Tissue kallikrein is synthesized and secreted by human vascular endothelial cells

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Abstract

The generation of kinins on the surface of vascular endothelium has been postulated in two pathways involving plasma kallikrein and tissue kallikrein; the former pathway has been well documented, but the latter is controversial. To clarify the presence of a kinin-generating system on endothelium, we examined whether human umbilical vein endothelial cells (HUVEC) synthesize and release tissue kallikrein in vitro. Kallikrein-like activity hydrolyzing a peptide Pro-Phe-Arg-4-methyl-coumaryl-7-amide was detected in the culture medium of HUVEC and was inhibited by aprotinin but not by soybean trypsin inhibitor. Western blotting of HUVEC medium using anti-human tissue kallikrein antibodies demonstrated the release of tissue kallikrein from HUVEC, and the reverse transcription-polymerase chain reaction (RT-PCR) followed by Southern blotting revealed the expression of tissue kallikrein mRNA in HUVEC. HUVEC metabolically labeled with [^{35}S]methionine released radioactive proteins corresponding to tissue kallikrein. RT-PCR also showed the expression of low-molecular-weight kininogen (L-kininogen) mRNA in HUVEC. The cGMP levels in HUVEC were significantly elevated by the incubation with angiotensin converting enzyme inhibitor, lisinopril, and the elevation was completely inhibited by aprotinin or bradykinin B2-receptor antagonist, FR172357. These results suggest that the endothelial cells continuously release an active form of tissue kallikrein which enables generation of kinins on the vascular endothelium.
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1. Introduction

The vascular tissue has been identified to be a paracrine and autocrine organ regulating blood flow by various vaso-active factors. Kinins, such as bradykinin and kallidin, generated in plasma mainly act on endothelial cells via B2-receptor to release various agents associated with vascular tonus and blood coagulation, such as prostaglandins, nitric oxide (NO), cytokines, and tissue plasminogen activator, resulting in the maintenance of circulation homeostasis [1].

Kinins are generated in the circulation by at least two different routes involving plasma kallikrein and tissue kallikrein, respectively. Plasma kallikrein (EC 3.4.21.34), which is activated from the circulating zymogen, prekallikrein, on the surface of endothelial cells during the initiation of intrinsic coagulation then cleaves high-molecular-weight kininogen (H-kininogen) to liberate bradykinin [2,3]. On the other hand, tissue kallikrein (EC 3.4.21.35) is a serine protease (hK1) encoded by one of the kallikrein gene family consisting of at least 15 genes (KLK1–KLK15) in humans [4]. Tissue kallikrein is distinct from plasma kallikrein in the origins of synthesis and the physicochemical properties, such as molecular size, substrate specificity, and the susceptibility to various inhibitors [1]. Tisssue kallikrein cleaves L- and H-kininogens to liberate Lys-bradykinin which is rapidly converted to bradykinin by aminopeptidases in plasma [1].

Low levels of tissue kallikrein have been detected in plasma [5]. Since the major sources of tissue kallikrein are exocrine glands such as the kidney, pancreas, and salivary gland, the origin of tissue kallikrein detected in plasma was

suggested to be these organs and tissues [6]. However, several findings have suggested that the tissue kallikrein–kinin system is present within the vascular wall; a kallikrein-like kininogenase activity is present in and released from vascular tissue [7,8], and the expressions of mRNAs for tissue kallikrein and kininogens were also demonstrated in vascular tissues [9]. Furthermore, the immunoreactivity of tissue kallikrein was detected in human endothelial cells [10,11], and the endothelial and smooth muscle cells in human blood vessels were demonstrated to be the sites of synthesis of tissue kallikrein by means of in situ hybridization [11]. Recently, Dedio et al. [12] reported that human umbilical vein endothelial cells (HUVEC) synthesized and stored tissue kallikrein with molecular sizes of about 50 kDa recognized by anti-tissue kallikrein antibodies, suggesting that the endothelial cells are a source of tissue kallikrein in the vascular wall. However, evidence that tissue kallikrein synthesized by HUVEC is released from the endothelial cells as an active form of the enzyme has not been provided. In the present study, we demonstrated that HUVEC synthesize and release an active form of tissue kallikrein with a molecular size of about 30 kDa, and therefore suggest that there is a tissue kallikrein-dependent kinin generation on the surface of endothelial cells, which probably plays important roles in the maintenance of circulation homeostasis.

2. Materials and methods

2.1. Materials

HUVEC, human coronary artery endothelial cells (HAEC), human coronary artery smooth muscle cells (HSMC), endothelial basal medium (EBM), and endothelial cell growth supplement were purchased from Clonetics; HeLa cells from Human Science Research Resources Bank (Osaka, Japan); fetal calf serum (FCS) from Invitrogen; rabbit anti-human tissue kallikrein from Calbiochem; Histofine SAB-PO Kit from Nichirei Co. (Tokyo, Japan); aprotinin, aprotinin-agarose, soybean trypsin inhibitor, and 3-isobutyl-1-methyl-xanthine (IBMX) from Sigma; Pro-Phe-Arg-4-methyl-coumaryl-7-amide (Pro-Phe-Arg-MCA) and 7-amino-4-methylcoumarin (AMC) from Peptide Institute, Inc. (Osaka, Japan). Tissue kallikrein purified from human urine was supplied by Japan Chemical Research Co. (Kobe, Japan). FR172357, a specific B2-receptor antagonist, and lisinopril were supplied by Fujisawa Pharmaceutical Co. (Osaka, Japan) and Shionogi & Co. (Osaka, Japan), respectively.

2.2. Cell cultures

HUVEC and HAEC were cultured in EBM supplemented with endothelial cell growth supplement and 10% FCS. After four to six passages of cultures, cells were seeded into 75-cm^2 dishes and used for experiments at confluence.

2.3. Assay of tissue kallikrein-like enzyme activity in the HUVEC-conditioned medium

The tissue kallikrein-like enzyme activity was assayed using the synthetic peptide Pro-Phe-Arg-MCA as the substrate [13]. HUVEC in 75-cm^2 dishes were washed twice with Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS), then cultured in EBM. The conditioned medium (about 7 ml) was dialyzed overnight against distilled water at 4 °C, then the dialyzate was lyophilized. The lyophilizate was dissolved in 0.5 ml of 0.1 M Tris–HCl, pH 7.8, containing 10 μg/ml of bovine serum albumin and 0.1 M NaCl (Buffer 1). The sample (50–100 μl) was mixed with Buffer 1 in a total volume of 0.5 ml and incubated for 5 min at 37 °C with or without 20 μg of aprotinin. Ten microliters of 5 mM Pro-Phe-Arg-MCA dissolved in dimethylsulfoxide was added, then the mixture was further incubated for 1 h. Reaction was terminated by adding 2 ml of 12% acetic acid, and the liberated AMC was determined using a spectrofluorometer (Hitachi F3000) at 380 nm (exitation) and 460 nm (emission) using AMC as a standard. The sample incubated in the presence of aprotinin was used as a blank. Enzyme activity was expressed as nmol AMC/mg protein of HUVEC/h. Cell protein levels were measured using the BCA Protein Assay Reagent (Pierce).

2.4. Western blots of human tissue kallikrein

Conditioned medium (500 ml) of HUVEC was harvested, then applied to a column of aprotinin–agarose (1.5×2.0 cm) equilibrated with 50 mM Tris–HCl, pH 8.0, containing 0.5 M NaCl. The column was washed with 20 ml of the equilibrium buffer, and the adsorbed proteins were eluted with 0.1 M glycine–HCl, pH 2.5. The eluate was neutralized immediately with 1 M Tris–HCl, pH 8.5. Fractions containing kallikrein-like activity on the synthetic peptide Pro-Phe-Arg-MCA were pooled, dialyzed against distilled water at 4 °C, then lyophilized. The lyophilizate was resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel, then electrophoretically transferred to Immobilon-PSQ membrane (Millipore). The membrane was then blocked with 10% skimmed milk followed by incubation with rabbit anti-human kallikrein antibody. Rabbit IgG on the membrane was detected by the avidin–biotin–peroxidase complex method according to the instruction manual issued by Bio-Rad Laboratories.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) Southern blot analysis of tissue kallikrein and low-molecular-weight kininogen (L-kininogen) mRNAs in HUVEC

Expressions of tissue kallikrein and L-kininogen mRNAs in endothelial cells were determined by RT-PCR followed by Southern blotting using an oligonucleotide probe (5′-ACTTGTTTGACGACGAAAAC-3′) for tissue kallikrein or

human L-kininogen cDNA for L-kininogen, as follows. Total RNA was extracted with acid guanidinium–phenol–chloroform from HUVEC, HSMC, and HeLa cells for tissue kallikrein or HUVEC, HAEC, and HepG2 cells for kininogen. Total RNA (0.5 μg) was reverse-transcribed in a 20-μl reaction mixture containing 50 pmol of the reverse primer, 2 μl of 10 mM dNTP, 2 μl of 10× PCR buffer (0.1 M Tris–HCl, pH 8.3, 500 mM KCl), 4 μl of 25 mM MgCl$_2$, 1 unit of RNase inhibitor, and 1 unit of cloned Moloney murine leukemia virus reverse transcriptase (Invitrogen). The RT reaction mixture was incubated at 42 °C for 15 min, 95 °C for 5 min, then 5 °C for 5 min to allow synthesis of the first strand of cDNA. The cDNA was amplified in a 100-μl reaction mixture containing 50 pmol of the forward primer, 8 μl of 10×PCR buffer, 4 μl of 25 mM MgCl$_2$, and 2.5 units of Taq DNA polymerase. Forty cycles proceeded as follows: denaturation at 95 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 3 min. RT-PCR products (8 μl) were Southern blotted and autoradiographed using a Fuji Film Bio Imaging Analyzer BAS1000 (Fuji Film, Tokyo). The forward and reverse primers for tissue kallikrein, L-kininogen, or H-kininogen were 5′-TGGGTGCTCACAGCTGCTCA-3′ (exon 2) and 5′-TTCTCCAGGAGGCTCATGTT-3′ (exon 3) [14], 5′-CAGACTGCAAGTCCCTTTGG-3′ (exon 5) and 5′-TCAAGAGACCTCCCTCTCAG-3′ (exon 11), or 5′-GCATACATCGATATTCAGCTACGAA-3′ (exon 7) and 5′-TGAATCCCGCTCTTCATCTTGTGCA-3′ (exon 10), respectively.

2.6. Radiolabeling of HUVEC and HAEC cultures and immunoprecipitations with anti-human tissue kallikrein antibodies

HUVEC and HAEC were grown to confluency in 75-cm^2 dishes and washed three times with methionine-deficient MEM. Methionine-deficient MEM supplemented with [^{35}S]methionine (3.7 MBq/m1) was added to the cultures, then cells were incubated for 24 h. The medium was harvested and centrifuged at 8000×g. The supernatant was mixed with an equal volume of 20 mM Tris–HCl buffer (pH 7.4) containing 0.28 M NaC1, 5 mM EDTA, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 2 pg/ml of leupeptin, and 0.2% bovine serum albumin. The sample (5 ml) was mixed with 0.5 ml of immunoprecipitation buffer (I-buffer, 10 mM Tris–HC1 (pH 7.4) containing 2.5 mM EDTA, 0.14 M NaCl and 0.5% Triton X-100), and 0.5 μl of rabbit anti-human tissue kallikrein serum. In some immunoprecipitation tubes, 200 ng of human urinary kallikrein was added to compete with radiolabeled tissue kallikrein for antibody binding. After incubation at 4 °C for 18 h, 200 μl of a 20% suspension of Protein A-agarose was added, mixed, and incubated for 2 h at 25 °C. The gel was washed five times with I-buffer, resuspended in 200 μl of 0.125 M Tris–HC1 (pH 6.8) containing 4% SDS, 10% 2-mercaptoethano1, 20% glycerol, and 0.2% bromophenol blue, then

heated in boiling water for 2 min. The supernatant (20 μl) was loaded onto a 10% polyacrylamide gel containing 0.1% SDS and resolved by electrophoresis. The gel was then fluorographed using sodium salicylate [15], then dried and autoradiographed using a Fuji Film Bio Imaging Analyzer BAS2000 (Fuji Film). Similar procedures of radiolabeling and immunoprecipitations with anti-human tissue kallikrein antibodies were carried out in HeLa cells as a positive control.

2.7. Immunostaining of tissue kallikrein in HUVEC using anti-human tissue kallikrein antibodies

HUVEC were cultured in glass disks coated with Cell-matrix type I-A (Nitta Gelatin Co., Japan). Cells were treated with paraformaldehyde and dehydrated using methanol, then immunostained with rabbit anti-human tissue kallikrein antibodies at dilution of 1:1000. Cells were incubated with a secondary biotinylated goat anti-rabbit IgG, then color development was performed by an immunoperoxidase staining procedure (Histofine SAB-PO Kit; Nichirei) using AEC substrate (Nichirei).

2.8. Measurement of cGMP levels in HUVEC after the addition of lisinopril

HUVEC were seeded in collagen type 1-coated plates (4 cm^2) and cultured until confluence in EBM supplemented with endothelial cell growth supplement and 10% FCS. The cells were washed with 2-ml HBSS twice, then incubated in 1-ml HBSS containing 0.1 mM IBMX at 37 °C for 15 min. Cells were incubated for 30 min with or without lisinopril (1 μM), FR172357 (1 μM), or aprotinin (10 μg/ml) at 37 °C. The reaction was terminated by aspirating HBSS followed by the addition of 0.1 N HCl (500 μl). The cell suspensions were snap-frozen and stored at −80 °C until assay. The cell suspensions were centrifuged, and cGMP measured from neutralized supernatant using cGMP RIA kits (Amersham, Buckinghamshire, UK).

3. Results

3.1. Kallikrein-like enzyme activity in the HUVEC-conditioned medium

We used a synthetic peptide Pro-Phe-Arg-MCA to detect tissue kallikrein activity in HUVEC culture medium. Although Pro-Phe-Arg-MCA is a relatively specific substrate for tissue kallikrein [13], the culture medium of HUVEC appeared to contain various proteases or peptidases hydrolyzing this peptide. Therefore, to estimate the tissue kallikrein-dependent hydrolyzing activity in the conditioned medium of HUVEC, we determined the aprotinin-susceptible hydrolysis of this susbstrate as a kallikrein-like activity. Since the kallikrein-like activity in conditioned medium was

very low, we concentrated the conditioned medium about 20-fold by lyophilization before the assay and found that greater than 80% of the hydrolyzing activity was inhibited by aprotinin (40 μg/ml). As shown in Fig. 1, the kallikrein-like activity was increased in the course of culture, suggesting that the hydrolyzing enzyme(s) is secreted from HUVEC. This enzyme activity was inhibited by serine protease inhibitors, such as phenylmethylsulfonylfluoride and benzamidine, but not by soybean trypsin inhibitor, a potent inhibitor for plasma kallikrein (data not shown). We could not detect a tissue kallikrein-like activity in lysates of HUVEC because greater than 90% of the hydrolyzing activity in lysates was not inhibited by aprotinin, probably due to the nonspecific hydrolysis by various proteases.

3.2. Presence of immunoreactive tissue kallikrein in HUVEC and their conditioned medium

To demonstrate the presence of tissue kallikrein in the culture medium of human endothelial cells, the kallikrein-like enzyme in the conditioned medium of HUVEC was concentrated by an aprotinin–agarose column, then resolved by Western blotting using a polyclonal antibody for human tissue kallikrein. As shown in Fig. 2, Western blotting using rabbit anti-human tissue kallikrein antibodies demonstrated that the conditioned medium contained a single broad immunoreactive band with an apparent molecular size of about 30 kDa that was indistinguishable from human urinary kallikrein. Culture medium alone before seeding cells showed no immunoreactive material for anti-human tissue kallikrein.
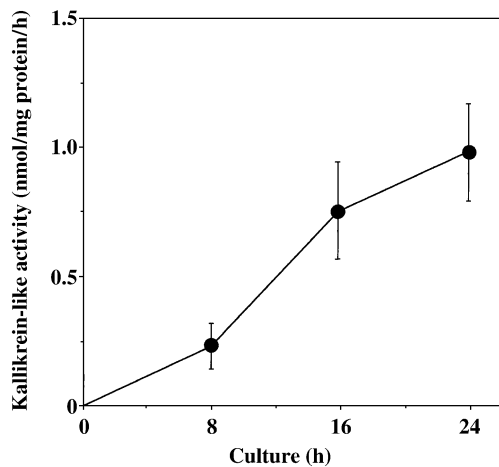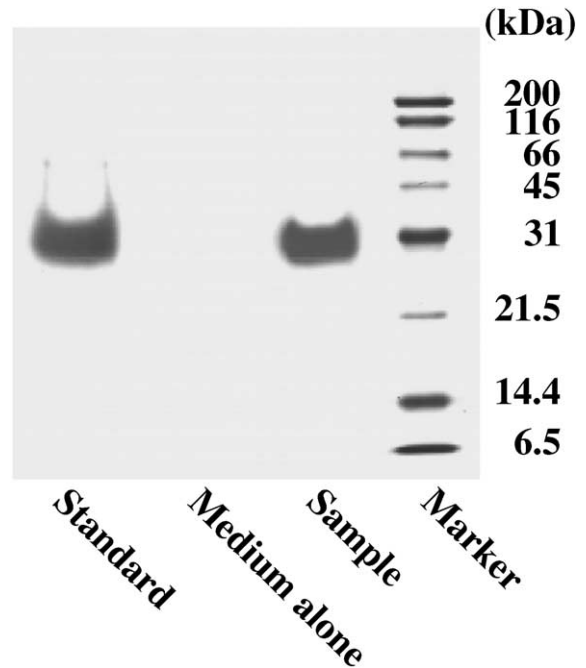

Fig. 2. Western blots of human tissue kallikrein in the conditioned medium of HUVEC. Kallikrein-like enzyme in the conditioned medium of HUVEC was concentrated by an aprotinin–agarose column, then resolved by Western blotting using rabbit anti-human tissue kallikrein antibodies, as described in the text. Sample lane contains a kallikrein-like protein corresponding to the amount obtained from the conditioned medium of two 75-cm^2 dishes in 24-h cultures. The culture medium alone without HUVEC was also applied to the aprotinin–agarose column, and the eluate was resolved by the same procedures, as a blank. Human urinary kallikrein (40 ng/lane) was used as a positive standard. The numbers to the right of the blot represent molecular mass standards in kDa.
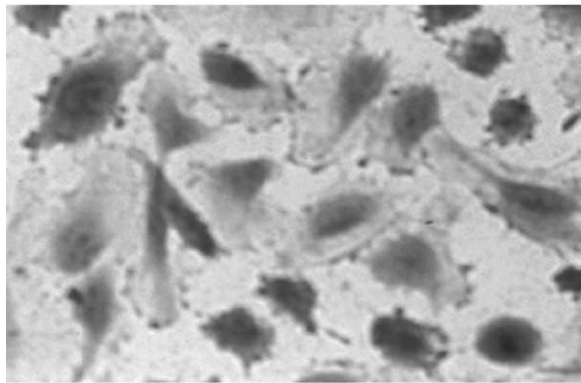
Fig. 3 illustrates the immunohistochemical staining of tissue kallikrein in HUVEC; the intensive immunoreactivity was seen in granules localized near the cell membranes.

3.3. Demonstration of mRNA expression of tissue kallikrein and L-kininogen in HUVEC

To clarify whether HUVEC express tissue kallikrein mRNA, RNA extracted from HUVEC was reverse-transcribed and amplified to the corresponding cDNA using primers specific for human tissue kallikrein cDNA [14]. The representative Southern hybridization demonstrated an amplified 155-bp fragment of tissue kallikrein cDNA from HUVEC RNA (Fig. 4). A similar amplification was also observed in the RNA sample from smooth muscle cells of human coronary artery or HeLa cells which was reported to express tissue kallikrein [11,14].

To determine whether HUVEC express kininogen mRNA, total RNA samples from HUVEC or HAEC were examined by RT-PCR followed by Southern blotting using primers specific for L-kininogen cDNA and H-kininogen cDNA. A clear signal was detected corresponding to the amplified product of 638 bp from L-kininogen mRNA in

Fig. 1. Kallikrein-like activity in the conditioned medium of HUVEC. HUVEC were cultured in 75-cm^2 dishes with EBM for 8, 16, and 24 h. The conditioned medium was dialyzed overnight against distilled water, then lyophilized. The lyophilizates were dissolved in 0.5 ml of 0.1 M Tris–HCl, pH 7.8, containing 10 μg/ml of bovine serum albumin and 0.1 M NaCl, and the kallikrein-like activity in samples was assayed using Pro-Phe-Arg-MCA as a substrate, as described in the text. Enzyme activity was expressed as nmol AMC/mg protein of HUVEC/h. Each point and vertical bar indicates the mean±S.D. of three dishes.

A) anti-human tissue kallikrein
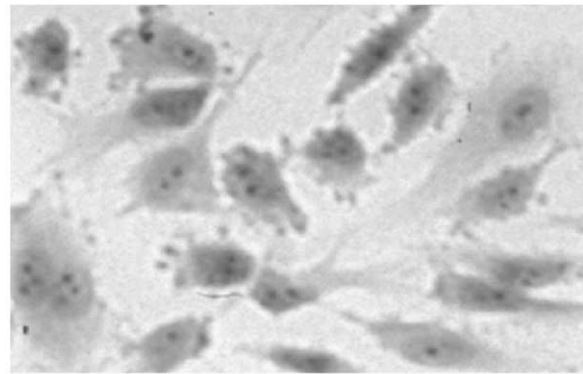
B) pre-immune serum

Fig. 3. Immunostaining of tissue kallikrein localized in HUVEC. The immunoreactive kallikrein was visualized with rabbit anti-human tissue kallikrein antibodies followed by an immunoperoxidase staining procedure using biotinylated goat anti-rabbit IgG/peroxidase-labeled streptoavidin (A). Equally diluted normal rabbit serum served as the negative control (B). Cells were counterstained with Mayer's hematoxylin, and the immunostaining viewed at ×400.

HUVEC and HAEC (Fig. 4), but no signals could be detected corresponding to H-kininogen mRNA in these cells (data not shown).
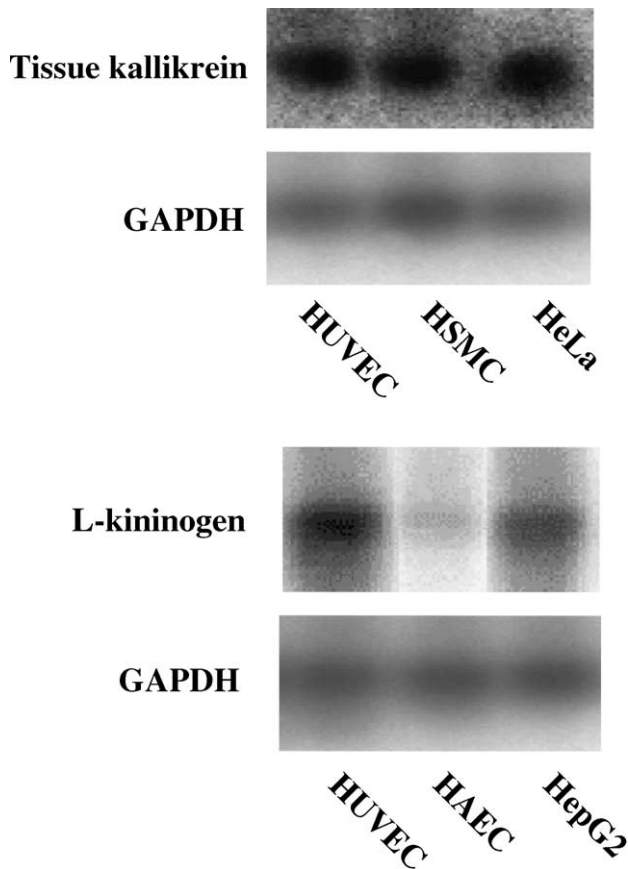

Fig. 4. Representative RT-PCR Southern blots of tissue kallikrein mRNA and L-kininogen mRNA in HUVEC. RT-PCR (40 cycles) was carried out using primers specific for human tissue kallikrein or human L-kininogen, then Southern hybridization of amplified products using an oligonucleotide probe for tissue kallikrein or human L-kininogen cDNA, respectively. Tissue kallikrein mRNA (upper panel) was analyzed in HUVEC, human coronary artery smooth muscle cells (HSMC) and HeLa cells, and kininogen mRNA (lower panel) in HUVEC, HAEC, and HepG2 cells. Each panel also shows RT-PCR Southern blots of GAPDH. Representative blots of RNA samples from three different cultures of cells are shown.
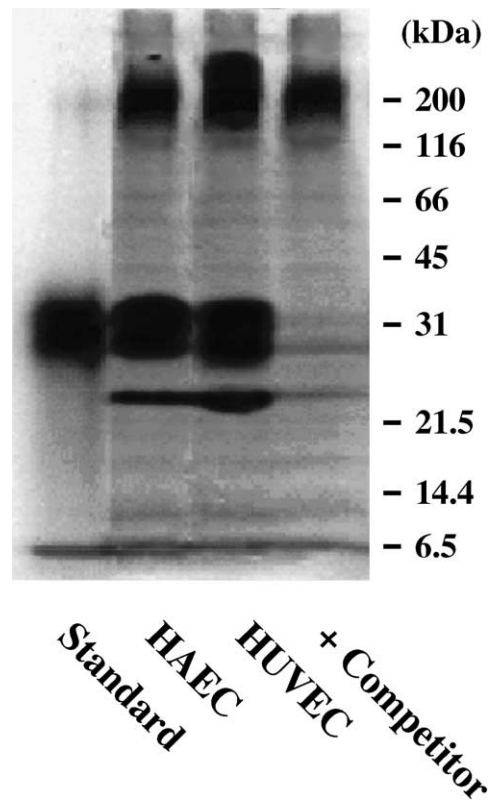

Fig. 5. De novo synthesis of tissue kallikrein in HUVEC. HUVEC and HAEC were metabolically labeled with [^{35}S]methonine for 24 h, then the conditioned medium was immunoprecipitated with rabbit anti-human tissue kallikrein antibodies. The immunoprecipitates were resolved on SDS-PAGE, then autoradiographed using a Fuji Film Bio Imaging Analyzer BAS2000. In the lane of 'competitor', the immunoprecipitates from radiolabeled HUVEC medium supplemented with 200-ng human urinary kallikrein were resolved with similar procedures. The numbers to the right of the blot represent molecular mass standards in kDa.

3.4. Demonstration of de novo synthesis of tissue kallikrein by endothelial cells

To confirm the de novo synthesis of tissue kallikrein by HUVEC, we performed metabolic labeling of the HUVEC or HAEC with [^{35}S]methionine; subsequently, tissue kallikrein in the conditioned medium was immunoprecipitated with rabbit anti-human tissue kallikrein antibodies. As shown in Fig. 5, the immunoprecipitates from the conditioned medium of either culture of human endothelial cells consisted of two major radioactive proteins between the molecular weights of 28 and 32 kDa. These two bands disappeared after the excess addition of human urinary kallikrein in the immunoprecipitation step. Since [^{125}I]-labeled human urinary kallikrein, as well as immunoprecipitates from conditioned medium of HeLa cells precultured with [^{35}S]methionine (data not shown), also exhibited two bands with similar molecular sizes, these two proteins released from endothelial cells appeared to be the active forms of tissue kallikrein with heterogeneity in their carbohydrate contents as reported previously [16].

3.5. Elevation of cGMP levels in HUVEC by lisinopril

To determine whether tissue kallikrein secretion from HUVEC is involved in the generation of kinins which in turn stimulates the NO/cGMP system, we examined the effect of the angiotensin converting enzyme inhibitor, lisinopril, on the cGMP levels in HUVEC in vitro (Fig. 6).
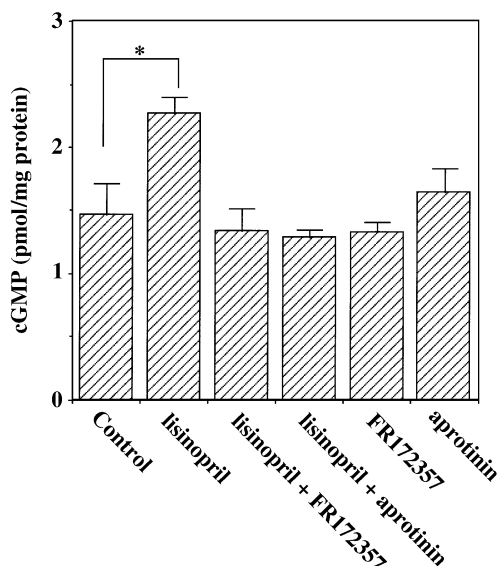

Fig. 6. Effect of lisinopril on cGMP levels in HUVEC. HUVEC in 4-cm^2 dishes were washed by HBSS, then incubated in HBSS containing 0.1 mM IBMX at 37 °C for 15 min. Cells were incubated for further 30 min with or without lisinopril (1 μM), FR172357 (1 μM), or aprotinin (10 μg/ml) at 37 °C. cGMP was extracted from cells with 0.1 N HCl (0.5 ml) and measured from neutralized supernatant by radioimmunoassay. Bars indicate means±S.D. of four to eight dishes. Significantly different from untreated control (*$P < 0.05$).

When HUVEC were incubated for 30 min in the presence of 1 μM lisinopril and IBMX, a phosphodiesterase inhibitor, the cGMP levels in HUVEC were significantly elevated. This elevation of the cGMP levels in HUVEC was completely inhibited by B2-receptor antagonist FR172357 (1 μM) or aprotinin (1 μg/ml), while FR172357 or aprotinin alone did not affect the cGMP levels (Fig. 6).

4. Discussion

Many studies have reported evidence that the vascular wall is a source of kallikrein: Oza et al. [7] demonstrated that rat vascular smooth muscle cells synthesized and secreted tissue kallikrein, and Nolly et al. [8] also demonstrated that the vascular tissues synthesize tissue kallikrein using rat vascular rings and isolated perfused hindquarters. Furthermore, Graf et al. [10] demonstrated that HUVEC, in addition to endothelial cells from human pulmonary arteries, contained a tissue kallikrein-like enzyme activity. The local distribution of tissue kallikrein has been demonstrated by immunostaining in endothelial and smooth muscle cells of human blood vessels, and the site of tissue kallikrein synthesis in blood vessels was also identified in these cells by in situ hybridization histochemistry [11]. In addition to these findings, the present study demonstrated that the conditioned medium of HUVEC or HAEC contained tissue kallikrein that had been de novo synthesized and secreted by endothelial cells, and that the proteins could not be distinguished from human urinary kallikrein in terms of the molecular size and susceptibility to protease inhibitors.

Recently, Dedio et al. [12] reported the presence of tissue kalikrein-like activity in lysates of HUVEC with a molecular size of about 50 kDa. On the basis of findings that the intracellular proteins having similar molecular sizes were radiolabeled in HUVEC prelabeled with [^{35}S]-cysteine/methionine and that an anti-peptide antibody against the activation site of prokallikrein did not detect traces of prokallikrein in HUVEC lysates, it was suggested that most of the de novo synthesized tissue kallikrein was stored within the cells as an activated form, not as an inactive prokallikrein form [12]. In contrast to their findings on the tissue kallikrein-like protease in HUVEC, we presently demonstrated the presence of an active form of tissue kallikrein with a molecular size of about 30 kDa in the conditioned medium of HUVEC: the tissue kallikrein-like enzyme secreted by HUVEC exhibited the same mobility on SDS-PAGE as that of human urinary kallikrein whose molecular weight was reported to be about 30 kDa [16]. Furthermore, the radiolabeled tissue kallikrein, synthesized de novo by HUVEC and HAEC, consisted of two major bands on SDS-PAGE which exhibited exactly the same profile as that of [^{125}I]-labeled human urinary kallikrein, suggesting that the enzyme from HUVEC had similar heterogeneity in carbohydrate content as urinary kallikrein. However, it is unknown why the molecular sizes of the

active form of tissue kallikrein secreted from HUVEC were different from those stored within HUVEC as reported by Dedio et al. [12].

When the conditioned medium of HUVEC was concentrated and incubated with a partially purified bovine kininogen, a kinin-like contractile activity on the rat uterus appeared during the course of incubation (data not shown). The kinin-like contractile activity was not generated in the presence of aprotinin during the incubation, suggesting that tissue kallikrein derived from HUVEC is responsible for kinin release from bovine kininogen. Thus, it is likely that the tissue kallikrein released from HUVEC has a kinin-generating activity. Therefore, it is hypothesized that an active form of tissue kallikrein released from HUVEC generated kinins, which in turn stimulated the B2-receptor on HUVEC. In fact, it was reported that the angiotensin converting enzyme inhibitor, ramiprilat, increases cGMP levels in HUVEC in vitro, and that the effect is inhibited by the B2-antagonist, Hoe-140, and the NO synthase inhibitor, N^G-nitro-L-arginine, suggesting that HUVEC generates kinins to enhance the NO/cGMP system [17]. To test the contribution of tissue kallikrein on the kinin generation by HUVEC, the cGMP levels in HUVEC were determined after the addition of the angiotensin converting enzyme inhibitor, lisinopril, in the presence or absence of aprotinin. We found that the cGMP levels in HUVEC were significantly increased by lisinopril, and that the addition of aprotinin or FR172357 completely inhibited the effect of lisinopril. These findings suggest that HUVEC continuously release an active form of tissue kallikrein which is responsible for the generation of kinins on HUVEC, and that lisinopril enhances the action of kinins on the B2-receptor of HUVEC by blocking their degradation.

In contrast to kallikrein, little evidence has been reported on the kininogen in vascular tissues. Although it was reported that rat vascular smooth muscle cells release a kininogen-like protein that liberates a kinin by trypsin digestion [7], our previous studies demonstrated that rat vascular smooth muscle cells express T-kininogen but not H-and L-kininogens [18]. Figueroa et al. [19] demonstrated that the immunoreactive H-kininogen localizes in the endothelial cells of human large vessels. It is well documented that high affinity binding sites for H- and L-kininogens are present on endothelial cells [20,21], and that kinin is generated from endothelial-bound H-kininogen by plasma kallikrein [22]. As shown in the present study, the incubation of HUVEC with lisinopril increased cGMP levels in vitro, probably by the action of kinins, but this does not clarify where kininogen comes from. There is a possibility that the H- and L-kininogens in FCS bound to the binding sites of the cell surface during subcultures with 10% FCS and remained there after washing. Another possibility is that kininogens are synthesized and released from HUVEC. In fact, we found that both HUVEC and HAEC express L-kininogen mRNA. However, since the H- and L-kininogens derived from the liver are abundant in the circulation, it is likely that the local generation of kinins on the endothelium in situ depends on the release of the active form of tissue kallikrein from endothelial cells. Recently, Bergaya et al. [23] reported that an exogenous infusion of kininogen in the carotid arteries caused a dilation of arteries in wild-type mice, but not in tissue kallikrein-knockout mice. Moreover, they demonstrated that the flow-dependent vasodilation response was impaired in tissue kallikrein-knockout mice [23], suggesting a physiological contribution of the vascular kallikrein–kinin system in the maintenance of circulation homeostasis. Thus, it will be important to elucidate the mechanisms by which the synthesis and release of tissue kallikrein from the endothelium are controlled for understanding not only the pharmacological actions of angiotensin converting enzyme inhibitors but also the pathophysiological role of kinins in cardiovascular tissues.

Acknowledgements

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