Regulation of Cardiovascular Signaling by Kinins and Products of Similar Converting Enzyme Systems

Endopeptidases 3.4.24.15 and 24.16 in endothelial cells: potential role in vasoactive peptide metabolism

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Endopeptidases 3.4.24.15 and 24.16 in endothelial cells: potential role in vasoactive peptide metabolism. Am J Physiol Heart Circ Physiol 284: H1978–H1984, 2003. First published February 27, 2003; 10.1152/ajpheart.01116.2002.—The closely related metalloendopeptidases EC 3.4.24.15 (EP24.15; thimet oligopeptidase) and 24.16 (EP24.16; neurolysin) cleave a number of vasoactive peptides such as bradykinin and neurotensin in vitro. We have previously shown that hypotensive responses to bradykinin are potentiated by an inhibitor of EP24.15 and EP24.16 (26), suggesting a role for one or both enzymes in bradykinin metabolism in vivo. In this study, we have used selective inhibitors that can distinguish between EP24.15 and EP24.16 to determine their activity in cultured endothelial cells (the transformed human umbilical vein endothelial hybrid cell line EA.hy926 or ovine aortic endothelial cells). Endopeptidase activity was assessed using a specific quenchable fluorescent substrate [7-methoxycoumarin-4-acetyl-Pro-Leu-Gly- d-Lys(2,4-dinitrophenyl)], as well as the peptide substrates bradykinin and neurotensin (assessed by high-performance liquid chromatography with mass spectroscopic detection). Our results indicate that both peptidases are present in endothelial cells; however, EP24.16 contributes significantly more to substrate cleavage by both cytosolic and membrane preparations, as well as intact cells, than EP24.15. These findings, when coupled with previous observations in vivo, suggest that EP24.16 activity is important in vivo.

Although both EP24.15 and EP24.16 are primarily soluble, cytosolic enzymes, membrane-associated forms have been demonstrated in a number of cell types. For example, Crack et al. (4) have detected immunoreactive EP24.15 on the extracellular membrane of intact corticotrophic tumor cells, whereas EP24.16 has been localized to the plasma membrane in neurons, both in vivo (9, 31) and in vitro (28), as well as in human kidney cells transfected with the peptidase (29). Given that neither enzyme possesses a transmembrane domain, the exact mechanism of association with the membrane is not understood. Furthermore, the expression and subcellular distribution of EP24.15 or EP24.16 in cells of the cardiovascular system has not been explored.

In the present study, we have used specific inhibitors that can distinguish EP24.15 and EP24.16 to characterize their presence in cultured vascular endothelial cells. The results show that both enzymes are present, although EP24.16 activity is higher, particularly in the membrane fraction. Furthermore, we present evidence that EP24.16 is present on the extracellular face of these cells, suggesting that it may indeed play a role in the metabolism of circulating peptides such as BK.

THE VASODILATORY PEPTIDE bradykinin (BK) is readily degraded in the circulation by a number of metalloendopeptidases, including angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP), aminopeptidase P, carboxypeptidases, and possibly endothelin-converting enzyme-1 (ECE-1) (12, 25). Recent evidence suggests that a portion of the hypotensive and particularly the cardioprotective effects of specific inhibitors of ACE and NEP are due to enhanced BK activity (5, 17, 22, 23). BK is also efficiently cleaved in vitro by the closely related peptidases EP24.15 (also known as thimet oligopeptidase) and EP24.16 (also known as neurolysin) (21). We have recently shown that hypotensive responses to BK are markedly potentiated by the administration of a stable inhibitor of these enzymes N-(1R,S)-carboxy-3-phenylpropyl-Ala-Aib-Tyr-p-aminobenzoate, termed JA2 (26). Unlike its predecessor N-(1R,S)-carboxy-3-phenylpropyl-Ala-Ala-Tyr-p-aminobenzoate (cFP), JA2 is resistant to proteolytic cleavage by NEP and does not form an ACE inhibitor within the circulation. These results suggested that EP24.15 and/or EP24.16 contribute to BK metabolism in the vasculature.
MATERIALS AND METHODS

Materials. The nonselective inhibitor cFP (K,[EP24.15] = 19 nM, K,[EP24.16] = 700 nM) was synthesized at the Baker Institute (3). JA2 (K,[EP24.15] = 23 nM, K,[EP24.16] = 690 nM) was synthesized by Dr. G. Abbenante [Centre for Drug Design and Development, University of Queensland, Australia (24)]. The specific inhibitors Pro-PheΨ(P(02CH2)3Leu-Pro-ΨNH2 (PFPC-LP-NH2; K,[EP24.15] = 66.5 μM, K,[EP24.16] = 12 nM), and Z-(L-D)-PheΨ(P(02CH2)3-(L-D)-Ala-Arg-Phe (Z-FPc, cARF; K,[EP24.15] = 0.16 nM, K,[EP24.16] = 530 nM) were synthesized as previously described (13, 30). BK, neurotensin (NT), and the specific inhibitors listed above. Cells were incubated for 60 min in a total volume of 100 μl TBS, in the presence or absence of cFP (10 μM), JA2 (10 μM), PFPC-LP-NH2 (10 μM), or Z-FPC (50 nM). In some cases, the NEP-ECE inhibitor phosphoramidon (50 μM) was also added. BK and NT (5–10 μg) were also incubated with crude endothelial membranes (5–10 μg = 20–40 μg protein) for 60–120 min in 100 μl TBS in the presence of phosphoramidon (50 μM) with or without each of the EP24.15 inhibitors listed above. Each inhibitor was tested in triplicate in each assay, and the percent inhibition was determined based on the area of the relevant product peak (BK1, NT, or NT2).

Hydrolysis of QFS by endothelial cells in culture. To assay EP24.15/EP24.16 activity on cells, EA.hy926 were plated onto six-well plates and grown to 80% confluence. Recombinant EP24.15 and EP24.16 were generous gifts of Dr. M. J. Glucksman (Finn University of Health Sciences, Chicago Medical School) and Dr. E. S. Ferro (University of Sao Paulo, Brazil), respectively.

Cell culture. Immortalized EA.hy926 endothelial cells (6) were grown to ~80% confluence on 100-mm tissue culture dishes in DMEM (ICN Biomedical; Aurora, OH) supplemented with 10% fetal bovine serum (CSL; Parkville, Victoria, Australia), antibiotics (0.5% penicillin, 0.5% streptomycin; Gibco, Grand Island, NY), 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT; Sigma Australia). Cells between passages 25 and 35 were used for experiments. Aortic endothelial cells from lambs (~1 yr old) were cultured as previously described (16) and used between passages 6 and 12. Positive staining for von Willebrand factor was obtained at both early and late passages, whereas staining for smooth muscle α-actin was negative, confirming the cells as endothelial and indicating minimal contamination by smooth muscle cells.

Preparation of soluble and membrane fractions. Confluent endothelial cells were washed in warm (37°C) Tris-buffered saline (TBS; 25 mM Tris·HCl and 125 mM NaCl, pH 7.4) and scraped off the culture plate into ice-cold TBS. The cell suspension was frozen and thawed three times, sonicated briefly on ice, and centrifuged at 100,000 g for 60 min at 4°C. The cytosolic supernatant was reserved, and the pellet was washed in fresh TBS, resuspended, and resuspended in TBS to form the crude membrane fraction. In some cases, membrane-associated enzyme activity was solubilized by resuspension of membranes in 1% (vol/vol) Triton X-100 in TBS and incubation on ice for 60 min with frequent vortexing. The solubilized membranes were then centrifuged at 100,000 g for 60 min at 4°C, and the supernatant was aliquoted and frozen. Protein content was determined by the Lowry (19) or bicinchoninic acid method (Micro BCA Protein Assay Reagent Kit, Pierce; Rockford, IL) (27).

QFS assay. Assays were performed in black flat-bottomed 96-well microtiter plates in a final volume of 250 μl TBS and included recEP24.15 (50 ng/well), recEP24.16 (20 ng/well), or EA.hy926 soluble or Triton X-100-solubilized membrane extract (~50 μg protein/well), together with 4.5 μM QFS and varying concentrations of peptidase inhibitor. In assays using recEP24.15, 0.1 mM DTT was added to activate the enzyme; preliminary experiments indicated that addition of DTT did not alter QFS cleavage by cell extracts. Reactions proceeded at 37°C for 30–120 min, within a thermostatted f/Max fluorescence microplate reader (Molecular Devices; Sunnyvale, CA) before the liberated fluorescence (λex = 320 nm, λem = 420 nm) was read. The extent of QFS degradation was <15%, as determined by comparison to a standard curve of fluorescent product (7-methoxycoumarin-4-acetyl-Pro-Leu-Calbiochem-Novabiochem, Alexandria, New South Wales, Australia).

Hydrolysis of BK and NT by cytosolic and membrane fractions. BK and NT (5–10 μg) were incubated separately at 37°C with 5–10 μl (~10–25 μg protein) of the endothelial cytosolic fraction for 60 min in a total volume of 100 μl TBS, in the presence or absence of cFP (10 μM), JA2 (10 μM), PFPC-LP-NH2 (10 μM), or Z-FPC (50 nM). In some cases, the NEP-ECE inhibitor phosphoramidon (50 μM) was also added. BK and NT (5–10 μg) were also incubated with crude endothelial membranes (5–10 μg ~20–40 μg protein) for 60–120 min in 100 μl TBS in the presence of phosphoramidon (50 μM) with or without each of the EP24.15 inhibitors listed above. Each inhibitor was tested in triplicate in each assay, and the percent inhibition was determined based on the area of the relevant product peak (BK1, NT, or NT2).

Hydrolysis of BK by endothelial cells in culture. Confluent ovine endothelial cells grown in 12-well plates were washed twice with warm HEPES-Krebs buffer [HKB: 20 mM HEPES, 103 mM NaCl, 4.77 mM KCl, 0.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 15 mM glucose, 0.25% (wt/vol) bovine serum albumin, pH 7.4], and incubated in 0.5 ml HKB per well containing 20 μg BK in the presence of 10 μM phosphoramidon with or without each of the EP24.15/16 inhibitors listed above. Cells were incubated for 4 h in triplicate in three separate experiments. Because the BK1 peak was obscured by a component of the medium, the percent inhibition was calculated based on the area of the corresponding BK6 peak.

HPLC and mass spectrometric (LC-MS) analysis. After incubation of BK or NT with subcellular fractions or with cells in culture, each reaction was stopped by addition of 4 vol methanol-1% TFA, and the samples were dried on a centrifugal vacuum evaporator (ThermoSavant; Holbrook, NY) before HPLC analysis using an Agilent 1100 series LC with on-line mass spectrometric detector (Agilent Technologies; Forest Hill, Victoria, Australia). Samples were loaded onto a Zorbax Eclipse C18 column (at 50°C) in 1.8% acetone-0.1% TFA-0.02% acetic acid at 0.15 ml/min and eluted with a 30-min linear gradient to 60% acetone-0.1% TFA. Peptide fragments were identified following mass spectral analysis using Agilent ChemStation deconvolution software.

RESULTS

Inhibition of recombinant EP24.15 and EP24.16 by specific inhibitors. The sensitivity of recombinant EP24.15 and EP24.16 to the inhibitors used in this study was confirmed by the generation of concentration-inhibition curves using the specific QFS assay. As shown in Fig. 1A, near-complete inhibition of
recEP24.15 was achieved with the specific inhibitor Z-FPCARF at just 50 nM, whereas 20% inhibition was seen at the highest concentration (50 μM) of the EP24.16 inhibitor PFPC-LP-NH2. Significant inhibition of EP24.15 was also seen with the less specific and less potent inhibitor JA2, with maximal inhibition occurring at micromolar concentrations. Recombinant EP24.16 was also inhibited by JA2 (Fig. 1B), but as reported previously (24), this peptidase is at least an order of magnitude less sensitive to this compound than is EP24.15. A similar concentration-inhibition curve was obtained with the more specific EP24.16 inhibitor PFPC-LP-NH2, whereas the EP24.15-specific compound Z-FPCARF was ineffective at the concentrations at which EP24.15 activity is substantially blocked.

Inhibition of QFS-cleaving activity in endothelial cell fractions. The effects of the EP24.15 and EP24.16 inhibitors on QFS-cleaving activity in cytosolic and solubilized membrane fractions of EA.hy926 cells are shown in Fig. 2. The sensitivity of the peptidase activity in both fractions to JA2 and PFPC-LP-NH2, together with the lesser sensitivity to Z-FPCARF, suggests that EP24.16 is the predominant QFS-cleaving enzyme in both the cytoplasm and membrane fractions of EA.hy926 cells, although EP24.15 is also likely to be present. Similar results were obtained using ovine aortic endothelial cells (data not shown).

Effect of EP24.15/16 inhibitors on BK cleavage by endothelial cell fractions. BK was readily degraded by the cytosolic fraction of both EA.hy926 and ovine aortic endothelial cells (~200 nmol·mg protein⁻¹·h⁻¹), primarily to the stable fragments BK₁₋₇ and BK₈₋₉, although BK₁₋₇ and BK₆₋₉ were sometimes also observed, particularly in EA.hy926 extracts (Fig. 3A). Because BK₁₋₇ partially coeluted with the BK₁₋₅ peak, making quantitation of the latter difficult, phosphoramidon (50 μM) was added to inhibit its generation by NEP-like enzymes. In the presence of phosphoramidon, the formation of BK₁₋₅ and BK₆₋₉ by EA.hy926 cytosol was inhibited 60–70% by cFP and JA2, and to a lesser extent (20–25%), by both PFPC-LP-NH2 and Z-FPCARF (Table 1). It should be noted that at the concentrations used, cFP and JA2 (10 μM) inhibit >95% of recombinant EP24.15 activity and >75% of EP24.16 activity; PFPC-LP-NH2 (10 μM) <15% vs. EP24.15 and >80% vs. EP24.16; and Z-FPCARF (50 nM) >85% vs. EP24.15 and <15% vs. EP24.16 (refer to Fig. 1). Thus it appears that both EP24.16 and EP24.15 are relatively minor contributors to BK degradation in the umbilical vein endothelial cell line. Indeed, other kininas, some of which appear to be cFP and JA2 sensitive, appear to be present in the cytoplasm of EA.hy926 cells.
Table 1. Bradykinin cleavage at the Phe²-Ser⁶ bond by endothelial cell fractions: effect of specific inhibitors of endopeptidases 24.15 and 24.16

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Peptidase</th>
<th>Soluble*</th>
<th>Membrane*</th>
<th>Soluble*</th>
<th>Membrane*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cFP (10 μM)</td>
<td>EP24.15/EP24.16</td>
<td>68.4±1.8</td>
<td>52.5±3.1</td>
<td>81.1±0.3</td>
<td>71.0±0.8</td>
</tr>
<tr>
<td>JA2 (10 μM)</td>
<td>EP24.15/EP24.16</td>
<td>62.2±2.1</td>
<td>43.7±1.6</td>
<td>75.7±1.3</td>
<td>58.9±3.4</td>
</tr>
<tr>
<td>Z-FPClARP (50 nM)</td>
<td>EP24.15</td>
<td>26.2±3.9</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>8.0±1.7</td>
</tr>
<tr>
<td>PFPClLP-NH₂ (10 μM)</td>
<td>EP24.16</td>
<td>21.1±3.1</td>
<td>32.2±3.2</td>
<td>81.7±1.0</td>
<td>78.1±1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. (n = 3 for EA.hy926 cells, n = 6 for ovine aortic endothelial cells) of percent inhibition, as determined from the area of the bradykinin (BK) 1–5 product peak in the presence or absence of each inhibitor at the concentration indicated. *Assayed in the presence of 50 μM phosphoramidon. See MATERIALS AND METHODS for definitions of inhibitors.
tion of cFP or JA2 significantly reduced the formation of both NT1–8 (~85% inhibition) and NT1–10 (~60% inhibition) (Table 2). Consistent with cleavage of NT at the Arg8-Arg9 bond by EP24.15, the appearance of NT1–8 was more sensitive to inhibition by Z-FPCARF than PFPCLP-NH2 (Table 2). Similarly, NT1–10 formation was inhibited to a greater extent by the EP24.16 inhibitor PFPCLP-NH2 than by the EP24.15 inhibitor Z-FPCARF (Table 2); however, in each case, some effect of the other inhibitor was observed.

NT was rapidly degraded by endothelial cell membranes, resulting in approximately equal amounts of NT1–10 and NT1–11, with a minor amount of NT1–8 (data not shown). Addition of phosphoramidon blocked the formation of NT1–11 (Fig. 4B) and was used in conjunction with the EP24.15 and EP24.16 inhibitors to limit the extent of degradation. Again, the inhibitor profile clearly suggests EP24.16 cleavage at the Pro10-Tyr11 bond (Table 2); however, formation of NT1–8 was too low to accurately assess the effects of each inhibitor.

Cleavage of QFS and BK by endothelial cells in culture. To determine whether either EP24.15 or EP24.16 is present on the extracellular surface of endothelial cells, we incubated either QFS or BK with intact cells in culture for up to 4 h. Because components of the serum-free medium interfered with LC-MS analysis of BK degradation, we used the QFS as substrate on EA.hy926 cells. Figure 5 shows the time-dependent cleavage of QFS by EA.hy926 cells, which was significantly attenuated at all time points by the addition of 10 μM JA2. Although both the EP24.15 inhibitor Z-FPCARF and the EP24.16 inhibitor PFPCLP-NH2 reduced QFS-degrading activity by

Table 2. Neurotensin cleavage by EA.hy926 cell fractions: effect of specific inhibitors of endopeptidases 24.15 and 24.16

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Peptidase</th>
<th>Soluble, %</th>
<th>Membrane NT1–10, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>cFP (10 μM)</td>
<td>EP24.15/EP24.16</td>
<td>NT1–8: 85.2 ± 0.3</td>
<td>NT1–10: 63.9 ± 2.0</td>
</tr>
<tr>
<td>JA2 (10 μM)</td>
<td>EP24.15/EP24.16</td>
<td>NT1–8: 85.2 ± 2.3</td>
<td>NT1–10: 60.1 ± 1.9</td>
</tr>
<tr>
<td>Z-FPCARF (50 nM)</td>
<td>EP24.15</td>
<td>NT1–8: 56.9 ± 6.5</td>
<td>NT1–10: 27.4 ± 2.2</td>
</tr>
<tr>
<td>PFPCLP-NH2 (10 μM)</td>
<td>EP24.16</td>
<td>NT1–8: 14.7 ± 3.5</td>
<td>NT1–10: 64.5 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 3 per group) of percent inhibition, as determined from the area of the indicated product peak in the presence or absence of each inhibitor at the concentration indicated. NT, neurotensin.
both the membrane and cytosolic preparations, cleaved no effect on BK degradation (data not shown). As in phoramidon (10^4 M) the absence of each inhibitor at the concentration indicated. Phosphoramidon (10^4 M) phosphoramidon inhibited BK1_7 production by ~60%; captopril had no effect on BK degradation (data not shown). As in both the membrane and cytosolic preparations, cleavage at the Phe^2-Ser^3 bond was significantly reduced by cFP and JA2 (~60%; Table 3). Again, the EP24.16 inhibitor was more effective than that specific for EP24.15 (45.2 ± 4.0% vs. 17.6 ± 5.5%; Table 3); however, the degree of inhibition was somewhat less than that seen with cFP or JA2. Interestingly, there was a greater effect of the EP24.15-specific inhibitor on BK degradation by the intact cells (17.6 ± 5.5%; Table 3) than by either of the subcellular fractions of ovine aortic endothelial cells (<10%; see Table 1).

**DISCUSSION**

The potentiation of hypotensive responses to exogenous BK on administration of a stable EP. 24.15/16 inhibitor JA2 (26) suggested for the first time that one or both of these metalloendopeptidases are involved in the degradation of this peptide in the circulation. However, neither the relative contributions of each enzyme nor their location within the vasculature could be determined from these in vivo studies. Because there is minimal EP24.15/16 activity present in plasma (M. U. Norman, R. A. Lew, and A. I. Smith, unpublished observations), we hypothesized that these enzymes, like many other metal-dependent kininases, are expressed by endothelial cells. The current study confirms the presence of both soluble and membrane EP24.16, and to a lesser extent EP24.15, in endothelial cells, and that these enzymes contribute to BK and NT metabolism by these subcellular fractions. Interestingly, EP24.16 was clearly more active than EP24.15 in the ovine aortic endothelial cells, whereas both peptidases contributed to BK breakdown in EA.hy926 cells. Thus endothelial cells from different vascular beds may express a different complement of peptidases. For example, ACE activity is markedly higher in arterial endothelial cells than in those from the venous circulation (14, 32), whereas NEP activity is greater in venous endothelial cells (18).

Extracellular EP24.16 activity was also observed in cultured endothelial cells, although the exact mechanism(s) by which the enzyme attains this position has yet to be determined. Studies aimed at determining the exact localization of both enzymes in endothelial cells, as well as the targeting motifs involved, are now underway. Extracellular EP24.16 has also been reported in both neurons and astrocytes in culture (28), whereas EP24.15 has been localized to the plasma membrane of AtT-20 corticotrophic tumor cells (4). Both peptidases have also been reported to be secreted from some, but not all, of these cell types (7, 8, 28). We have also reported (20) secreted QFS-cleaving activity in medium conditioned by ovine aortic endothelial cells; however, in EA.hy926 cells, activity in the medium was not consistent and did not increase with time of incubation. Thus in these cells, constitutive secretion is unlikely. It also remains possible that BK may be degraded by intracellular EP24.16 following receptor internalization. Such a scenario has been proposed for insulin degradation by insulysin, another soluble metalloendopeptidase (1), although any mechanism by which receptor-bound peptide could reach the cytoplasmic enzyme is completely unknown.

In summary, the present work demonstrates the functional presence of active EP24.16 in endothelial cells and supports the hypothesis that this enzyme may be involved in the physiological metabolism of BK. Future whole animal studies using stable, specific inhibitors of EP24.16 are necessary to confirm its precise contribution to the degradation of BK or other peptides in vivo.

**Table 3. Bradykinin degradation by intact ovine aortic endothelial cells: effect of specific inhibitors of endopeptidases 24.15 and 24.16**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Peptidase</th>
<th>Inhibition of BK1_7 Generation, %</th>
</tr>
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<tbody>
<tr>
<td>cFP (10 μM)</td>
<td>EP24.15/EP24.16</td>
<td>65.6 ± 3.6</td>
</tr>
<tr>
<td>JA2 (10 μM)</td>
<td>EP24.15/EP24.16</td>
<td>59.9 ± 4.7</td>
</tr>
<tr>
<td>Z-FP3-ARG (50 nM)</td>
<td>EP24.15</td>
<td>17.6 ± 5.5</td>
</tr>
<tr>
<td>PFP3-LP-NH2 (10 μM)</td>
<td>EP24.16</td>
<td>45.2 ± 4.0</td>
</tr>
</tbody>
</table>

Values are means ± SE of percent inhibition (n = 6 per group, derived from 2 separate experiments performed in triplicate), as determined from the area of the BK1_7 product peak in the presence or absence of each inhibitor at the concentration indicated. Phosphoramidon (10 μM) was present in the medium during incubation.

The authors thank Dr. E. S. Ferro (University of Sao Paulo, Brazil) for providing recombinant EP24.16, Dr. M. J. Glucksman (Finch University of Health Sciences, Chicago Medical School) for recombinant EP24.15, Dr. G. Abbenante (University of Queensland) for JA2 synthesis, Dr. P. J. Little (Baker Institute) for the initial isolation of ovine endothelial cells, and Dr. R. Dilley (Baker Institute) for immunohistochemistry.

This work was funded by a block grant awarded to the Baker Institute by the National Health and Medical Research Council of Australia.

_AJP-Heart Circ Physiol_ • VOL 284 • JUNE 2003 • www.ajpheart.org
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