significantly diminished the bradykinin degradation after 30 and 60 minutes, although angiotensin converting enzyme activity was not blocked. The time curve of bradykinin degradation indicates that the major decrease during the first 30 minutes is not observed in the presence of the angiotensin converting enzyme inhibitor, lisinopril. We therefore attributed this fall of bradykinin concentration to the dominant role of angiotensin converting enzyme in the endothelial bradykinin degradation.

By calculating the half-life of bradykinin in presence of intact endothelium, we detected a significant effect of phosphoramidon. This effect was pronounced when the angiotensin converting enzyme inhibitor, lisinopril, was present, indicating a physiological role of neutral endopeptidase in the degradation of bradykinin during angiotensin converting enzyme inhibition. The combination of lisinopril and phosphoramidon almost prevented the bradykinin degradation, indicating the presence of a low undefined additional

kininase activity on intact endothelial cells. These activities cannot be attributed to aminopeptidase or carboxypeptidase, since their inhibitors, amastastin and *DL*-2-mercaptomethyl-3-guanidinoethyl-thiopropionic acid, are without effect. The present data are consistent with the hypothesis of an additional role of endothelial neutral endopeptidase in the local degradation of bradykinin, besides angiotensin converting enzyme, in intact endothelium.

In a recently published study (13), which investigated bradykinin-induced vascular permeability in the hamster cheek pouch, phosphoramidon, as well as the angiotensin converting enzyme inhibitor, captopril, significantly augmented the effect of bradykinin on vascular leakage. Furthermore, neutral endopeptidase located in the vascular endothelium is involved in the cleavage of several other vasoactive peptides including substance P, chemotactic peptide (N-Formyl-Met-Leu-Phe), endothelin and angiotensin I (1, 14−18). Recently, *Solheilac* and coworkers identified neutral endopeptidase as a M_r 94000 membrane protein in vascular membranes from rat and rabbit aorta. In addition, they demonstrated the degradation of atrial natriuretic peptide by neutral endopeptidase (19).

Thus, the neutral endopeptidase, which is present in membranes of human endothelial cells, may contribute to the local degradation of bradykinin and other vasoactive peptides by the vascular endothelium, particularly when angiotensin converting enzyme inhibitors are present.

Acknowledgement

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