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ORIGINAL PAPER

Prostate-specific membrane antigen (PSMA)-mediated laminin proteolysis generates a pro-angiogenic peptide

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Abstract Prostate-specific membrane antigen (PSMA) is a membrane-bound glutamate carboxypeptidase expressed in a number of tissues. PSMA participates in various biological functions depending on the substrate available in the particular tissue; in the brain, PSMA cleaves the abundant neuropeptide *N*-acetyl-aspartyl-glutamate to regulate release of key neurotransmitters, while intestinal PSMA cleaves polyglutamated peptides to supply dietary folate. PSMA expression is also progressively upregulated in prostate cancer where it correlates with tumor progression as well as in tumor vasculature, where it regulates angiogenesis. The previous research determined that PSMA cleavage of small peptides generated via matrix metalloprotease-mediated proteolysis of the extracellular matrix protein laminin potently activated endothelial cells, integrin signaling and angiogenesis, although the specific peptide substrates were not identified. Herein, using enzymatic analyses and LC/MS, we unequivocally demonstrate that several laminin-derived peptides containing carboxy-terminal glutamate moieties (LQE, IEE, LNE) are bona fide substrates for PSMA. Subsequently, the peptide products were tested for their effects on angiogenesis in various models. We report that LQ, the dipeptide product of PSMA cleavage of LQE, efficiently activates endothelial cells in vitro and enhances angiogenesis in vivo. Importantly, LQE is not cleaved by an inactive PSMA enzyme containing an active site mutation (E424S). Endothelial cell activation by LQ was dependent on integrin beta-1-induced activation of focal adhesion kinase. These results characterize a novel PSMA substrate, provide a functional rationale for the upregulation of PSMA in cancer cells and tumor vasculature and suggest that inhibition of PSMA could lead to the development of new angiogenic therapies.

Keywords PSMA · GCPII · Peptides · Angiogenesis · Extracellular matrix · Laminin

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Introduction

Tumor growth is critically dependent on establishing a functional blood supply to provide oxygen and nutrients to the developing tumor. Initially, the preexisting tissue vasculature can be co-opted by the tumor without the need for new vessel formation, but eventually the tumor

overwhelms this supply. The resulting hypoxic conditions evoke a secondary angiogenic response where the tumor cells elicit proangiogenic molecules to activate endothelial cells to support new vessel growth and nourish the expanding tumor. In addition to the tumor cells themselves, the tumor microenvironment is an active contributor to tumor development and neovascularization, in part due to its significant modification during disease progression. Activated tumor cells release growth factors and cytokines that induce the expression or activation of extracellular matrix (ECM)-modifying proteases of varying specificities, primarily members of the matrix metalloprotease (MMPs) and "a disintegrin and metalloproteinase with thrombospondin motifs" (ADAMTS) [1–11] families. These endopeptidases hydrolyze large molecules at internal cleavage sites to orchestrate the local degradation of intact ECM proteins into smaller bioactive fragments such as endostatin [6] and angiostatin [10]. These and similar ECM-derived fragments have been shown to regulate tumor and stromal cell activation, proliferation, migration, invasion, angiogenesis and eventual metastatic escape [5–8, 12–20]. In addition to the endopeptidases, a wide array of membrane-bound, extracellular exopeptidases are also abundantly upregulated in tumor vasculature and have been shown to contribute significantly to the angiogenic process [18, 21–23]. The fact that these exopeptidases sequentially cleave one or two amino acids from small peptide substrates suggests that in a manner analogous to other well-characterized enzyme/peptide cascades, they may modify or degrade bioactive peptide fragments produced during degradation of the extracellular matrix to regulate tumor and endothelial cell invasion [24].

Prostate-specific membrane antigen (PSMA) is a widely expressed cell surface metallopeptidase that hydrolyzes C-terminal glutamate residues of small peptides in the extracellular space [25]. Two primary PSMA substrates have been identified and include the neurotransmitter *N*-acetyl-L-aspartyl-L-glutamate (NAAG) and the precursor of vitamin B, polygamma-glutamate folate. Although it is the same protein, PSMA enzymatic activity is typically referred to as glutamate carboxypeptidase II (GCPII) in the brain and folate hydrolase I (FOLH1) in the intestine. Of interest to this study, PSMA expression is induced on the neovasculature of solid tumors [26, 27] where we have shown that PSMA functionally regulates integrin activation and signal transduction in a laminin-specific manner to direct angiogenic endothelial cell adhesion and invasion [11]. Further studies demonstrated that mechanistically, PSMA hydrolyzes laminin fragments generated by MMP-2 to promote α6β1 integrin activation and stimulate angiogenesis via focal adhesion kinase (FAK) [16], thereby establishing a functional connection between proteolytically generated peptides, cell surface peptidases and angiogenesis. However, while we previously predicted potential peptide substrates based on the location of canonical MMP-2 cleavage sites in the laminin-10 alpha chain [16], the identity or activity of these putative peptide substrates has not been elucidated. In the current study, we synthesized a panel of predicted MMP-2-generated laminin peptides and functionally assessed their angiogenic potential using PSMA/laminin as a model system. Surprisingly, one of the most pro-angiogenic candidates was found to be the PSMA-induced dipeptide LQ that promotes endothelial adhesion in vitro and angiogenesis in vivo via activation of laminin-binding integrin β1 and phosphorylation of the focal adhesion kinase FAK. Therefore, these studies define a member of a novel class of small molecule angiogenic regulators representing new targets for directed anti-angiogenic therapies.

Methods

Synthesis of laminin peptides

Syntheses of the laminin peptides IEE, LQE, LNE, LE, LAE, GAGE, YQLGE, ASQRISE, VDRTLSE and VIIAE were carried at the synthesis and sequencing core facility at Johns Hopkins University using an AAPPTec Focus Synthesizer utilizing Fmoc chemistry. After cleaving the peptide from the resin, the peptide was purified by reverse-phase HPLC utilizing an XBridge Peptide BEH C18 Prep Column. Chromatograms of the purified synthetic peptides showed one peak indicating 100 % purity. Molecular weight of each peptide was confirmed by MALDI-TOF. Pure peptides were collected, pooled and lyophilized before use.

Wild-type PSMA and PSMA (E424S) inactive mutant

Wild-type PSMA (N-terminally flanked by the Strep-tag) was heterologously expressed and purified from insect S2 cells as described previously [28]. The E424S PSMA (E424S) mutant was constructed by a quick change site-directed mutagenesis protocol using the plasmid encoding wild-type PSMA as a template together with a pair of mutagenic primers 5′-GCAAGCTGGGATGCATCAGAA TTTGGTCTTCTTG-3′ and 5′-CAAGAAGACCAAATTC TGATGCATCCCAGCTTGC-3′. The PSMA (E424S) mutant was expressed and purified in a manner identical for wild-type PSMA. Analysis of the enzymatic activity of PSMA (E424S) revealed <0.1 % NAAG-hydrolyzing activity compared to wt PSMA (data not shown).

Competition between NAAG and laminin peptides for PSMA

PSMA enzymatic activity measurement ± laminin peptides were based on published procedures [28–30]. Briefly, the reaction mixture (total volume of 90 µL) contained radiolabeled substrate *N*-acetyl-L-aspartyl-L-[3,4-^{3}H]-glutamate ([^{3}H]-NAAG) (30 nM, 30 Ci/mmol) and recombinant human PSMA (40 pM) in Tris–HCl (pH 7.4, 40 mM) containing 1 mM $CoCl_2$. The reaction was carried out at 37 °C for 20 min and terminated with ice-cold sodium phosphate buffer (pH 7.4, 0.1 M, 90 µL). Blanks were obtained by incubating the reaction mixture without PSMA. Duplicate aliquots of 90 µL from each reaction mixture were transferred to a well in a 96-well spin column containing AG1X8 ion exchange resin; the plate was centrifuged at 1000 rpm for 5 min using a Beckman GS-6R centrifuge equipped with a PTS-2000 rotor. [^{3}H]-NAAG bound to the resin and [^{3}H]-glutamate eluted in the flow through. Columns were washed twice with formate (1 M, 90 µL) to ensure complete elution of [^{3}H]-glutamate. Flow through and washes were collected in a deep 96-well block; from each well with a total volume of 270 µL, a 200 µL aliquot was transferred to a glass scintillation vial, to which 10 mL of Ultima Gold (PerkinElmer) was added. The radioactivity in each vial corresponding to [^{3}H]-glutamate was determined via a Beckman LS-6000IC scintillation counter.

PSMA-catalyzed hydrolysis of laminin peptides

Peptides were incubated with 25 µg/mL recombinant PSMA (wild-type or mutant E424S) in Tris buffer (pH 7.7, 60 mM) containing 1 mM $CoCl_2$. During the experiment using LQE and mutant E424S, both mutant and controls (recombinant PSMA ± 2-PMPA) were incubated for 24 h; all other experiments used 20 or 60 min incubations as indicated. Following incubation, samples were frozen until analysis. An aliquot (10 µL) was removed and derivatized with butanol containing 3 N-HCl which esterifies carboxylic acid moieties to the corresponding butyl ester [31]. Aliquots of derivatized samples (10 µL) were injected and separated on an Agilent 1290 UPLC system with a 1 × 150 mm, 3.5 micron c18 column using a mobile phase of water and acetonitrile +0.1 % formic acid with gradient run of 2.5–95 % acetonitrile over 3 min and detected on an Agilent 6520 QTOF mass spectrometer in positive ion mode.

Tissue culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in EBM media containing the EBM bullet kit. Cells were maintained in a 37 °C incubator at 5 % CO_2. All cells used in this study were passaged fewer than 10 times.

Endothelial cell adhesion assay

Synthetic peptides or free glutamate (E) (0–100 µg) were diluted in 100 mM sodium carbonate buffer, pH 9.6, pipetted onto Nunc Immobilizer Amino 96-well plates (Thermo Scientific) and incubated at 4 °C overnight to bind the peptide (or free glutamate) to the bottom surface of the wells. For no-peptide control wells, only carbonate buffer was added to each well, according to the manufacturer's recommendations. The following day, wells were blocked with 0.5 % BSA/PBS for 1 h at 37 °C and washed with 0.1 % BSA/PBS. HUVECs were then released from tissue culture plates using non-enzymatic cell dissociation solution (ATCC) according to the manufacturer's protocol. HUVECs were centrifuged, counted and resuspended to 500,000 cells/mL in serum-free and phosphate-free DMEM (Life Technologies). 100 µL of the cell suspension was added to each well of the peptide-coated nunc immobilizer plate and incubated at 37 °C for 30 min. Following this incubation period, non-adherent cells were removed by washing with warm 0.1 % BSA/PBS three times. To quantitate adherent cells, calcein–AM diluted in PBS to 5 µM was added to each well and incubated for 30 min at 37 °C before visualization and quantification using an inverted fluorescent microscope and 4× objective. Adherent cells were manually counted, and relative adhesion was calculated by normalizing adhesion values to no-peptide control wells. All experiments were performed in technical quadruplicates over three independent experiments.

Integrin neutralization assay

The adhesion assay described previously was followed for these experiments, with the following exceptions. After releasing and counting cells, HUVECs were incubated with neutralizing antibodies from EMD Millipore specific for integrin beta-1 (clone 4B7, 1:50 dilution), integrin alpha-6 (MAB-1378; 40 µg/mL), integrin alpha-2 (Abcam ab24697, 10 µg/mL) or integrin alpha-3 (EMB Millipore MAB1952Z, 10 µg/mL) at room temperature for 15 min (for integrins beta-1 and integrin alpha-6) or 30 min (for integrins alpha-2 and alpha-3) prior to adding the cells to the 96-well plate coated with LQ/carbonate buffer. Quantitation of adhesion was performed as described above.

Flow cytometry

HUVECs were released from culture dishes using cell dissociation buffer (Thermo Fisher), washed and

centrifuged. 1×10^5 cells were resuspended in flow cytometry staining buffer (R&D) stained with isotype control or primary antibody: anti-alpha2 (BV421, BD Biosciences, 5 µl per test), anti-alpha3 (Millipore MAB 1952Z, 1 µg per 100,000 cells), anti-beta1 (BV510, BD Biosciences, 5 µl per test) and anti-alpha6 (Affymetrix eBioscience, CD49f-PE, 0.25 µg per 100,000 cells) and incubated on ice for 30 min. Cells were washed twice and resuspended in flow cytometry staining buffer; flow cytometry was done on the BD LSR II or the BD Accuri C6 (alpha3 only) and analyzed with FlowJo software.

Matrigel morphogenesis assay

250 µl Matrigel (BD Biosciences) was plated on 24-well plates and incubated at 37°C for 1 h. HUVECs were detached from plates, counted and resuspended in growth media to a concentration of 400,000 cells/mL. 300 µl cell suspension with or without 50 µM glutamate (E) or LQ was carefully pipetted onto each well, and plates were incubated at 37 °C for 18 h. The next day, wells were rinsed with PBS and stained with calcein–AM for 30 min. Fluorescent images were taken with an inverted fluorescent microscope with a 10× objective, and the three most highly branched fields were imaged with a Moticam Pro282B camera (Motic, Richmond, British Columbia) and quantitated. Number of tubes, branch points and complete loops (tubes connected to form a continuous circle) were quantified and averaged. Three independent experiments were performed in technical duplicate.

2-MPPA adhesion assay

The adhesion assay described previously was followed, with the following exceptions. To increase basal adhesion, wells were coated with 0.5 mg/mL Matrigel (BD Biosciences) and sodium carbonate buffer with or without the addition of 5 µg of LQ or glutamate (E). Plates were incubated overnight at 4 °C. After counting and resuspending the cells in serum-free, phosphate-free DMEM, 100 µM 2-MPPA or vehicle was added to the cell suspension and incubated at room temperature for 15 min. Cells were then added to prepared and blocked plates, and the adhesion assay was continued as described above.

Matrigel implant assay and Drabkin's assay

For the in vivo angiogenesis assay, we followed the procedure described previously for Matrigel implants [16]. Briefly, 50 µg E or LQ diluted in carbonate buffer was mixed with each 1 mL of Matrigel. As a negative control, equal volume of carbonate buffer was added to the Matrigel. 0.5 mL Matrigel was injected subcutaneously into the hind flanks of 6-week-old female C57/Bl6 mice and left for 7 days. Mice were killed, and implants were excised and fixed for paraffin sections or homogenized in PBS for hemoglobin quantitation. 5-µm sections of paraffin embedded implants were stained with hematoxylin and eosin and visualized microscopically for the presence of microvessels. Microvessel density (MVD) from H&E slides was determined by counting the number of microvessels (defined as lumens surrounded by cells and containing erythrocytes) per 20× field; MVD across the three most vascularized fields per implant were averaged, and the averages across multiple animals were determined and graphed. Paraffin sections from Matrigel implants were also stained with anti-CD31 antibody to quantitate CD31-positive microvessels. CD31-positive lumens were quantified by counting the three most vascularized 20× fields per implant; averages across multiple animals in the same group were graphed. Additionally, slides were stained with anti-CD45 to quantitate hematopoietic cells; the three most densely CD45+ fields were quantified for each implant and averaged. Total nuclei per 20× field (three fields per implant) were quantified and averaged to reflect total cell infiltration. Hemoglobin quantitation was analyzed in the PBS-homogenized implants using Drabkin's assay (Sigma-Aldrich) according to the manufacturer's protocol. Following homogenization, implant solutions were centrifuged and equal volumes of supernatant were analyzed for hemoglobin content using human hemoglobin as a standard (Sigma-Aldrich); the implant supernatant was also analyzed for total protein content using the Bradford reagent (Bio-Rad) and hemoglobin values were normalized to total protein content of each implant. A minimum of five animals per group were analyzed as indicated in the figure legend. These methods were reviewed and approved by the Institutional Animal Care and Use Committee.

P-FAK activation assay

To measure levels of total FAK and phosphorylated FAK in whole HUVECs in the presence and absence of LQ, we performed the basic adhesion assay as described above (in the absence of Matrigel), except that the adhesion incubation time was increased to 2 h. Cells were fixed with fresh paraformaldehyde, and FAK and P-FAK were immunologically measured using the Fast Activated Cell-based ELISA (FACE) kit for P-FAK Activation Kit (Active Motif) according to the manufacturer's protocol. Absorbance was measured using an Infinite 200 Pro plate reader (Tecan).

Statistics

Cell-based experiments were performed in quadruplicate over a minimum of three independent experiments. Matrigel implant studies were performed in a minimum of five mice per group (as stated in figure legends), and technical replicates were performed as described in the specific methods section. The Student's *T* test was used to calculate significance between sets of data, and resulting *p* values and significance ($p \leq 0.05$) are included in the figure legends.

Results

IEE, LQE and LNE compete with [^{3}H]-NAAG for hydrolysis catalyzed by PSMA

The previous proteolysis mapping analysis (PMAP) of laminin 511 chains generated amino acid fragments with a C-terminal glutamate that were predicted to serve as substrates for PSMA [16]. Consequently, these peptides were synthesized and evaluated for their ability to compete with PSMA-catalyzed [^{3}H]-NAAG hydrolysis. NAAG affinity for PSMA is in the 87–540 nM range [28, 29, 32]. Control experiments in the present studies using NAAG to compete with [^{3}H]-NAAG gave an IC_{50} value of 200 nM. Peptides that were most potent at inhibiting [^{3}H]-NAAG hydrolysis were the tripeptides IEE (IC_{50} 3 μM), LQE (IC_{50} 50 μM) and LNE (IC_{50} 80 μM) (Fig. 1a). All other peptides tested showed IC_{50} values ≥100 μM (Fig. 1a). Dose response curves ranged from 0 to 100 % inhibition of PSMA activity when using IEE, LQE and LNE as the peptide concentrations were increased. In contrast, VDRTLSE at 1 mM did not inhibit 50 % of PSMA activity (Fig. 1b).

IEE, LQE and LNE are hydrolyzed by PSMA

IEE, LQE and LNE were further analyzed to determine if their ability to inhibit [^{3}H]-NAAG hydrolysis was the result of their being substrates for PSMA. IEE, LQE and LNE (10 μM) were incubated for 12 h with recombinant PSMA (25 μg/mL). After incubation, reaction mixtures were subjected to derivatization and products of hydrolysis were analyzed by LC–MS (Fig. 2a). All three peptides were hydrolyzed in the presence of PSMA. The different steps of hydrolysis, derivatization and characterization by LC–MS are illustrated for LQE (Fig. 2a). LQE was hydrolyzed to LQ + E and its hydrolysis could be inhibited by 2-PMPA (Fig. 2b). Upon derivatization, *n*-butyl esters of molecular species in reaction mixture will exhibit higher molecular weights due to the addition of the *n*-butyl group (Fig. 2c–e left panels). Chromatograms of reactant and products showed disappearance of LQE and appearance of LQ and glutamate after incubation with PSMA (Fig. 2c–e middle panels, red traces). Mass spectra analysis confirmed the identity of derivatized LQE, LQ and E as the molecular species corresponding to the chromatographic peaks (Fig. 2c–e right panels).

LQ and E increase endothelial cell adhesion and morphogenesis

Increased cell adhesion is one of the earliest indicators of pro-angiogenic endothelial cell activity and is commonly used to initially test angiogenic activity in vitro. To determine whether any of the above products of PSMA hydrolysis may affect angiogenesis, we initially performed in vitro adhesion assays using primary human endothelial cells (HUVECs) in the presence of the synthetic peptides hydrolyzed by PSMA (Fig. 2). As a control, we also included VIIAE, which has a C-terminal glutamate but was not hydrolyzed by PSMA. We also included glutamate (E) in these assays as it logically would be released upon PSMA hydrolysis of its substrates and may be a potential activator as well. Of the peptides tested, only the dipeptide LQ (released from LQE) and glutamate (E) consistently and significantly increased endothelial cell adhesion in a dose-dependent manner (Fig. 3a).

To further confirm that PSMA's previously described role as an angiogenic regulator is contingent on generating LQ and E, we treated HUVECs using a PSMA-specific chemical inhibitor 2-MPPA; this resulted in a significant loss of cell adhesion. Importantly, including LQ or E in these adhesion assays rescued the loss of adhesion in the presence of PSMA inhibition (Fig. 3b), suggesting that once PSMA hydrolyzes its substrate to generate LQ and E, it is no longer required for endothelial cell adhesion.

Angiogenesis can be further modeled in vitro with extracellular matrices, and HUVECs are known to form organized tubes and net-like structures on matrices reminiscent of vessel formation. We next incubated HUVECs on Matrigel, a collagen and laminin-rich matrix, in the presence of E or LQ. The addition of LQ significantly increased the number of independent tubes formed (Fig. 3c), while glutamate had no effect on tube formation. Branch points did not significantly differ between the groups, but LQ stimulated a significant increase in the formation of complete loops compared to both control and E-containing wells. Interestingly, we observed a statistically significant decrease in complete loops in the glutamate-containing wells compared to controls (Fig. 3c). Together, this suggests that while both LQ and E increase the initial endothelial cell adhesion, only LQ results in sustained and organized pro-angiogenic activities in vitro.

LQ increases angiogenesis in vivo

While in vitro endothelial cell assays are an accepted and effective measure of pro-angiogenic activity, the process of angiogenesis is complex and requires multiple coordinated steps. To better assess if the increase in endothelial adhesion and tube formation by LQ is sufficient to activate the angiogenic process, we performed Matrigel implant assays in mice in the presence and absence of LQ and E. Although supplementing implants with glutamate alone (E) had no effect on microvessel density quantified from H&E slides (Fig. 4a), CD31-positive lumens (Fig. 4b) or hemoglobin content (Fig. 4c) when compared to controls, LQ augmentation significantly increased all measures of angiogenesis in the implants (Fig. 4a–c). Importantly, no difference in total cell infiltration or hematopoietic cell infiltration (CD45-positive cells) was observed between any of the implant groups, suggesting that inflammatory processes are not responsible for the observed increase in microvessels (Supplementary Fig. 1). Therefore, LQ is capable of enhancing angiogenesis in vivo.

Fig. 2 Hydrolysis of LQE catalyzed by PSMA. Laminin peptide (LQE) was incubated with recombinant PSMA (25 μg/mL) ± 2-PMPA (1 μM) for 12 h. After incubation, carboxylic acids of analytes in the reaction mixture were derivatized to the corresponding butyl esters and analyzed by LC–MS (**a**). Products of hydrolysis after incubation of LQE with PSMA, extent of conversion of LQE to products and inhibition in the presence of 1 μM 2-PMPA are shown in **b**. Structure of molecule with *n*-butyl additions from derivatization reaction in *red* (*left panel*), chromatogram before (*black trace*) and after (*red trace*) incubation with PSMA (*middle panel*) and mass spectra of molecules giving rise to chromatographic signal (*right panel*) for LQE (**c**), LQ (**d**) and E (**e**). (Color figure online) ▶

LQE peptide is hydrolyzed by PSMA but not by PSMA (E424S) mutant

To confirm that LQE was being hydrolyzed by PSMA, LQE was incubated with PSMA ± 2-PMPA, a potent and

(A)	IEE	LQE	LNE	LE	LAE	GAGE	YQLGE	ASQRISE	VDRTLSE	VIIAE
IC_{50} (μM)	3	50	80	100	100	100	> 100	> 100	> 1 mM	> 100

(B)

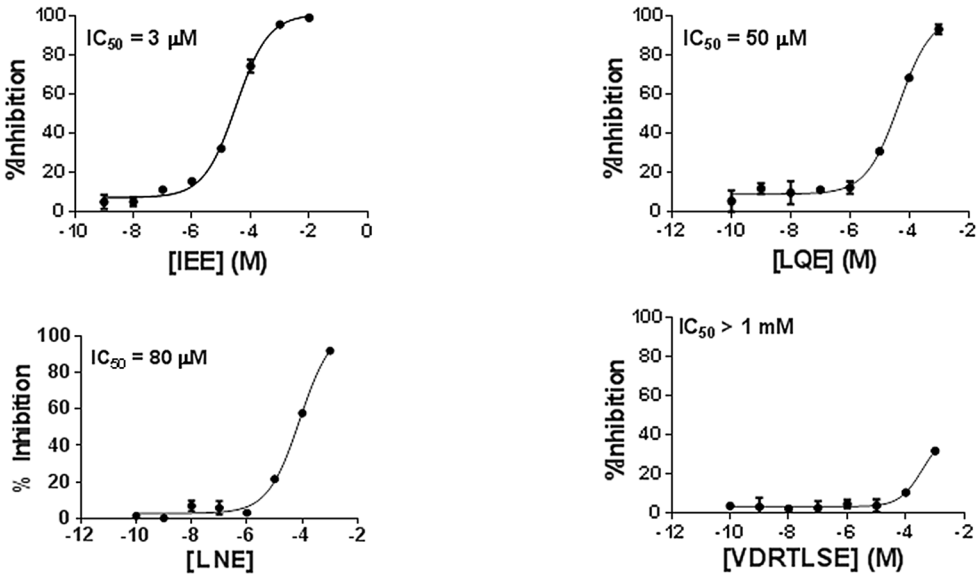
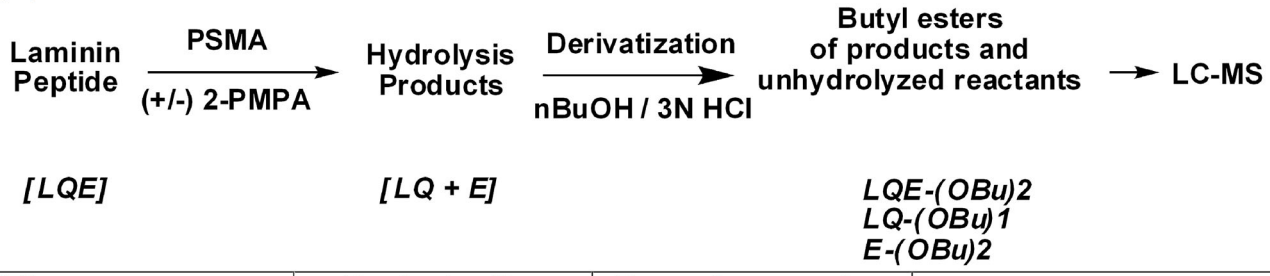


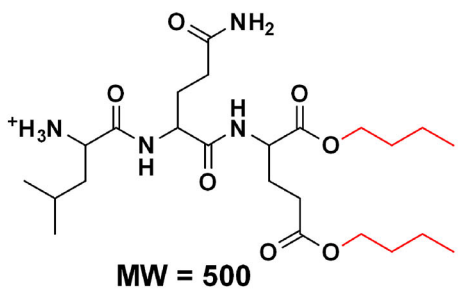
Fig. 1 Competition with [^{3}H]-NAAG for PSMA-catalyzed hydrolysis of laminin fragments. *N*-acetyl-L-aspartyl-L-[3,4-^{3}H]-glutamate ([^{3}H]-NAAG) and PSMA were incubated in the presence of increasing concentrations of laminin fragments for 20 min. Reaction was terminated with phosphate buffer; [^{3}H]-glutamate was separated from [^{3}H]-NAAG by ion exchange and measured in scintillation counter. [^{3}H]-glutamate production in the absence of peptide fragments is a measure of PSMA hydrolytic activity. IC_{50} is the concentration of peptide needed to inhibit PSMA hydrolytic activity by 50 %. **a** IC_{50} values for all laminin peptides evaluated. **b** Dose response curves for selected peptides (three most potent and one least potent). Label on *y*-axis is given as percent inhibition where 100 % corresponds to full inhibition of enzyme activity in the absence of inhibitor. Label on *x*-axis is the concentration of inhibitor (mol/L). Each *data point* is the average of two replicates; *error bars* correspond to standard deviation. When *error bars* are not seen, standard deviation was smaller than symbol representing the average

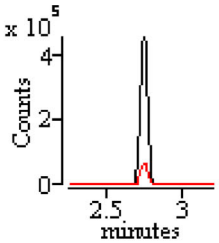
(A)

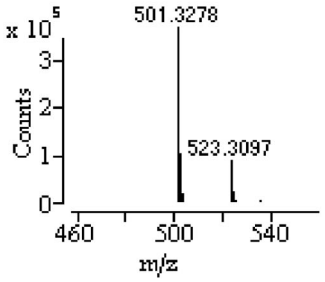


(B) Peptide	Products after PSMA incubation	Percent conversion to products	% Inhibition by 2 -PMPA
LQE	LQ + E	86	100

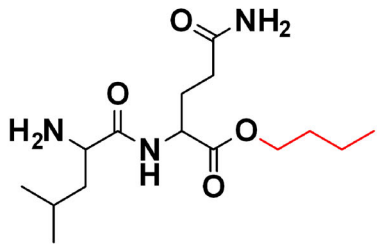
(C) Derivatized LQE

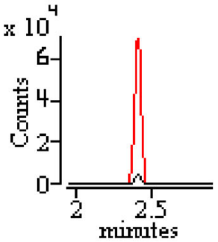


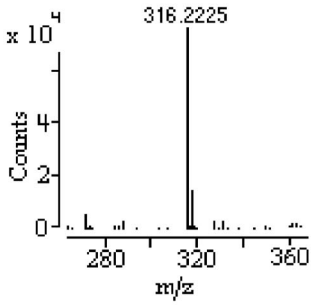




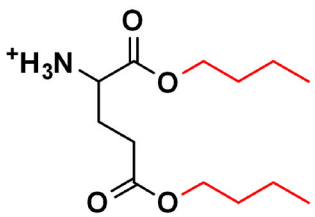
(D) Derivatized LQ

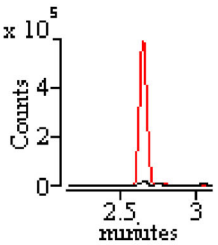


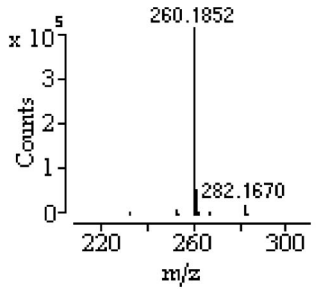




(E) Derivatized E







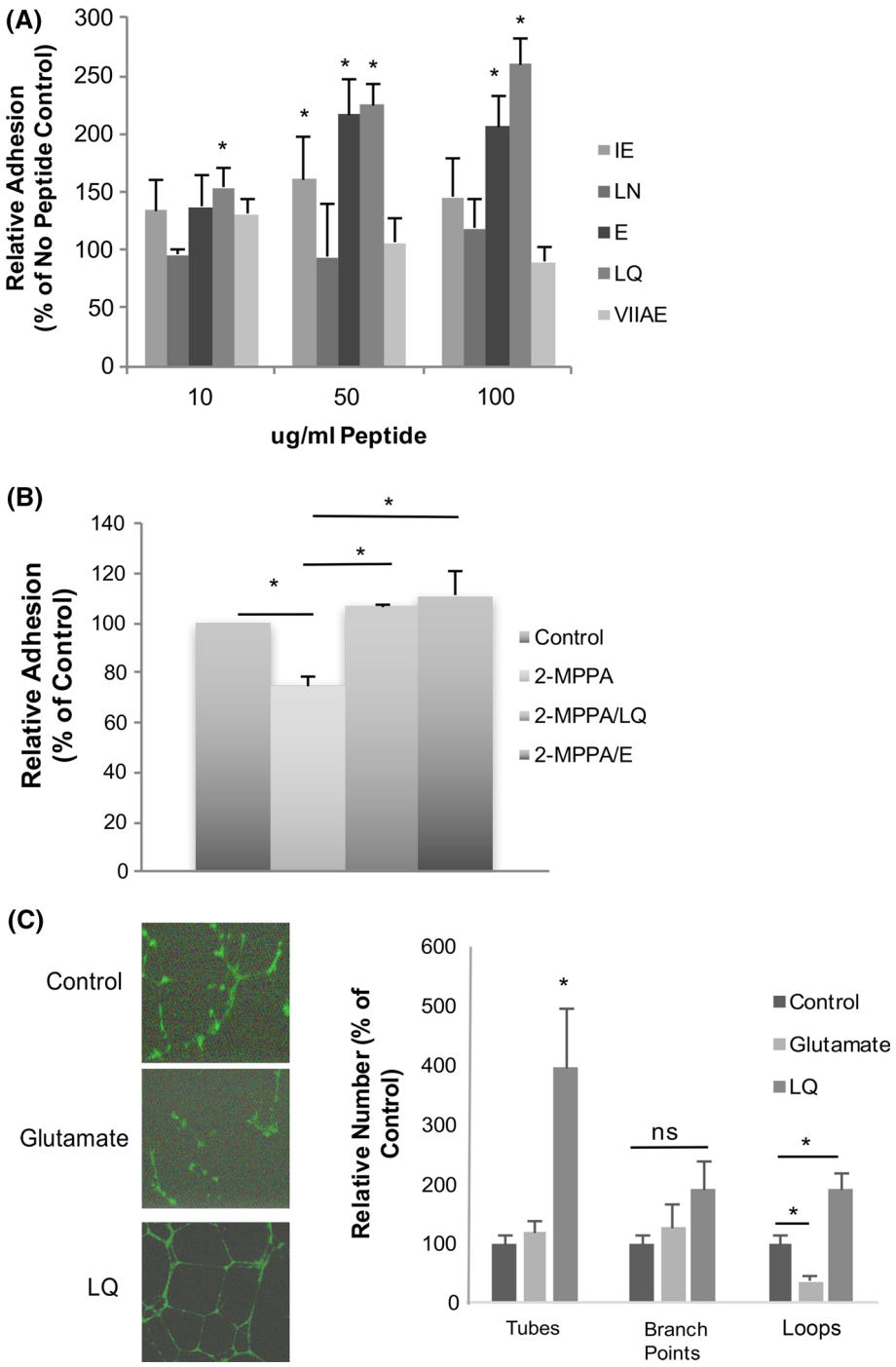


Fig. 3 PSMA peptide hydrolysis products activate endothelial cell adhesion. **a** HUVECs were tested for adhesion to 10, 50 and 100 μg/mL of the listed peptides (*p value <0.05). **b** HUVEC adhesion was tested in the presence 100 μM 2-MPPA, in the presence or absence of 50 μg/mL LQ or E (*$p < 0.05$). **c** HUVEC tube formation on Matrigel in the presence of 50 μg/mL LQ or E was quantified by counting number of tubes, branch points and loops per field. *Left* representative images of calcein–AM-stained cells; *right* graphical representation of data from three independent experiments. LQ significantly increases number of tubes (LQ vs. control $p = 0.026$; LQ vs. E $p = 0.033$) and loops (LQ vs. control $p = 0.014$; LQ vs. E $p = 0.002$), and E decreases loop formation (E vs. control $p = 0.0024$)

selective PSMA inhibitor [32], or with E424S, an enzymatically inactive PSMA mutant [33]. Incubation was carried out for 24 h and analyzed by LC/MS for the presence of LQE peptide. In the absence of PSMA, LQE remained intact. In the presence of PSMA, <5 % of LQE remained, whereas LQE was not hydrolyzed in the presence of 2-PMPA. Further, after LQE was incubated for 24 h with the inactive PSMA (E424S) mutant, 92 % of the intact tripeptide was still present (Fig. 5).

LQ stimulates endothelial cells in an integrin beta-1-dependent manner

In our previous study, we found that small laminin-derived peptides generated by PSMA stimulated endothelial cells via the α6β1 integrin pair [16]. To determine whether production of LQ underlies this α6β1-dependent, PSMA-mediated endothelial cell activation, we performed adhesion assays in the presence of a panel of integrin blocking

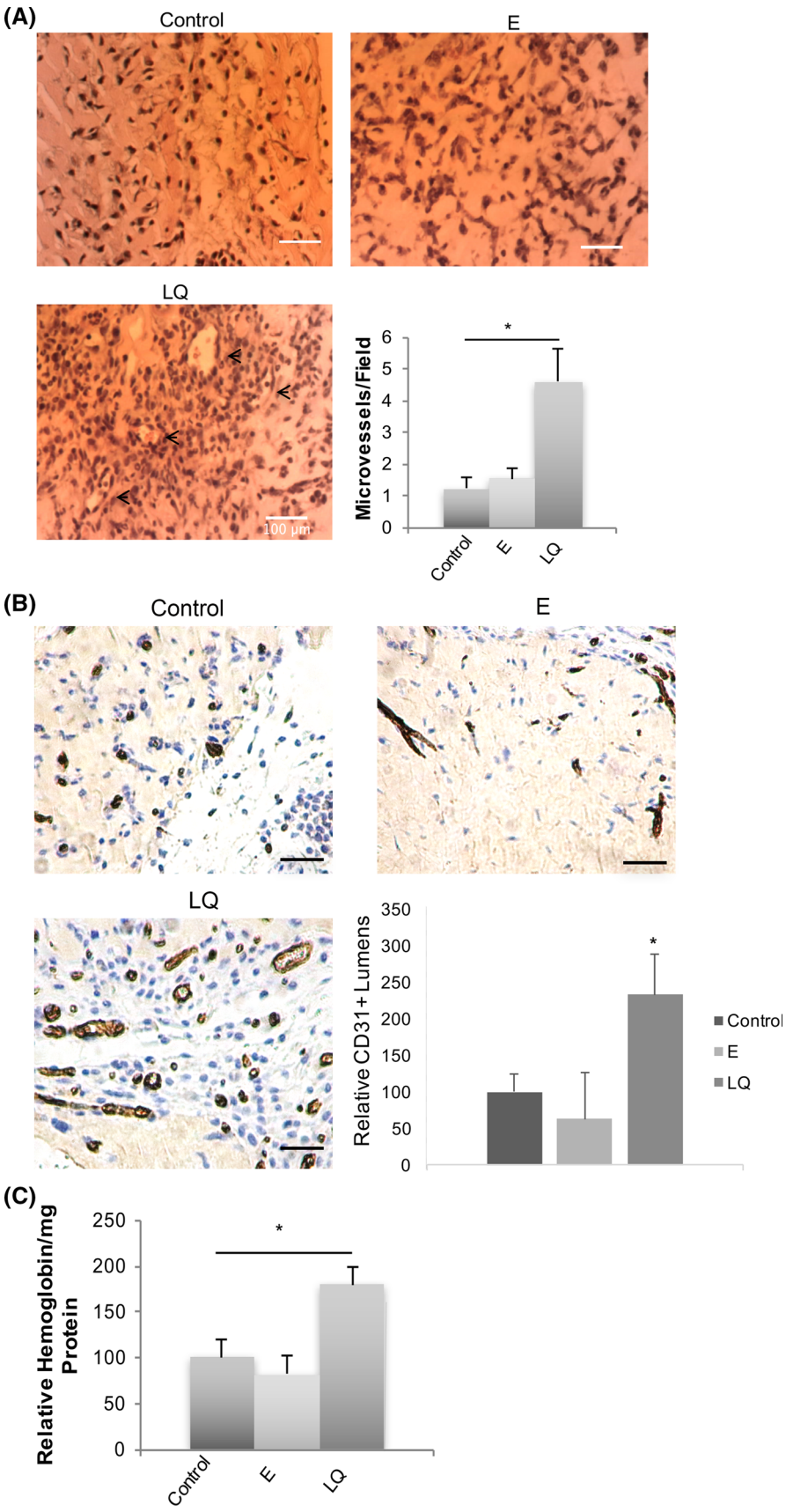


Fig. 4 Addition of LQ to Matrigel implant assays increases microvessel density and hemoglobin. Matrigel implants were prepared by adding 50 μg LQ or E (diluted in carbonate buffer) or carbonate buffer alone to 1 mL of Matrigel and injected subcutaneously into 6-week-old mice. **a** Implants were excised, paraffin embedded, sectioned and stained with H&E. Representative images are shown of H&E-stained Matrigel implant sections from control, LQ or E implants (*asterisk* microvessels, defined as lumens surrounded by cells and containing red blood cells); average microvessel density from three fields each of control ($n = 9$), E-containing ($n = 9$) or LQ-containing ($n = 5$) Matrigel implants (*$p = 0.02$) is shown. **b** CD31-positive lumens were imaged and quantitated; representative images is shown, and average CD31-positive lumens are graphically represented (counted from the three highest fields per implant and averaged across mice from the same group) (LQ vs. control $p = 0.026$; LQ vs. E $p = 0.046$). *Scale bars* 100 μm. **c** A graph representing average normalized hemoglobin in the Matrigel implants measured by Drabkin's assay is shown (*$p = 0.013$). Hemoglobin concentration was normalized to total protein content per plug using Bradford's reagent. For each implant ("n" for each group is reported above), the assay was performed in triplicate; the averages across each biological replicate are shown. *Error bars* represent SEM

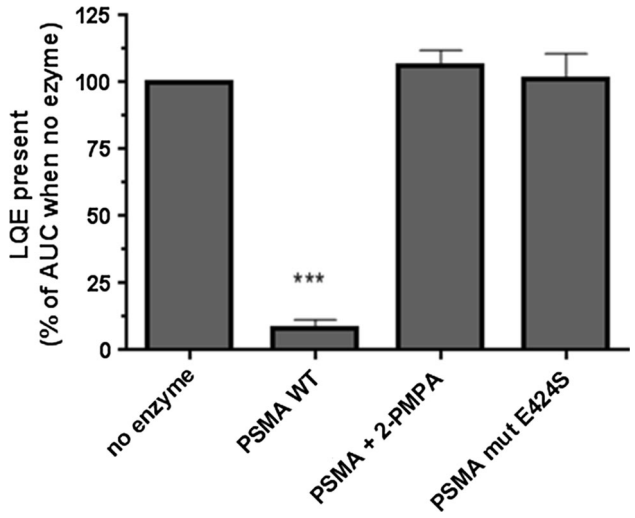


Fig. 5 Hydrolysis of LQE by PSMA and PSMA mutant. LQE was incubated with PSMA ± 2-PMPA (1 μM) or with the PSMA mutant E424S for 24 h. Incubation mixtures were analyzed by LC/MS for the presence of LQE peptide. Data were normalized to area under the curve (AUC) for LQE when incubated alone. *Error bars* correspond to S.E.M. ***$p < 0.0001$ when compared to LQE incubated with no enzyme

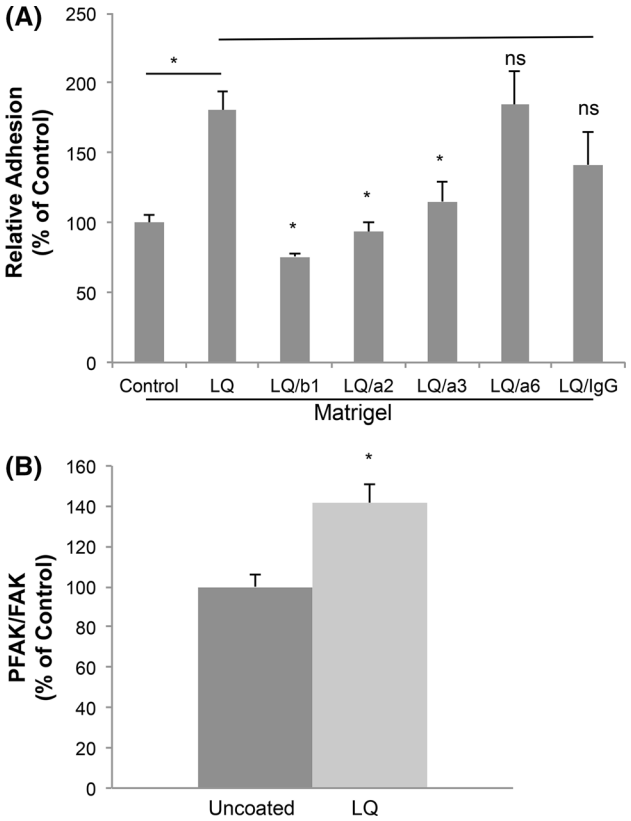


Fig. 6 LQ activates endothelial cells through integrin signaling and FAK. **a** HUVEC adhesion to wells coated with Matrigel ± LQ in the presence of neutralizing antibodies for integrin β1, α2, α3 and α6, along with IgG control demonstrates LQ activation requires integrin β1, α2 and α3 (*$p < 0.01$) but not integrin α6. **b** Ratios of P-FAK/FAK, measured from HUVECs adhered to uncoated or LQ-coated wells, demonstrate activation of FAK by LQ (*$p = 0.003$)

antibodies. Antibody neutralization of integrin β1 abrogates endothelial cell adhesion to LQ, but surprisingly, treatment with an integrin α6 neutralizing antibody had no effect on adhesion (Fig. 6a). The α2β1 and α3β1 integrin pairs also mediate endothelial cell binding to laminin [34–39], and neutralizing both α2 and α3 integrin subunits inhibited LQ-stimulated adhesion (Fig. 6a). Flow cytometry analysis of HUVECs confirmed strong expression of all tested integrins on the endothelial cells (Supplementary Fig. 2). In agreement with these findings, focal adhesion kinase (FAK) phosphorylation measured using a validated P-FAK specific in-cell ELISA assay [40] was increased in the presence of LQ alone (Fig. 6b), confirming that LQ activates endothelial cell integrin signaling. Therefore, PSMA regulates angiogenesis in part via hydrolysis of the laminin fragment LQE to generate a small, angiogenically active dipeptide that activates endothelial integrin signal transduction cascades.

Discussion

PSMA is widely distributed [27, 41, 42], but only two biological substrates have been described. The first, *N*-acetyl-L-aspartyl-L-glutamate (NAAG) [28], is one of the most abundant peptides in the mammalian brain which serves as both a neurotransmitter at NMDA and mGluR3 receptor [43, 44] as well as storage form of glutamate. The second, pteroylpolygamma-glutamate exists in the jejunum where it undergoes PSMA-catalyzed hydrolysis to folate and glutamate enabling folate transport across the intestine by the reduced folate carrier [45]. The observations that PSMA is upregulated both in prostate cancer and in tumor vasculature [46] together with evidence that PSMA promotes tumor angiogenesis in a laminin-dependent manner [16] suggests that additional, previously uncharacterized PSMA substrates may be functional in these tissues. Here, we tested laminin-derived peptides and found that PSMA efficiently hydrolyzes three of these novel peptides: IEE, LQE and LNE (Figs. 1, 2). However, only one of the novel hydrolysis products tested, LQ, efficiently activated both endothelial cells and angiogenesis. This finding not only characterizes LQE as a novel angiogenic PSMA substrate, but also suggests the possibility that additional substrates produced by exopeptidases that are upregulated during angiogenesis and other diseases could be important biological regulators.

PSMA hydrolysis of its substrates generates free glutamate. In the brain, glutamate release from NAAG

following injury or stroke is associated with ischemia and neuronal death; inhibiting PSMA prevents the increase in glutamate associated with ischemia [32]. More recently, it has been shown that free glutamate can activate T-cell adhesion to fibronectin and laminin [47] and neutrophil migration [48]. Interestingly, our results demonstrate that free glutamate significantly increases endothelial adhesion (Fig. 3), though it fails to alter tube formation in vitro (Fig. 3c) and angiogenesis in vivo (Fig. 4). The mechanism by which glutamate increases endothelial cell adhesion is unclear, but the presence of glutamate receptors on endothelial cells from the brain, skin and respiratory tissues [49, 50] suggests that glutamate could interact with endothelial glutamate receptors and stimulate glutamate signaling and endothelial cell activation. However, glutamate alone is apparently insufficient for stimulating the complex process of angiogenesis. Thus, it is the production of LQ, rather than the release of glutamate, which is primarily responsible for the increased angiogenesis we observed.

As a small molecular weight dipeptide, it is possible that LQ could either be further metabolized or diffuse out of the Matrigel implant, diminishing its efficacy as a pro-angiogenic therapy (Fig. 4). However, previous in vivo studies have shown that small peptides can readily support angiogenesis over extended periods of time [51, 52]. Additionally, Matrigel and other hydrogels have been demonstrated to prolong the stability of small peptides in vivo [54–56], suggesting that injecting LQ with Matrigel limits its diffusion and protects against degradation. Taken together with our current data, these observations support our hypothesis that the LQ dipeptide is capable of eliciting sustained pro-angiogenic activity in vivo via activation of integrins and FAK signal transduction (Fig. 6).

LQ activation of integrins and FAK signaling (Fig. 6) provides a mechanism for sustained pro-angiogenic signaling in vivo. While it is possible that as a dipeptide, LQ could either diffuse away from the Matrigel implant or be metabolized after injection in the in vivo Matrigel implant assay (Fig. 4), efficient activation of pro-angiogenic integrins by LQ would likely lead to prolonged angiogenic effects, consistent with our observation that LQ increases vascularization of Matrigel implants. In support of this, other in vivo studies have shown that specific small peptides lead to sustained angiogenesis [51–53]. Additionally, injecting LQ with Matrigel would likely limit its diffusion, as Matrigel and other hydrogels have been shown to prolong the stability of small peptides in vivo [54–56]. Our data support our hypothesis that LQ activates sustained pro-angiogenic activity in vivo.

While further investigation is required to determine the presence and activity of LQ in angiogenic tissues, it is interesting to note the prevalence of the tripeptide LQE across various laminin isoforms. All laminin alpha and beta chain isoforms and two gamma chain isoforms have at least one LQE sequence, and some isoforms have as many as 4–5 LQE sequences. Conversely, LQE is rarely found in collagen sequences. This observation is consistent with our earlier finding that PSMA specifically regulates endothelial cell activation in the presence of laminin but not collagen or other ECM proteins [11]. Thus, it is likely that LQE would be present in tissues containing abundant laminin, MMPs and PSMA; angiogenic tissues are known to express high amounts of all three of these proteins.

A number of extracellular matrix protein fragments have been characterized as powerful negative regulators of angiogenesis [5, 6, 10, 57, 58]. ECM fragments endostatin, arresten and tumstatin share similar mechanisms of action by binding to specific integrins, inhibiting integrin interaction with ECM substrates and subsequent FAK activation and abrogating endothelial cell migration or proliferation [58, 59]. In contrast, we found that LQ activates α2β1, α3β1 and FAK signaling and promotes endothelial cell activation and angiogenesis (Fig. 6). Although the precise mechanistic differences between pro-angiogenic LQ activity and anti-angiogenic ECM fragments is not known, it is feasible that as a small peptide, LQ could bind to integrins even in a closed, inactive conformation to stimulate integrin signaling and endothelial cell activation, while larger fragments require integrins to be in an open conformation [60]. Additionally, small peptide substrates would likely bind to integrins with lower affinity than larger fragments, and strong integrin/substrate interactions often result in increased cell attachment and decreased motility. This idea is consistent with our previous finding that PSMA is required for the initial integrin β1 signaling and inducing an open integrin confirmation [11].

We previously found that laminin peptides generated by MMP/PSMA hydrolysis increased endothelial cell adhesion in an integrin α6β1-dependent manner. However, we could not rule out the involvement of other integrins [11]. In this study, we found that while LQ-dependent endothelial activation requires integrin β1, it is independent of integrin α6 and instead relies on active integrins α2 and α3. It is therefore plausible that the previously tested laminin hydrolysis products [11] contained a mixture of pro-angiogenic peptides that activate distinct integrins. While both α2β1 and α3β1 have been associated with angiogenesis, the literature reports both pro- and anti-angiogenic functions for these integrins [61–65]. The dual functions of these integrins could depend on the substrate availability and possibly, substrate affinity with the integrins. Our findings implicate LQ as a pro-angiogenic regulator of these integrins.

Our results provide the framework for two potential therapeutic applications. Inhibition of LQE hydrolysis by a

PSMA inhibitor could be of use as an anti-angiogenic in cancer treatment. In support of this notion, our previous reports demonstrated that the potent PSMA inhibitor 2-PMPA or PSMA null mice exhibit reduced angiogenesis [8, 11]. On the other hand, agents capable of stimulating angiogenesis have been used clinically to treat ischemia and wound healing, and have been proposed to stimulate vascularization of engineered tissues. Small peptides, such as LQ, generally have better stability, are less antigenic and are synthesized more efficiently than large peptides and proteins. For these reasons, small pro-angiogenic peptides have been suggested as efficient activators of therapeutic angiogenesis [54]. For example, the copper-binding tripeptide GHK, derived from the secreted protein rich in cysteine (SPARC), has been shown to induce in vivo vascularization and tissue remodeling [53]. LQ may prove to have similar utility in promoting therapeutic angiogenesis.

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Compliance with ethical standards

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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