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RESEARCH PAPER

Prolyl oligopeptidase induces angiogenesis both *in vitro* and *in vivo* in a novel regulatory manner

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BACKGROUND AND PURPOSE

A serine protease, prolyl oligopeptidase (POP) has been reported to be involved in the release of the pro-angiogenic tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (Ac-SDKP) from its precursor, 43-mer thymosin β4 (Tβ4). Recently, it was shown that both POP activity and the levels of Ac-SDKP are increased in malignant tumours. The aim of this study was to clarify the release of Ac-SDKP, and test if POP and a POP inhibitor, 4-phenyl-butanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (KYP-2047), can affect angiogenesis.

EXPERIMENTAL APPROACH

We used HPLC for bioanalytical and an enzyme immunoassay for pharmacological analysis. Angiogenesis of human umbilical vein endothelial cells was assessed *in vitro* using a 'tube formation' assay and *in vivo* using a Matrigel plug assay (BD Biosciences, San Jose, CA, USA) in adult male rats. Moreover, co-localization of POP and blood vessels was studied.

KEY RESULTS

We showed the sequential hydrolysis of Tβ4: the first-step hydrolysis by proteases to <30-mer peptides is followed by an action of POP. Unexpectedly, POP inhibited the first hydrolysis step, revealing a novel regulation system. POP with Tβ4 significantly induced, while KYP-2047 effectively prevented, angiogenesis in both models compared with Tβ4 addition itself. POP and endothelial cells were abundantly co-localized *in vivo*.

CONCLUSIONS AND IMPLICATIONS

We have now revealed that POP is a second-step enzyme in the release of Ac-SDKP from Tβ4, and it has novel autoregulatory effect in the first step. Our results also advocate a role for Ac-SDKP in angiogenesis, and suggest that POP has a pro-angiogenic role via the release of Ac-SDKP from its precursor Tβ4 and POP inhibitors can block this action.

Abbreviations

ACE, angiotensin-converting-enzyme; Ac-SDKP, acetyl-N-Ser-Asp-Lys-Pro; AMC, amino methyl coumarin; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; EIA, enzyme immunoassay; HUVEC, human umbilical vein endothelial cell; KYP-2047, 4-phenyl-butanoyl-L-prolyl-2(*S*)-cyanopyrrolidine; POP, prolyl oligopeptidase; Tβ4, thymosin β4; VEGF, vascular endothelial growth factor

Introduction

The growth of vessels is an essential physiological function in embryogenesis, development, growth and in the maintenance of adult tissues. Angiogenesis has also been implicated in various pathological conditions such as malignant tumours, wound healing and inflammation, and in the restoration of ischaemic damage (for review, see Carmeliet and Jain, 2000). In malignant tumours specifically, angiogenesis plays a key role in uncontrolled growth and metastasis because a vascular supply is necessary above a tissue size of 2–3 mm^3 (Folkman, 1971; Carmeliet and Jain, 2000). Several

physiological pro- and anti-angiogenic factors have been identified, and the families of vascular endothelial growth factor (VEGF) and angiopoietin have been well characterized (Carmeliet and Jain, 2000; Furuya *et al.*, 2009). However, other molecules and peptides can affect angiogenesis as VEGF-independent angiogenesis can be induced in cells treated with VEGF inhibitors (Shojaei *et al.*, 2007).

Thymosin β4 (Tβ4) has been shown to be associated with anti-fibrosis (Rossdeutsch *et al.*, 2008), wound healing (Malinda *et al.*, 1999), immunomodulation (Rossdeutsch *et al.*, 2008) and angiogenesis (Malinda *et al.*, 1997; Smart *et al.*, 2007a). The therapeutic potential of Tβ4 has been studied, for example, in the recovery of cardiac muscle after myocardial infarction (for reviews, see Cavasin, 2006; Rossdeutsch *et al.*, 2008). At the cellular level, Tβ4 is able to regulate the dynamics of G-actin and also affect the transcription of various genes, including those of VEGFs (Sun and Yin, 2007; Smart *et al.*, 2007a). The tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (Ac-SDKP) is released from its precursor Tβ4 by a chain of peptidases, and after its release, is rapidly inactivated by angiotensin-converting-enzyme (ACE) (Lenfant *et al.*, 1991; Cavasin *et al.*, 2004). Although the mechanism for Ac-SDKP release from Tβ4 is not clear, one of the enzymes that has been suggested to be involved in the cleaving of Tβ4, is prolyl oligopeptidase (POP; EC 3.4.21.26) (Cavasin *et al.*, 2004; Hannappel, 2010). The limit of POP hydrolytic activity is 30 amino acids (see next), and a previous cleavage step of the 43-mer Tβ4 is required to produce a suitable substrate for POP. The exact cellular effects of Ac-SDKP have not been elucidated, but it is a negative physiological regulator of haematopoiesis (Monpezat and Frindel, 1989; Bonnet *et al.*, 1992) that even has anti-inflammatory and anti-fibrotic activities (Peng *et al.*, 2001; Cavasin *et al.*, 2007). Moreover, it has been identified as a pro-angiogenic factor both *in vitro* and *in vivo* and to mimic the effects of Tβ4 (Liu *et al.*, 2003; Wang *et al.*, 2004).

POP is an 80 kDa enzyme that belongs to the POP family of serine proteases (family S9 of clan SC) (Rawlings and Barrett, 1994). POP is widely distributed in the CNS and peripheral tissues and it has been implicated in the hydrolysis of under 30-mer, proline-containing bioactive peptides, such as angiotensins, arginine-vasopressin, substance P, neurotensin and thyrotropin releasing hormone (for reviews, see Garcia-Horsman *et al.*, 2007; Myöhänen *et al.*, 2009a). Several potent substrate-like POP inhibitors have been developed, and Z-Pro-prolinal, JTP-4819, S 17092, ZTTA and 4-phenylbutanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (KYP-2047) are the most potent and widely studied *in vitro* and *in vivo* (for review, see Männistö *et al.*, 2007). In addition to cognitive disorders, POP has also been shown to be involved in several other physiological and pathological functions such as inflammation (Brandt *et al.*, 2007), multiple sclerosis (Tenorio-Laranga *et al.*, 2010), inositol-1,4,5-triphosphate (IP_3) (Williams *et al.*, 2002) and neuronal signalling in general (Schulz *et al.*, 2005; Myöhänen *et al.*, 2009b), and the regulation of the cell cycle and differentiation (Ohtsuki *et al.*, 1997; Ishino *et al.*, 1998; Moreno-Baylach *et al.*, 2008; Myöhänen *et al.*, 2008b).

Recently, Liu *et al.* (2008) observed that the protein levels of Ac-SDKP and POP are significantly increased in several malignant tumours. Previously, increased POP activities have been shown in various cancers (Goossens *et al.*, 1996; Larrinaga *et al.*, 2010), and we have also found that the POP protein levels are high in various malignant tumours (T.T. Myöhänen and P.T. Männistö, unpubl. data). Increased levels of Ac-SDKP have been found to be associated with the malignant thyroid gland cancer (Kusinski *et al.*, 2006) and acute myeloid leukaemia (Liu *et al.*, 2006). In addition, in various other solid tumours, Ac-SDKP levels are increased at least after chemotherapy (Liozon *et al.*, 1995; Comte *et al.*, 1997). Also, administration of Ac-SDKP during chemotherapy has been shown to reduce the haemotoxicity of the treatment (Bogden *et al.*, 1991). However, even though Ac-SDKP is able to inhibit cell proliferation in normal cells, it does not have a similar effect on cancerous cells (Bonnet *et al.*, 1992; Cashman *et al.*, 1994). This indicates that the increased levels of Ac-SDKP in malignant tumours are associated with angiogenesis rather than inhibition of cell proliferation.

The objective of this study was to clarify the role of POP in Tβ4 processing and its consequent involvement in the angiogenic processes using three approaches. Firstly, we wanted to prove that an initial hydrolysis of the 43-mer Tβ4 is required before POP can affect the tetrapeptide releasing pathway. Secondly, we studied the effects of the active POP protein itself and a specific and well-characterized POP inhibitor 4-phenyl-butanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (KYP-2047; Venäläinen *et al.*, 2006; Jalkanen *et al.*, 2007) on the angiogenesis of endothelial cells *in vitro*. Finally, we determined whether POP is also able to induce vessel formation *in vivo* using the Matrigel plug assay in rats, and if so whether this could be reversed by KYP-2047. In conclusion, in this study we revealed that POP is a second-step enzyme in the release of Ac-SDKP from Tβ4, and has novel autoregulatory effects in the first step of this process. Our results also indicate the role of Ac-SDKP in angiogenesis, and suggest that POP has a pro-angiogenic effect via the release of Ac-SDKP from its precursor Tβ4 that can be blocked by POP inhibitors.

Methods

Chemicals

All chemicals used were purchased from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. Ethanol was purchased from Altia (Helsinki, Finland). Human recombinant POP was prepared as described previously (Venäläinen *et al.*, 2006). Synthetic Tβ4 was purchased from Bachem (Product# H-2608; Bubendorf, Switzerland) and KYP-2047 was synthesized in the University of Eastern Finland as previously described (Jarho *et al.*, 2004).

The concentrations of KYP-2047 (0.1 μM and 0.5 μM in tissue homogenates, and 5 μM and 10 μM in cellular and *in vivo* studies) were high enough to fully inhibit POP activity (K_i value of KYP-2047 is 0.023 nM; Venäläinen *et al.*, 2006).

The drug/molecular target nomenclature conforms to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2009).

Animals and tissue preparation

Young Wistar rats (aged 3–4 months; weight 250–350 g; n = 30; 5 for the Ac-SDKP enzyme immunoassay determinations and POP enzyme activity measurements, 25 for the Matrigel

plug assay) were supplied by the National Laboratory Animal Centre, University of Helsinki. Room temperature was 22°C and light/dark cycle was 12 h/12 h. Animals had free access to food and water.

For Ac-SDKP determinations and enzyme activity measurements, rats were deeply anaesthetized using pentobarbital (100 mg·kg^{-1}; Orion Pharma, Espoo, Finland) and then perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) for 5 min to reduce the possible background level induced by POP in the plasma. Kidneys were removed, quickly frozen in liquid nitrogen and thereafter stored at –70°C until homogenized with an ultrasound homogenizer (RincoUltrasonics, Arbon, Switzerland) in 5 volumes of assay buffer (0.1 M Na–K-phosphate buffer, pH 7.0) containing 10 μM lisinopril (Toronto Research Chemicals, North York, Canada). The purpose of adding lisinopril, a selective ACE inhibitor, was to prevent Ac-SDKP degradation caused by ACE during the sample processing. The homogenate was centrifuged at 10 000× *g*, 4°C, for 20 min. Aliquots of supernatant were frozen and stored at –70°C.

All animal procedures were conducted according to the Council of Europe (directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Southern Finland.

Peptide digestion assay

The assay mixture (140 μL) was composed of 50 mM Tris-HCl (pH 7.4) and recombinant POP [0.625 μM, equivalent to an activity of 4 nmol of amino methyl coumarin (AMC) released·min^{-1}; see below activity assay], in the presence or absence of KYP-2047 (10 μM). A 30 min pre-incubation of the kidney homogenate was performed in the reaction buffer with or without recombinant POP (0.625 μM) or KYP-2047 (10 μM) prior to the addition of the pre-warmed (30°C) synthetic Tβ4 at a final concentration of 50 μM. The reaction was carried out at 30°C for 60 min and stopped by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.1%. The resultant mixture was centrifuged for 30 min at 10 000× *g* and the supernatant was filtered and applied to a reversed-phase HPLC column C-18 5 μm (Licrospher; Merck, Darmstadt, Germany) and peptides were eluted with a 25 min linear gradient of acetonitrile (10–80%) in 0.1% TFA. To test the effect of caspase inhibition on the reaction, 25 μM of Boc-Asp(OMe)-CH2F (BAF) (Calbiochem, Merck) was included during the pre-incubation. Peptides were identified by electrospray ionization combined to tandem mass spectrometry at the Protein Chemistry Core Facility, Institute of Biotechnology, University of Helsinki.

Effect of KYP-2047 on Ac-SDKP synthesis in vitro

For Ac-SDKP determinations by enzyme immunoassay (EIA, see next) and POP activity measurements, rat kidney homogenates (approximately 1.55 mg of protein in 100 μL per well) were used in the following groups and incubated at 37°C for 80 min: (i) 0.5 μM KYP-2047 + 2 μM Tβ4; (ii) 0.1 μM KYP-2047 + 2 μM Tβ4; (iii) 2 μM Tβ4; and (iv) tissue homogenate alone (negative control). Stock solutions of KYP-2047 (1 mM) were prepared in 5% Tween 80 and then diluted to their final concentration with PBS. The final concentration of Tween 80 was under 0.005%. Determinations (Ac-SDKP and POP activity) were made at 0, 20, 40 and 80 min of incubation. The experiments were repeated with homogenates from the kidneys of five different animals, and the assay was replicated three times. Kidney homogenates were chosen because the Ac-SDKP that has been shown has a POP-related anti-fibrotic effect in the kidney cortex (Cavasin *et al.*, 2007).

Ac-SDKP

Ac-SDKP in tissue homogenates was measured as described previously (Cavasin *et al.*, 2004) using a commercially available EIA kit (Product# A05881, SPIBio, Montigny le Bretonneux, France). An aliquot of tissue homogenate was diluted 1:5 in assay buffer (SPIBio), and thereafter extracted and processed according to the EIA kit manufacturer's instructions (SPIBio). Fluorescence was read at 405 nm wavelength by a Wallac Victor2 fluorescence plate reader (PerkinElmer, Waltham, MA, USA) and Ac-SDKP concentrations were calculated by using GraphPad Prism (version 5.0, GraphPad Software, Inc., San Diego, CA, USA). The average amount of Ac-SDKP (nmol·mg^{-1} tissue) was calculated from four different measurements.

POP enzyme activity assay

The level of POP activity in kidney homogenates and human umbilical vein endothelial cells (HUVECs) was determined as described previously (Myöhänen *et al.*, 2008b). Briefly, enzyme solution (approximately 155 μg of protein in 10 μL of tissue homogenates) was pre-incubated with 465 μL of assay buffer for 30 min at 30°C. The reaction was initiated by adding 25 μL of substrate (4 mM Suc-Gly-Pro-AMC) and the plates were incubated for 60 min at 30°C. The reaction was terminated by the addition of 500 μL of 1 M sodium acetate buffer (pH 4.2). The formation of AMC was measured fluorometrically using a Wallac Victor2 fluorescence plate reader (PerkinElmer). The excitation and emission wavelengths were 360 and 460 nm respectively. The POP activities in HUVEC cells were analysed using a method described by Moreno-Baylach *et al.* (2008). The protein concentration in the enzyme preparation was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) based on the method of Bradford (Bradford 1976) with bovine serum albumin as the standard.

HUVEC line

HUVECs were purchased from 3H Biomedical (Uppsala, Sweden; Product# 3000). Cells were cultured with endothelial cell medium (Product# SC1001; 3H Biomedical) containing 1% endothelial cell growth supplement (Product# 1052; 3H Biomedical), 5% fetal bovine serum (FBS; Gibco/Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (3H Biomedicals). Cells were used at passages 3 to 5.

Matrigel 'tube formation' assay

The Matrigel tube formation assay (Liu *et al.*, 2003; Wang *et al.*, 2004; Smart *et al.*, 2007a), a commonly used cellular model of angiogenesis where spontaneous formation of capillary-like structures by endothelial cells on a basement membrane matrix preparation occurs (Matrigel, Catalog no. 356237, BD Biosciences, San Jose, CA, USA), was used to

assess the effect of POP and KYP-2047 on angiogenesis. The 48-well plates (Lab-Tech, Nunc, Roskilde, Denmark) were coated with 150 μL Matrigel [diluted 1:1.5 with Dulbecco's Modified Eagle Medium (DMEM); Gibco/Invitrogen] that was allowed to solidify for 30 min at 37°C. HUVEC (50 000 cells per well; 3H Biomedical) were plated onto the surface of the Matrigel in DMEM containing 1.5% FBS. The following groups were studied: (i) 10 μM KYP-2047 + 4 μM Tβ4 (Bachem); (ii) 5 μM KYP-2047 + 4 μM Tβ4; (iii) 0.625 μM human recombinant POP + 4 μM Tβ4; (iv) 4 μM Tβ4 (positive control); and (v) DMEM alone (negative control). The effect of KYP-2047 (10 and 5 μM) on tube formation without Tβ4 addition was also tested. A stock solution of KYP-2047 was made with 5% Tween 80 and then diluted to final concentrations with PBS. POP-immunofluorescence was determined in the HUVEC and Matrigel using the method described previously.

After 4 and 6 h incubation at 37°C in a 5% CO_2 humidified atmosphere, cellular organization into tubular structures was investigated using a Nikon Eclipse TE300 microscope (Nikon Corporation, Tokyo, Japan) with Cool Snap Pro digital camera (Meyer Instruments, Houston, TX, USA); two different areas of each well were photographed. Each study group had three different wells in each assay and four assays were performed. Formed endothelial tubes were counted and averaged by two independent observers. The addition of KYP-2047, POP or Tβ4 did not affect cell viability (observed for up to 8 h).

Matrigel plug assay

The Matrigel plug assay was used to assess the effect of KYP-2047 and POP on angiogenesis *in vivo*. The experiment was performed as previously described (Liu *et al.*, 2003). Similar treatments to those used in the Matrigel tube formation assay were prepared and added to 0.8 mL of Matrigel (BD Biosciences). Matrigel containing the test substances was injected s.c. to the back of 8- to 12-week-old male Wistar rats (300–440 g, $n = 25$, five per group). After 5 days, the rats were decapitated, and the Matrigel plugs were removed and fixed in 4% paraformaldehyde. The plugs were embedded in paraffin, sectioned using a microtome (Leica SM2000R, Leica Microsystems Inc., Wetzlar, Germany), and stained for CD-31 immunofluorescence and POP/CD-31 double-label immunofluorescence as described next. Four sections from each series were haematoxylin-eosin (H&E) stained to detect the Matrigel plug. H&E stained sections were examined by light microscopy (Nikon Eclipse TE300 microscope, Nikon Corporation) and photomicrographs were taken by a CoolSnapPro digital camera (Meyer Instruments) attached to the microscope. Immunofluorescence micrographs of CD-31 and POP were obtained as described next, and the number of CD-31 immunoreactive cells from four to seven fields of each section were counted and averaged; four to six sections of each animal were taken for counting.

Immunofluorescence

CD-31 immunofluorescence for the Matrigel plug assay (see previous) was performed, modifying the earlier protocol (Myöhänen *et al.*, 2008b). CD-31 is a commonly used endothelial cell marker that is expressed specially in developing tumour vessels. Commercial rabbit anti-CD-31 antibody (Product# ab28364, AbCam, Cambridge, UK) has been tested for specificity using Western blot and used before in similar studies (Wake *et al.*, 2009).

Briefly, the sections were deparaffinized and the antigen retrieval was processed in a microwave oven in citrate buffer (pH 6.0). Non-specific binding was blocked with 10% normal goat serum (Product# S-1000; Vector Laboratories, Burlingame, CA, USA) in PBS, pH 7.4. The slides were incubated overnight at room temperature with rabbit anti-CD-31 (dilution 1:200 in 1% goat normal serum; Vector Laboratories), followed by washing with PBS. The slides were then incubated with the goat anti-rabbit IgG fluorescein-conjugated secondary antibody (dilution 1:300 in 1% goat normal serum; Product# 31583, Pierce Biotechnology, Rockford, IL, USA) for 60 min at room temperature. After being washed with PBS, the slides were mounted with Vectashield with 4′,6-diamidino-2-phenylindole (DAPI; Product# H-1200; Vector Laboratories) to detect the nuclei of the cells. Control stainings for immunofluorescence were carried out by omission of primary antibodies. No evidence of any staining was observed in these negative controls (data not shown).

Immunofluorescence photomicrographs were captured by an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) and MicroFire True Color digital camera (Optronics, Goleta, CA, USA) with PictureFrame imaging software (Optronics). Only minor corrections to brightness and contrast of the pictures were made with Adobe Photoshop CS2 software (version 9.0, Adobe Systems Incorporated, San Jose, CA, USA).

Double-label immunofluorescence

In the co-localization studies of CD-31 and POP in the Matrigel plug assay, a double-label immunofluorescence was used, as previously described, using a POP-specific chicken anti-POP antibody prepared and characterized by Myöhänen *et al.* (2008a). Briefly, after a CD-31 immunofluorescence procedure with goat anti-rabbit IgG fluorescein-conjugated secondary antibody, POP immunofluorescence was measured as described previously (Myöhänen *et al.*, 2008a). Non-specific binding was blocked with 15% rabbit normal serum rabbit (Product S-5000; Vector Laboratories) after which the sample was incubated overnight with the chicken anti-POP antibody (dilution 1:250 in 1% rabbit normal serum). After being washed with PBS, the slides were incubated with anti-chicken IgY Texas Red-conjugated secondary antibody (dilution 1:500 in 1% rabbit normal serum; Product# ab6751, rabbit anti-chicken IgY Texas Red-conjugated, Abcam) for 60 min at room temperature. The slides were then washed with PBS and mounted with Vectashield with DAPI (nuclear marker; Vector Laboratories). Wavelengths for fluorescein were 494 nm (excitation) and 512 (emission), and for Texas Red 596 nm and 620 nm respectively.

Double-label immunofluorescence photomicrographs were captured and modified as described previously. The co-localization of CD-31 with POP was assessed by merging immunofluorescence pictures with Adobe Photoshop CS2 software (version 9.0, Adobe Systems Incorporated).

Data analysis and statistical procedures

Statistical analyses were performed using GraphPad Prism (version 5.0, GraphPad Software, Inc.). To detect differences between the groups in Ac-SDKP formation EIA assay, and in tube formation and Matrigel plug assays, one-way ANOVA with Newman–Keuls multiple comparison *post hoc* test was used. Differences with P values <0.05 were considered to be statistically significant.

Results

Bioanalytical evaluation of the thymosin β4 processing in the kidney homogenate

The ability of recombinant POP to hydrolyze pure Tβ4 was tested *in vitro* by analysing the incubation products by HPLC. Somatostatin-28 (1–12) was used as a positive control as it has been previously described as a POP substrate (Tenorio-Laranga *et al.*, 2009). We found that the recombinant POP was not able to cleave whole 43-mer Tβ4, while it was effective at hydrolyzing somatostatin-28 (1–12) (Figure 1). This result is in agreement with the fact that POP can only cleave peptides <30-mer (Polgar, 1994).

Using the same technique, we analysed the degradation products of Tβ4 after incubation with kidney homogenate in the presence of an ACE inhibitor lisinopril (10 μM) to inhibit the metabolism of Ac-SDKP. We observed that kidney homogenate was able to cleave Tβ4 efficiently, and we identified four products of this reaction (Figure 2A). Peak 1 corresponded to the 38-mer peptide Met-8-Ser-44 derived from Tβ4 (Tβ4 7–44). The peak 2 corresponded to a peptide with the same sequence as Tβ4 7–44 but was lacking the first Met residue (Tβ4 8–44). Peak 3 was the N-terminal sequence

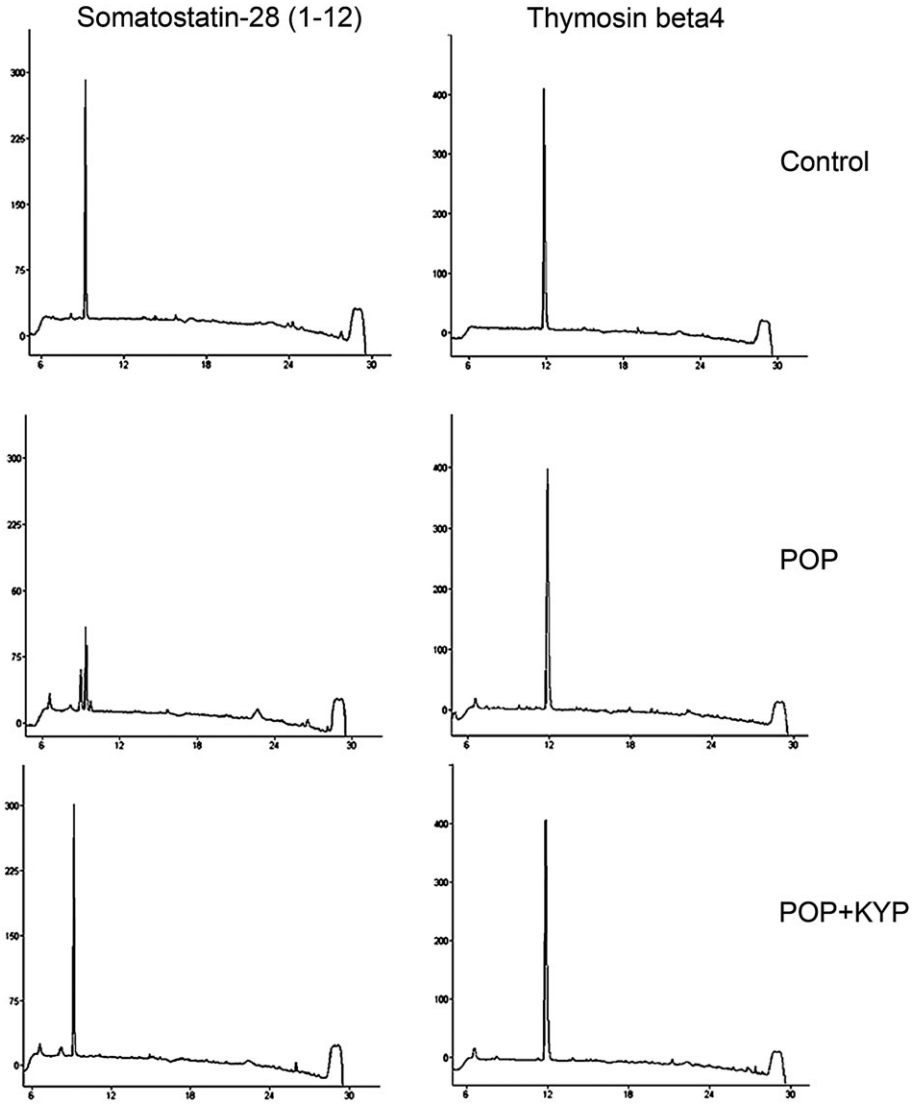

Figure 1

Full-length thymosin β4 (Tβ4) is not cleaved by recombinant prolyl oligopeptidase (POP) *in vitro*. RT-HPLC profiles of the peptide mixture, containing only pure Tβ4 peptide (control), peptide and recombinant POP (0.625 μM; POP) and peptide, recombinant POP and 4-phenyl-butanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (KYP-2047; 0.5 μM; POP + KYP). Somatostatin-28 (1–12) was degraded by POP and used as a positive control.

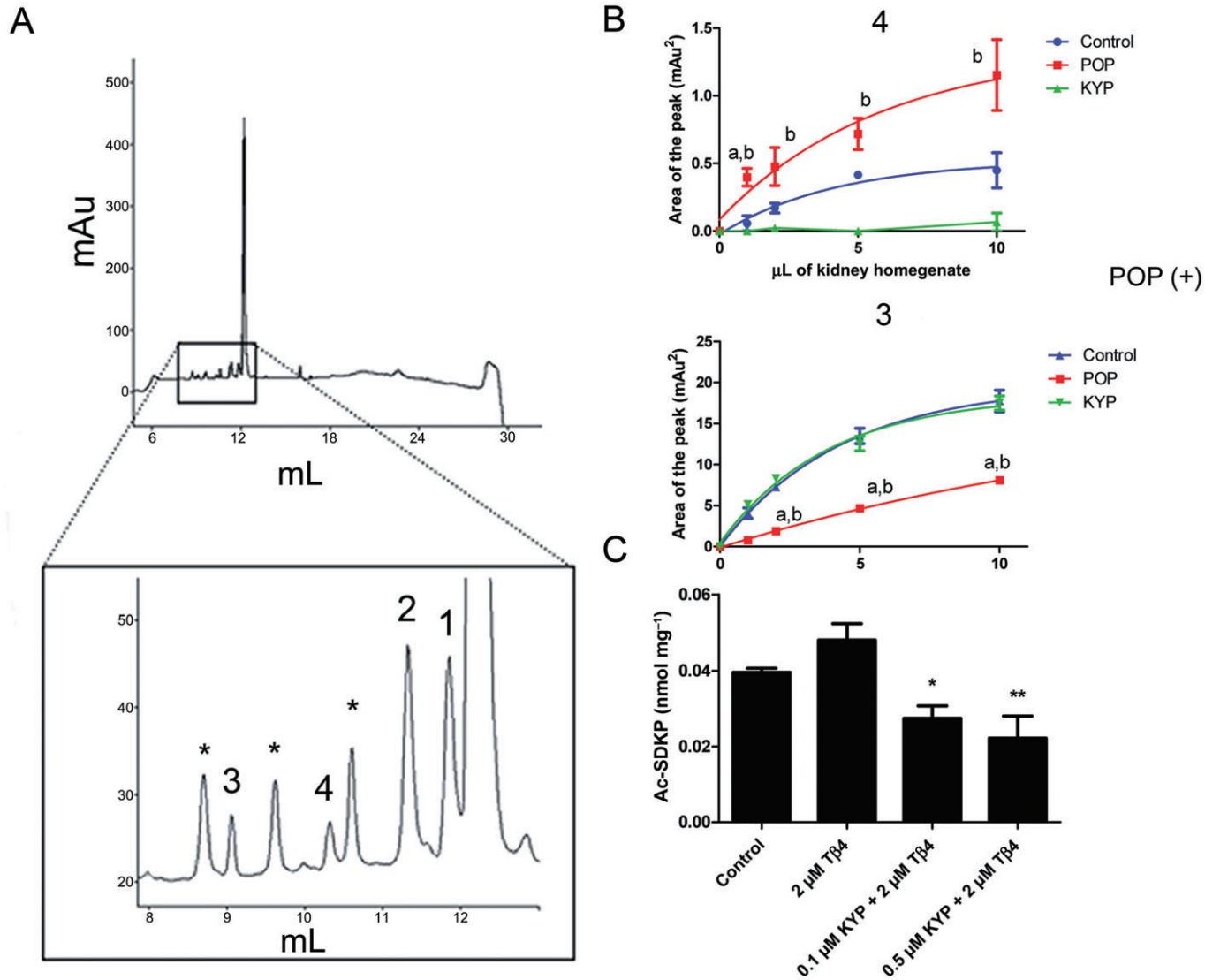

Figure 2

Characterization of the main products of thymosin β4 (Tβ4) after incubation with kidney homogenate and the effect of prolyl oligopeptidase (POP) on acetyl-N-Ser-Asp-Lys-Pro (Ac-SDKP) release. Identification of the HPLC elution peaks corresponding to 50 µM Tβ4 degradation products in the presence of kidney homogenates (A). Sequences from Tβ4 degradation (1–4, * unrelated peptides present in kidney homogenate control) have been identified by mass spectrometry and Edman N-terminal sequencing. (B) The profiles of the two last products in Tβ4 degradation at different amounts of kidney homogenate. POP clearly increases the amount of Ac-SDKP (4) while a POP inhibitor, 4-phenyl-butanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (KYP-2047), decreases the Ac-SDKP levels. At the previous degradation step (Tβ4 2–7; 3), the addition of POP lowers the levels of the substrate. The results of HPLC analysis and the effect of POP inhibitor on Ac-SDKP levels were confirmed by enzyme immunoassay (C). After an 80 min incubation of kidney homogenates with Tβ4 addition (2 µM), KYP-2047 significantly reduced the amount of Ac-SDKP at concentrations of 0.1 µM ($P < 0.05$ compared with 2 µM Tβ4 group) and 0.5 µM ($P < 0.01$ compared with 2 µM Tβ4 group). (B) Control, pure kidney homogenate; POP, kidney homogenate in presence of 0.625 µM recombinant POP; KYP, kidney homogenate in presence of 0.5 µM KYP-2047. a, $P < 0.05$ POP addition compared with control; b, $P < 0.05$ POP addition compared with KYP-2047. *$P < 0.05$; **$P < 0.01$.

Ac-SDKPDM which corresponded to the acetylated peptide containing the first six residues of Tβ4 (Tβ4 2–7). According to these results, Tβ4 has two initial cleavage sites; one between Asp-6 and Met-7, and the other between Met-7 and Ala-8 of Tβ4, producing two different N-terminal fragments, Tβ4 (2–6) and Tβ4 (2–7) (peak 3), both containing the Ac-SDKP (Tβ4 2–5) tetrapeptide (peak 4). In order to clarify the possible proteases cleaving between residues 6 and 7, or 7 and 8 of Tβ4, we analysed the sequence using the MEROPS database (Rawlings *et al.*, 2010). This analysis gave high scores for digestion with caspases 1, 3, 6, 7 for the cleavage in those positions. Accordingly, we determined whether the caspases are involved in the generation of these peptides by using specific inhibitors. Incubation of the digestion mixture in the presence of BAF, a general caspase inhibitor, had no effect on the digestion pattern of Tβ4 or on the levels of the peptide, as determined by HPLC, indicating that caspases have no role in the processing of Tβ4 (data not shown).

We also investigated the effect of POP or POP inhibitors on the processing of Tβ4 by kidney homogenate. We measured, in the presence of lisinopril (10 μM), the formation of various peptides that were identified by their relative amounts in HPLC, after incubations of Tβ4 (50 μM), plus the kidney homogenate in the presence or absence of either recombinant POP or KYP-2047 (Figure 2B). Synthetic Ac-SDKP was used as a standard for the calculation of peptide levels. After a 30 min incubation with increasing amounts of kidney homogenate, the addition of recombinant POP decreased the levels of Tβ4 2–7 (peak 3) and increased considerably the levels of Tβ4 2–5 (Ac-SDKP, peak 4; Figure 2B). On the other hand, KYP-2047 did not have any effect on the Tβ4 2–7 peptide but dramatically decreased the level of the Tβ4 2–5 peptide (Figure 2B). These results demonstrate that POP is indeed responsible for the formation of the tetrapeptide Ac-SDKP from Tβ4. When the changes in the larger fragments, that is, whole Tβ4, Tβ4 7–44 and Tβ4 8–44, were analysed after the incubations with kidney homogenates and upon the addition of POP or KYP-2047, an opposite effect of these peptide levels occurred (Figure 3A–C.) For example, the disappearance of the whole-length Tβ4 upon incubation with the homogenate, caused by unidentified endogenous proteases, was significantly reduced when measured in the presence of POP (Figure 3A–C), while KYP-2047 did not have a clear effect on Tβ4. Similarly, the release of the initial cleavage products, Tβ4 7–44 and Tβ4 8–44, were significantly reduced when POP was added (Figure 3B–C). These observations strongly suggest that POP has a negative effect on the proteolytic activity responsible for the initial cleavage at sites Asp6-Met7 and Met7-Ala8 of Tβ4.

Pharmacological evaluation of Tβ4 processing in the kidney homogenate: effect of KYP-2047 on the release of Ac-SDKP in tissue homogenates

We wanted to confirm the effect of KYP-2047 on the formation of pro-angiogenic Ac-SDKP from its precursor, Tβ4. To this end, the Ac-SDKP concentrations from rat kidney homogenates were measured by a specific EIA. Also, the enzymatic activity of POP was followed.

Both KYP-2047 concentrations (0.1 μM and 0.5 μM) significantly inhibited POP enzyme activity in tissue homogenates ($P < 0.01$, data not shown). Similar to the results from the HPLC assay, both concentrations of KYP-2047 significantly ($P < 0.05$ compared with the addition of 2 μM Tβ4 without KYP-2047) prevented the release of Ac-SDKP from Tβ4 after 80 min of incubation with rat kidney homogenates (Figure 2C). At 0.5 μM, the effect was significant ($P < 0.05$) even at 40 min (data not shown). However, the addition of exogenous Tβ4 (2 μM) did not increase the Ac-SDKP levels over the negative control levels (Figure 2C).

Effect of KYP-2047 on HUVEC angiogenesis in the 'tube formation' assay

We tested the effect of KYP-2047 on angiogenesis *in vitro* by measuring the tube formation of HUVEC, a commonly used cellular model of angiogenesis (Liu *et al.*, 2003; Wang *et al.*, 2004). Of note, these cells have a fairly high capacity to form tubes spontanenously. The addition of Tβ4 (4 μM), that is itself pro-angiogenic, only slightly increased tube formation compared with the negative control group in this model (Figure 4A,B). However, when 0.625 μM POP was added

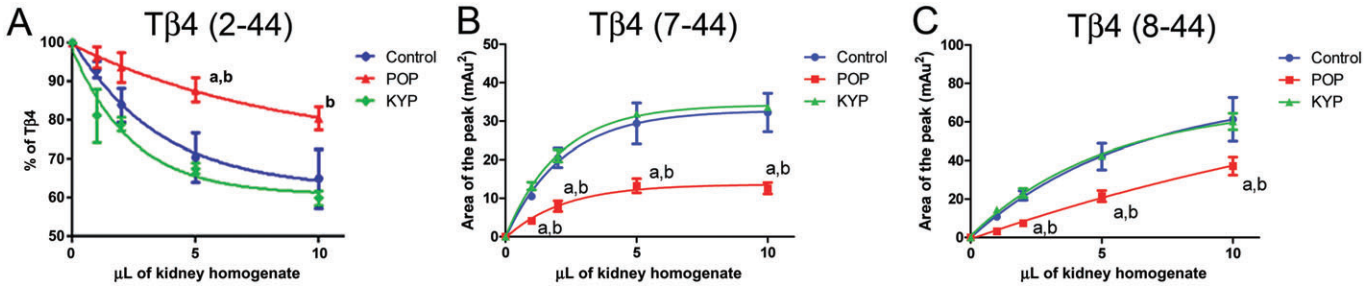

Figure 3

Prolyl oligopeptidase (POP) inhibits the first cleavage during the processing of thymosin β4 (Tβ4) in rat kidney homogenates. The addition of recombinant POP (0.625 μM) inhibited the degradation of exogenously added Tβ4 (50 μM; A), and also decreased the amounts of its two main fragments, Tβ4 (7–44) (B) and Tβ4 (8–44) (C) (see sequences and details in Figure 2). A specific POP inhibitor, 4-phenyl-butanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (KYP-2047), did not have any effect on degradation of Tβ4 or its fragments. The conditions were: control, pure kidney homogenate; POP, kidney homogenate in presence of 0.625 μM recombinant POP; KYP, kidney homogenate in presence of 0.5 μM KYP-2047. a, $P < 0.05$ POP addition compared with control; b, $P < 0.05$ POP addition compared with KYP-2047.

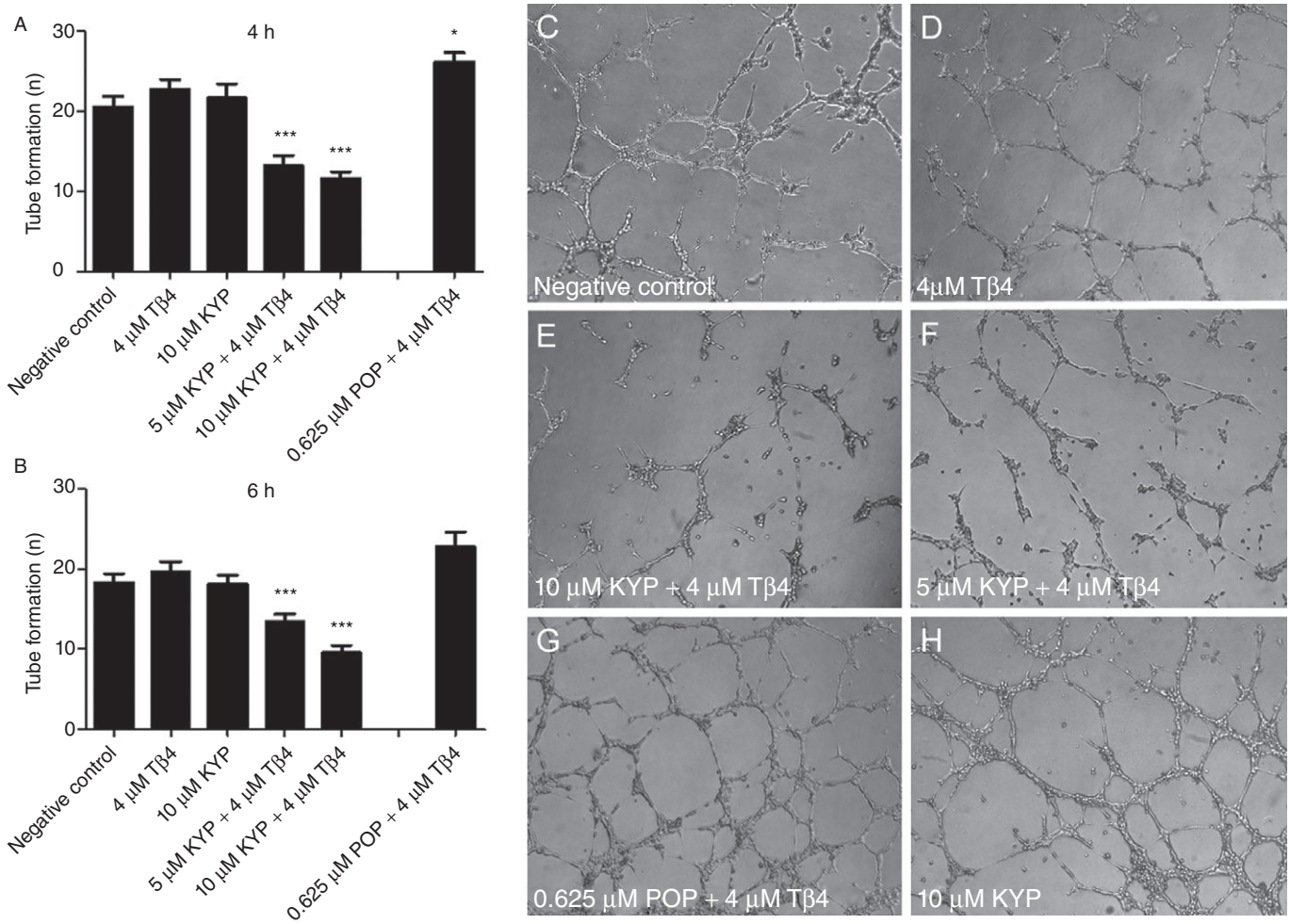

Figure 4

The effect of KYP-2047 and human recombinant prolyl oligopeptidase (POP) protein on tube formation of human umbilical vein endothelial cell (HUVEC) *in vitro*, assessed using the Matrigel tube formation assay. Both doses of 4-phenyl-butanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (KYP-2047) with 4 μM thymosin β4 (Tβ4) significantly decreased the endothelial tube formation when compared with 4 μM Tβ4 group (A, B; $P < 0.001$). The addition of 0.625 μM POP with 4 μM Tβ4 clearly increased the tube formation at 4 h time point (A; $P < 0.05$) but not at 6 h time point. Interestingly, the addition of Tβ4, thought to serve as a positive control, did not increase the tube formation above the control group at either time point (A,B), probably due to the lack of internal POP in these cells. When 10 μM of KYP-2047 was added without Tβ4, it had no effect on tube formation (A,B,H; 10 μM KYP). Representative photomicrographs are shown in the right panel (C–H), depicting the tube formation of HUVEC under the different conditions at the 4 h time point. ***$P < 0.001$; *$P < 0.05$.

together with 4 μM Tβ4, there was a significant elevation of tube formation at the 4 h time point ($P < 0.05$ compared with 4 μM Tβ4 alone; Figure 4A,B,G). The small effect of Tβ4 itself on tube formation may be explained by low amounts of POP protein and POP enzyme activity in HUVEC (and none in Matrigel; data not shown), preventing the effective release of Ac-SDKP from the intermediates. The addition of recombinant POP increases the processing of intermediate peptides and the release of Ac-SDKP, leading to elevated angiogenesis of HUVEC.

At both concentrations (5 and 10 μM), KYP-2047 significantly ($P < 0.001$ compared with 4 μM Tβ4 alone) reduced the tube formation of HUVEC to below the basal level both after 4 and 6 h (Figure 4A,B,E,F). There was no significant difference between the effects of the two KYP-2047 concentrations at 4 h (Figure 4A), but after 6 h, 10 μM KYP-2047 was more effective than 5 μM ($P < 0.05$, data not shown). KYP-2047 alone, without the addition of Tβ4, did not have an effect on tube formation (Figure 4A,B,H), excluding possible off-target effects. A sufficient amount of Tβ4 is needed to produce enough intermediates to act as POP substrates, and therefore the role of active POP in the formation of Ac-SDKP is critical.

Effect of KYP-2047 on angiogenesis in vivo *in Matrigel plug assay*

The effects of POP and KYP-2047 on angiogenesis *in vivo* were determined by the use of the Matrigel plug assay (Liu *et al.*, 2003; Wang *et al.*, 2004). CD-31 immunofluorescence was used to monitor the formation of endothelial cells in the Matrigel plug assay (He *et al.*, 2009; Wake *et al.*, 2009). At both KYP-2047 concentrations (5 and 10 μM), CD-31 immunoreactivity was significantly reduced when compared with

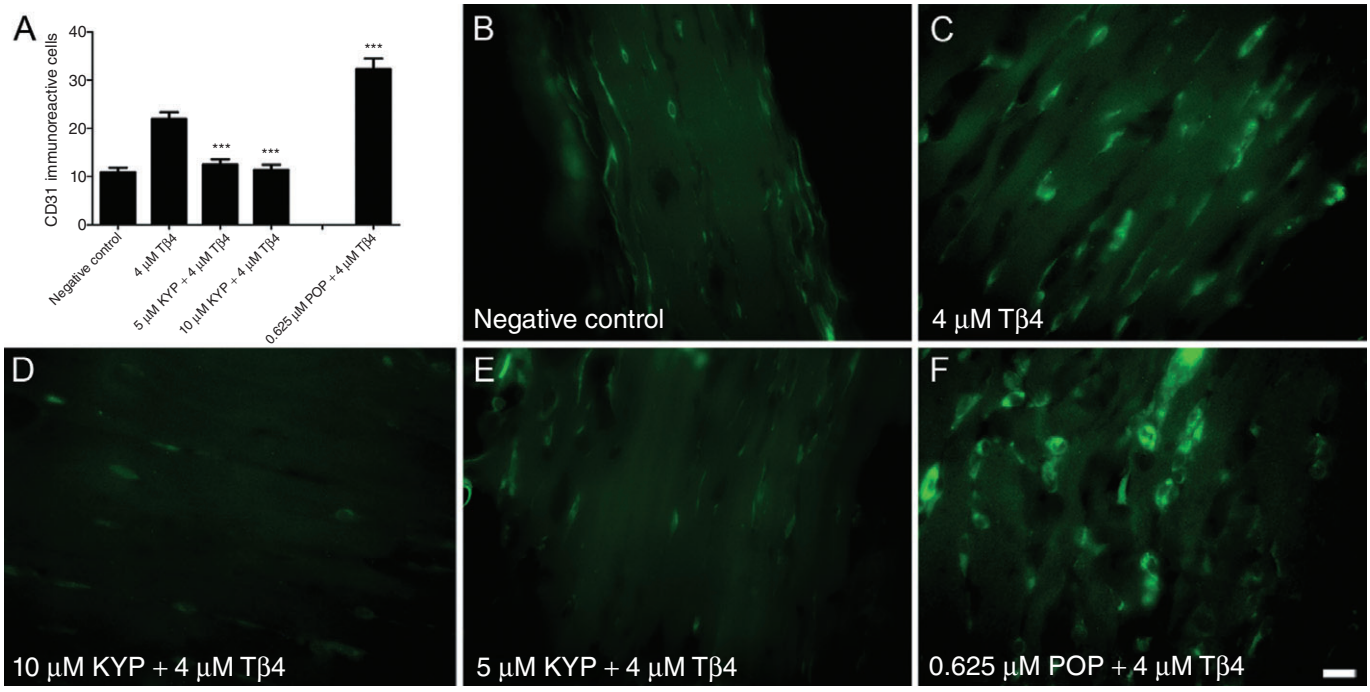

Figure 5

The effect of 4-phenyl-butanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (KYP-2047) and human recombinant prolyl oligopeptidase (POP) protein on angiogenesis *in vivo* in Matrigel plug assay. Both doses of KYP-2047 inhibited angiogenesis measured by CD-31 immunoreactivity to the control level [A; $P < 0.001$ compared with thymosin β4 (Tβ4) group] in Matrigel plug assay. Moreover, the addition of 0.625 μM POP with 4 μM Tβ4 clearly increased angiogenesis compared with 4 μM Tβ4 itself (A; $P < 0.001$). Representative fluorescent micrographs (B–F) depict the fluorescein-stained immunoreactive CD-31 cells in the Matrigel plug assay in the different treatment groups. ***$P < 0.001$.

the 4 μM Tβ4 group (Figure 5A–B, $P < 0.001$). There was no significant difference between results with the KYP-2047 groups and the negative control, demonstrating that the addition of KYP-2047 reduces angiogenesis to the level of the negative control (Figure 5A–F).

The addition of 0.625 μM POP + 4 μM Tβ4 significantly increased the angiogenesis compared with that of the Tβ4 group (Figrue 5A, $P < 0.001$). In contrast to the results obtained in the 'tube formation' assay, Tβ4 (4 μM) alone also increased angiogenesis (Figure 5A). Taken together, these *in vivo* results confirm those obtained *in vitro* in the tube formation assay.

Co-localization of CD-31 and POP in Matrigel plug assay

We also determined whether POP is co-localized with CD-31, a marker of endothelial cells, in the Matrigel plugs *in vivo* using a double-label immunofluorescence method. In all the groups, a substantial co-localization of POP and CD-31 was seen (Figure 6A–F), supporting the hypothesis that POP participates in the angiogenesis of endothelial cells.

Discussion and conclusions

POP has traditionally been considered a brain enzyme hydrolyzing <30-mer neuropeptides. However, POP is also widely distributed in peripheral tissues and even found in body fluids (for review, see Myöhänen *et al.*, 2009a), and has been shown to be associated with several pathological conditions outside of neurological diseases (for review, see Brandt *et al.*, 2007). Tβ4 has been suggested to be a precursor of the pro-angiogenic Ac-SDKP through a process in which POP has a major role (Cavasin *et al.*, 2004). However, until now the direct effect of POP on Tβ4 processing and the identification of the intermediates had not been investigated. In this study, using *in vitro* and *in vivo* models, we have shown for the first time that POP has a pro-angiogenic role, probably via the release of angiogenic Ac-SDKP from its precursor Tβ4. Furthermore, to support this conclusion, we demonstrated that a specific POP inhibitor, KYP-2047, inhibits angiogenesis both *in vitro* and *in vivo*.

It has been claimed that POP induces the release of Ac-SDKP from its precursor Tβ4 (Cavasin *et al.*, 2004), even though Tβ4 is considerably larger than the hydrolytic limit of POP (Polgar, 1994). This proposition is based on the finding that two substrate-like POP inhibitors, Z-Pro-Prolinal and S-17902, restored the enhanced release of Ac-SDKP in tissue homogenates (Cavasin *et al.*, 2004). We have now shown that POP is not able to cleave the full-length Tβ4. Using tissue homogenate analysis, we demonstrated that Tβ4 is cleaved at amino acids 6–7 and 7–8 from the peptide sequence of Tβ4 by unknown first step protease(s). From the resulting N-termini peptides, POP is then able to release Ac-SDKP tetrapeptide in the second-step hydrolysis. The addition of exogenous POP

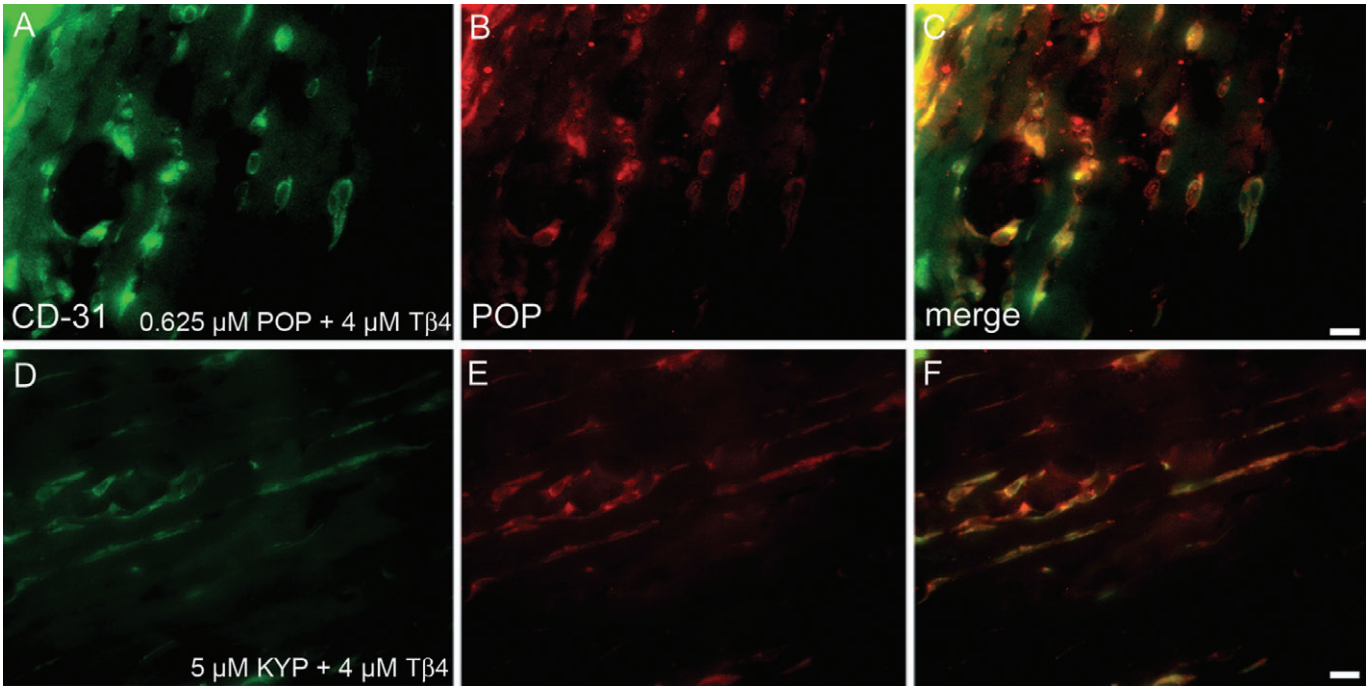

Figure 6

The co-localization of prolyl oligopeptidase (POP) with CD-31 in the Matrigel plug assay. CD-31 (green; fluorescein label) was shown to co-localize with POP (red; Texas Red label) in a double-label immunofluorescence of Matrigel plugs in POP group (A–C) and in KYP 5 μM group (D–F). The orange/yellow colour indicates co-localization. Scale bar is 20 μm. Tβ4, thymosin β4.

clearly increased the amount of Ac-SDKP in kidney homogenates. On studying the mechanism of cleavage, the most important finding was that POP is able to inhibit the first-step proteases, hence self-regulating the final release of Ac-SDKP (Figure 7). Although the MEROPS database (Rawlings *et al.*, 2010) predicted that the first-step protease(s) could belong to a group of caspases, a pan-caspase inhibitor BAF, even at high concentrations, did not inhibit Ac-SDKP formation in our experimental set-up and the identity of the primary proteases remains to be clarified.

Nevertheless, our findings reveal that the generation of Ac-SDKP is tightly regulated by a negative feedback mechanism. This strategy of regulation is employed by nature in a number of pathways where fine-tuning is dictated by the metabolic state of the cell. Furthermore, this negative control pathway could explain the dual effects of Ac-SDKP on cell proliferation and cancer (Bonnet *et al.*, 1992; Cashman *et al.*, 1994). The formation of Tβ4 7–44 seems to accord with that of Tβ4 8–44, and both processes are inhibited by POP in a very similar manner, which suggests that these peptides are the products of a single enzyme. One possible mechanism by which POP inhibits the process could be by degradation of the first-step protease(s). However, because the sizes of proteases are generally too large (much over 30-mer) to fit to the active site of POP, the inhibitory action is likely to be beyond the hydrolytic functions of POP. There is increasing evidence that POP participates in protein–protein interactions and thus regulates different functions of which at least some can be blocked by POP inhibitors (Brandt *et al.*, 2008; Di Daniel *et al.*, 2009). On the other hand, we now have data indicating

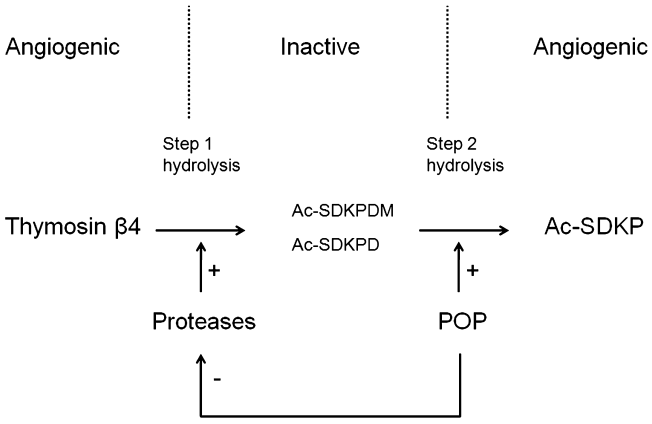

Figure 7

A schematic representation of the two-step hydrolysis of thymosin β4 to acetyl-N-Ser-Asp-Lys-Pro (Ac-SDKP). The first step involves degradation by currently unidentified proteases, and the second step is the hydrolysis by prolyl oligopeptidase (POP). POP itself is able to inhibit the first step proteases and thus autoregulate the whole hydrolysis chain.

that many *in vivo* substrates of POP include regulatory peptides (J. Tenorio-Laranga *et al.*, submitted). Therefore, we propose that POP generates peptide products, which in turn inhibit the protease(s) responsible for the initial cleavage of full-length Tβ4.

In line with the results of the analysis of peptide digestion, in the tube formation and Matrigel plug assays the addition of POP increased, and a POP inhibitor decreased, the angiogenesis. Moreover, the addition of Tβ4 itself did not significantly increase the angiogenesis in HUVEC. Several studies have shown that Tβ4 is pro-angiogenic (Malinda *et al*., 1997; 1999; Smart *et al*., 2007b), and even in our hands, the pro-angiogenic effect of Tβ4 was seen *in vivo* in Matrigel plug assay. Similarly, in the cellular model, when KYP-2047 was added without Tβ4, it had no effect on angiogenesis, but when Tβ4 was added with KYP-2047, it was found to have an inhibitory effect on angiogenesis. This suggests that in the cell culture environment, there is not enough active POP for the final cleavage action, whereas *in vivo* the tissue POP is available to compensate for this shortage. It seems that in the cellular model, the addition of Tβ4 increases the amount of inactive Tβ4 intermediates. The administration of a POP inhibitor blocks both the negative autoregulatory effects of POP on the first-step enzymes and the formation of the pro-angiogenic Ac-SDKP (Figure 7). The former event leads to reduced angiogenic Tb4 levels and may in part explain why the tube formation drops to below basal levels. Liu *et al*. (2008) have shown that a high level of POP activity is associated with an elevated expression of Ac-SDKP in various malignant tumours. They hypothesized that the elevated POP activity may increase the angiogenesis of malignant tumours via the release of Ac-SDKP. Our results prove that POP can indeed induce angiogenesis and its inhibition can significantly reduce this effect both *in vitro* and *in vivo*. In both our models, the addition of POP with Tβ4 significantly increased the angiogenesis above the effect of Tβ4 alone. These findings support the notion that POP participates in the release of pro-angiogenic Ac-SDKP, and that Ac-SDKP, rather than Tβ4 itself, induces angiogenesis. Moreover, the finding that POP is mainly co-localized with CD-31, a marker of immunoreactive vascular endothelial cells, fits well with this proposed role of POP in vessel formation.

However, as the POP inhibitor was found to reduce the angiogenesis in the tube formation assay to below the control levels, an alternative hypothesis is needed to explain how POP regulates cell growth and angiogenesis. POP inhibitors have previously been shown to prevent cell proliferation and differentiation in a Swiss 3T3 cell line and *Sarcophaga peregrine* (fresh fly) imaginal disks (Ohtsuki *et al*., 1994; 1997; Ishino *et al*., 1998). The exact mechanism is not known, but according to these studies, POP inhibitors may be able to interfere and stop the DNA synthesis, at least at high doses (Ishino *et al*., 1998). In addition, the nuclear localization of POP in peripheral tissues and in developing cell cultures supports a role for POP in cell proliferation/differentiation as well (Myöhänen *et al*., 2008b), although the mechanisms have not been elucidated (Moreno-Baylach *et al*., 2008). Nevertheless, POP has been shown to increase the inflammatory and collagen-cleaving effects of matrix metalloproteases that are important in tumour metastasis (Roy *et al*., 2009), by releasing the extracellular matrix-derived neutrophil chemoattractant, proline-glycine-proline (Gaggar *et al*., 2008), and these events may be associated with the increased POP activity and expression found in malignant tumours.

In conclusion, we have shown that POP is involved in angiogenesis evidently by participating in the second step of the release of pro-angiogenic Ac-SDKP tetrapeptide from its precursor Tβ4. Moreover, we have found that POP is able to inhibit the first-step proteases, degrading Tβ4 to smaller peptides suitable for the POP substrates and thus POP autoregulates the release of Ac-SDKP (Figure 7). Importantly, a specific POP inhibitor, KYP-2047, was able to prevent robustly the release of Ac-SDKP and angiogenesis both *in vitro* and *in vivo*. These findings, and an apparent effect of KYP-2047 in an *in vivo* model of angiogenesis, opens possibilities for further research into the effects of POP and POP inhibitors in tumour models.

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Conflicts of interest

The authors state that they have no conflicts of interests.

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