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Suppression of Vascular Endothelium Hyperpermeability by Cell-Permeating Peptide Inhibitors of Myosin Light Chain Kinase[1]

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Abstract—Novel peptides originating from the peptide inhibitor of myosin light chain kinase (MLCK), L-PIK (Arg-Lys-Lys-Tyr-Lys-Tyr-Arg-Arg-Lys), have been studied for their ability to attenuate the thrombin-induced hyperpermeability of an endothelial cell monolayer in culture. Peptides [N^{α}MeArg^{1}]-Lys-Lys-Tyr-Lys-Tyr-Arg-(D)Arg^{8}-Lys and H-Arg(NO_2)Lys-Lys-Tyr-Lys-Tyr-Arg-Arg-Lys-NH_2 (designated PIK2 and PIK4, respectively) appeared to be the most effective inhibitors of endothelial cell monolayer hyperpermeability, and surpassed other known peptide inhibitors of MLCK derived from original L-PIK. Our results validate PIK2 and PIK4 as the leading molecules for the development of novel drugs intended to counteract pathological hyperpermeability of vascular endothelium.

Keywords: peptide, inhibitor, myosin light chain kinase, hyperpermeability, vascular endothelium

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INTRODUCTION

The vascular endothelium acts as a barrier between blood and tissues and also performs a variety of important functions: it regulates vascular smooth muscle tone, hemodynamics, immune reactions, angiogenesis, hemostasis, etc. In capillaries, the endothelium controls the exchange of substances between the blood and tissue compartments. The selectivity of exchange is an important feature of the endothelial monolayer. Dysfunction of the endothelial barrier results in increased permeability of the vascular wall and tissue edema. The severity of edema varies from mild to life-threatening in cases of affliction of lung, brain, or other vital organs. The problem is commonly associated with various clinical conditions like intoxication, acute cardiac failure, cardiac valve abnormality, renal failure, cirrhosis, cancer, etc. Despite the progress in modern medical treatment, edema is still associated with high morbidity and mortality rates, even in intensive care units. Diuretics, crystalloids, and steroid hormones are widely used to fight edema. These agents improve fluid excretion but do not act on the molecular mechanisms of edema—the increase in microvascular endothelium permeability caused by the contractile activity of endothelial cells.

In cells, including endothelial ones, a key regulator of contractile activity is myosin light chain kinase (MLCK). The enzyme is activated by various edemagenic factors, such as thrombin, bradykinin, histamine, proinflammatory interleukins, tumor necrosis factor, vascular endothelium growth factor, reactive oxygen species, and excessive physicochemical stimuli. The importance of MLCK in the development of pulmonary edema was convincingly demonstrated in an endothelial MLCK knockout mouse model [1]. Through other approaches, MLCK was identified as a molecular target for preventing acute inflammatory lung injury [2].

However, the majority of known MLCK inhibitors are not suitable for human use because of severe adverse reactions. Therefore, of immediate interest is the search for MLCK inhibitors with pharmacologically privileged structure and high selectivity. One such example is a low-molecular aminopyridazine-based inhibitor [1, 3, 4]. Recent studies reported development of peptide inhibitors of MLCK based on the autoinhibitory domain of the enzyme [5]. Nonapeptide H-Arg-Lys-Lys-Tyr-Lys-Tyr-Arg-Arg-Lys-NH_2 (L-PIK) [6] was able to penetrate the plasma membrane and regulate epithelial permeability in vitro [7]. However, because of its chemical structure, L-PIK is highly susceptible to proteolysis [8], which puts in doubt its possible use in clinical practice. Although modification of L-PIK structure by replacement of L-amino acids with their D-analogs resulted in an expected increase of proteolytic stability [7, 9], the inhibitory activity of D-PIK was inferior to that of L-PIK, apparently because D-PIK is a complete enantiomer of L-PIK and has

[1] Translation of the text provided by the authors; redaction imposed solely for consistency and comprehensibility.

Modifications of MLCK peptide inhibitor (L-PIK) used in the present study

Peptide ID	Modification
L-PIK [6]	No modifications
PIK2	(N^{α})methyl-Arg in position 1, D-Arg in position 8
PIK3	(N^{α}) methyl-Arg in position 1, the peptide chain is elongated at the C-terminus by Pro-Gly-Pro tripeptide
PIK4	Arg residue in position 1 has a nitroguanidine group
PIK5	Pseudopeptide bond between Arg^1 and Lys^2, D-Arg in position 8
PIK6	Pseudopeptide bond between Arg^1 and Lys^2, the peptide chain is elongated at the C-terminus by Pro-Gly-Pro tripeptide
Cyclo-PIK	Cyclic analog of L-PIK where free N^{α}-amino group of Arg^1 and carboxyl group of Lys^9 are linked together by a peptide bond

another spatial structure [9]. Nevertheless, intravenous administration of D-PIK effectively attenuated the severity of lung edema induced in rats by intravenous injection of oleic acid [10].

Among the peptide analogs, there is no clear correlation between the chemical structure and biological activity. Therefore, modifications of structure may result in drastic alteration of the inhibitory properties of a peptide. Indeed, we reported previously that among the peptide inhibitors of MLCK obtained by modification of L-PIK structure (D-PIK, [N^{α}Me-Arg^1]-PIK, [εAca^1]-PIK, [Cit^1]-PIK, [Cit^1,Orn^3]-PIK), only [N^{α}Me-Arg^1]-PIK (designated PIK1) showed in vitro inhibitory activity comparable to that of L-PIK [9]. In vitro, the half-life of PIK1 in human plasma was extended 3-fold relative to L-PIK; moreover, 100 μM PIK1 attenuated the thrombin-induced permeability of the endothelial monolayer [11].

In this study, using models of stress-induced hyperpermeability of cultured endothelial cells, we compared the inhibitory properties of the novel and known derivatives of L-PIK. Based on these data, we have selected the peptide inhibitors most promising in further development of drugs to prevent/attenuate the vascular endothelium hyperpermeability.

EXPERIMENTAL

On the basis of the L-PIK sequence, a set of peptide inhibitors of MLCK were synthesized at the Russian Cardiology Research and Production Centre (Moscow) (table). All reagents were of analytical grade and were purchased from DiaM (Russia), Sigma (USA), Serva (Germany), Fluka (Switzerland), Bio-Rad (USA). Alpha-thrombin was purchased from Enzyme Research Laboratories (USA), fluorescein isothiocyanate (FITC)-conjugated albumin, *Escherichia coli* lipopolysaccharide (LPS), and gelatin were from Sigma.

Endothelial cell culture. EA.hy926 (ATCC, USA) and HMVEC-L (Human Microvascular Endothelial Cells – Lung) (Lonza, USA) were used in the study. The cells were cultured at 37°C in a 5% CO_2 atmosphere in the presence of antibiotics and 10% fetal calf serum (HyClone, USA). EA.hy926 cells were cultured in DMEM (PanEco, Russia), HMVEC-L cells were cultured in EGM 2 MV (Lonza). The medium was changed every 2 days.

FITC-albumin permeability across the endothelial cell monolayer in vitro. For experiments, we used 24-well plates (Greiner Bio-one, Germany) equipped with special inserts to form inner (upper) chambers with a porous bottom (pore diameter 0.4 μm). EA.hy926 cells were seeded at the density of $2 \cdot 10^4$ cells per membrane pre-coated with 0.2% gelatin and cultured at 37°C in a 5% CO_2 atmosphere to confluence. On the day of experiment, the cells were deprived for 1 h in the medium with 0.1% fetal calf serum, followed by 1-h incubation with 20–100 μM peptide inhibitors that were added to both lower and upper compartments. Then FITC-albumin was added to the upper compartment to a final concentration of 1 mg/mL. One hour later, a 10 μL aliquot was withdrawn from the lower compartment to estimate the endothelial monolayer baseline permeability. The hyperpermeability response was induced by alpha-thrombin at the final concentration of 100 nM. Every 30 min during the next 4 h, the intensity of FITC fluorescence was measured in the aliquots from the lower compartment, using a Victor 3X plate reader (Perkin-Elmer, USA) at λ_{ex} = 495 nm and λ_{em} = 535 nm. Experiments were carried out in triplicates, data were presented as mean ± standard deviation.

Measurement of transendothelial electric resistance in vitro. For experiments, HMVEC-L cells were cultured to early confluence in 100-mm dishes coated with 0.2% gelatin. The cells were detached with 0.15% trypsin-Versene solution and seeded at a density of $0.5 \cdot 10^5$ cells per well in 8W1E multiwell arrays (ECIS Cultureware, Applied BioPhysics, Inc., USA) on a gold-plated electrode precoated with 0.2% gelatin. Two arrays with cells were fitted in a holder connected to an ECISz (Applied BioPhysics) instrument, and following 1–1.5 h data collection, peptide inhibitors of MLCK were added to a final concentration of 100 μM. Forty five minutes later, LPS from *E. coli* was added to a final concentration of 100 ng/mL. Transen-

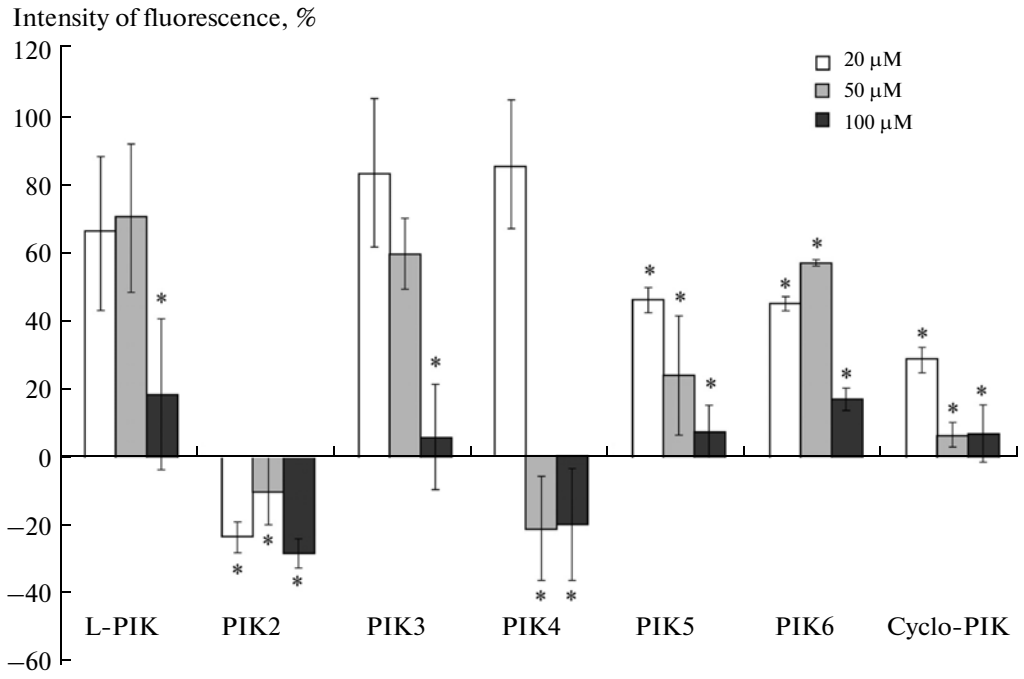

Fig. 1. Effects of PIK-family peptide inhibitors on thrombin-induced FITC-albumin flux across the endothelial cell monolayer. The monolayer of EA.hy926 cells was incubated with MLCK peptide inhibitors at final concentration of 20 μM (white), 50 μM (gray), or 100 μM (black) for 1 h and stimulated with 100 nM alpha-thrombin. Four hours after thrombin addition, the intensity of FITC fluorescence in the lower compartment was measured. The results are expressed as percent of monolayer permeability in the absence of peptide inhibitors, where 100% is the response to 100 nM thrombin stimulation and 0% is the basal permeability without thrombin stimulation. Data are presented as mean ± standard deviation; $n = 3$, $^*p < 0.05$ vs. control in the presence of 100 nM thrombin.

dothelial electric resistance changes were measured for 16–20 h. Experiments were carried out in triplicates, the values of normalized electrical resistance were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Previously we reported that peptide derivatives of L-PIK can permeate the human umbilical vein endothelial cells [10] and EA.hy926 endothelial cells [11]. In the present study, the EA.hy926 cell line was used for modeling the barrier function of endothelium in vitro.

We modified the original structure of L-PIK (table), using modifications known to increase the stability of peptides in biological fluids [12–15]. The effect of the modifications on the biological activity of the peptides was analyzed in a model of endothelial hypermeability in vitro. As shown in Fig. 1, 1-h preincubation of EA.hy926 cell monolayer with peptide inhibitors of MLCK augmented the endothelial barrier function as judged by a decrease in FITC-albumin leakage induced by the treatment of cells with 100 nM thrombin. All the peptides tested showed concentration-dependent ability to prevent the thrombin-induced hyperpermeability of endothelial monolayer. For the peptide inhibitors tested, the inhibitory activities were not inferior to that of L-PIK in the working concentration range (20–100 μM). This result indicates that the modifications we introduced (table) did not affect the active groups important for the inhibitory properties of L-PIK.

At the concentration of 20 μM, the inhibitory efficiency of PIK3 and PIK6 was relatively low. Apparently, in these analogs, incorporation of the C-terminal Pro-Gly-Pro extension (see table) increased the molecular dimensions and, possibly, hindered the translocation of the peptides across the plasma membrane. Presumably, the same effect was exerted by the incorporation of a nitro group in the guanidine moiety of Arg^1 (PIK4). This suggestion is based on the known importance of basic amino acid residues for peptide translocation across the plasma membrane [16]. Nevertheless, at higher concentrations (50 and 100 μM), PIK4 effectively prevented the thrombin-induced endothelial cell hyperpermeability.

Interestingly, among the peptide inhibitors tested, PIK2 was clearly superior in attenuating the FITC-albumin flux across the endothelial cell monolayer and showed complete restoration of the barrier function even at the concentration of 20 μM. The distinguishing feature of PIK2 is the methylated amino group of Arg^1 and the D-Arg at position 8 (table). We showed previously that peptide inhibitors obtained by replacement of Arg^1 with either citrulline or Na-methylated arginine

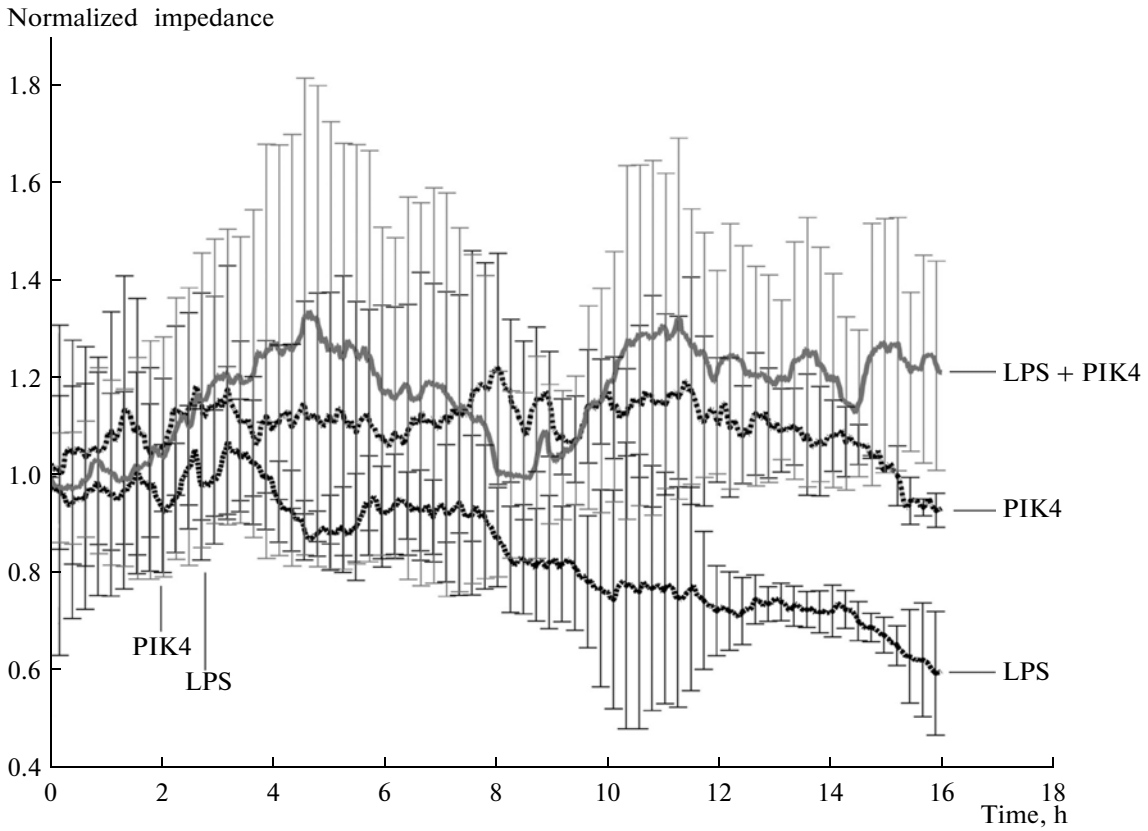

Fig. 2. Effects of PIK-family peptide inhibitors on LPS-induced changes in transendothelial electric resistance of human endothelial cells. HMVEC-L cells were plated on gold-plated electrodes and cultured to confluence. Basal transendothelial resistance was measured, and the cells were challenged (indicated by vertical arrows) with LPS at a final concentration of 100 ng/mL; 45 min later, PIK4 was added at a final concentration of 100 μM. Data are presented as mean ± standard deviation ($n = 3$).

were comparable to L-PIK in their ability to inhibit MLCK in vitro and attenuate the thrombin-induced endothelial monolayer hyperpermeability [11]. These data in combination with the results for PIK4 (present study) indicate that modification of Arg^1 does not interfere with the ability of PIK-family peptide inhibitors to restore the barrier function of endothelial monolayer. These results are supported by the effective inhibition of FITC-albumin flux by PIK5 containing a pseudopeptide bond between amino acid residues 1 and 2. Altogether, it seems plausible to conclude that an increase in the inhibitory properties of PIK2 is due to the replacement of L-Arg with D-Arg at position 8. Possibly, the change in the spatial orientation of the charged moiety of Arg^8 either led to tighter binding of the peptide to MLCK or abolished the steric interference that could be caused by L-Arg at position 8.

The cyclic analog of L-PIK effectively attenuated the FITC-albumin flux through the EA.hy926 cell monolayer (Fig. 1). While at the concentration of 20 μM the inhibition was 70%, 50 μM cyclo-PIK almost completely abolished the thrombin-induced hyperpermeability of the endothelial monolayer. These findings suggest that the restriction of possible conformations imposed by the cyclization of L-PIK did not interfere with effective binding of the peptide to MLCK and its inhibitory effect. These findings may indicate that despite the more rigid structure of cyclo-PIK, this peptide preserved the relative spatial position of the active groups necessary for effective inhibition of MLCK and/or only a part of the peptide is needed to exert the inhibitory effect.

The EA.hy926 cell line is a convenient model of vascular endothelium; however, some important functional features of the endothelium are not reproduced in these cells [17]. Indeed, we observed that the transendothelial electric resistance of EA.hy926 cell monolayer was not altered by a clinically relevant edemagenic agent, bacterial endotoxin LPS (data not shown). In further experiments, in order to test the possible protective effects of PIK against the LPS-induced endothelial hyperpermeability, we used HMVEC-L cells that are responsive to LPS. We analyzed the effect of PIK4 (one of the most effective peptides in the model of the thrombin-induced FITC-albumin flux across the EA.hy926 monolayer) on the changes in transendothelial electric impedance of HMVEC-L cell monolayer in response to LPS stimulation (Fig. 2). As shown in Fig. 2, LPS induces a slow decrease in electric resistance reflecting an increase in

the endothelial monolayer permeability. In the presence of 100 μM PIK4, the time course of impedance changes was not significantly altered by LPS administration and was similar to that in control cells. Similar results were obtained when HMVEC-L were pretreated with 100 μM PIK2 (data not shown).

Thus, peptide inhibitors of MLCK—PIK4 and PIK2—have demonstrated effective reduction of vascular endothelial hyperpermeability both in the model of thrombin-induced FITC-albumin flux across EA.hy926 cell monolayer (Fig. 1) and in the model of LPS-induced decline of transendothelial electric resistance of HMVEC-L cell monolayer (Fig. 2).

Previous reports demonstrated that MLCK inhibitors L-PIK [2] and D-PIK [10] may be protective against acute lung injury. In the model of EA.hy926 cell permeability, 100 μM D-PIK was shown to inhibit the FITC-albumin flux by 50% [10]. At 100 and 50 μM, L-PIK attenuated the flux of FITC-albumin by 80 and 40%, respectively (Fig. 1). It is noteworthy that at the concentration of 100 μM, peptides PIK3, PIK5, PIK6, and cyclo-PIK (table) almost completely inhibited the FITC-albumin flux (Fig. 1), indicating that they are not inferior to either L-PIK or D-PIK. Moreover, peptides PIK2 and PIK4 were superior to both L-PIK and D-PIK as evidenced by the ability of either 50 μM PIK2 or 50 μM PIK4 to abolish the thrombin-induced FITC-albumin flux across the EA.hy926 cell monolayer (Fig. 1).

The data presented herein demonstrate that structural changes, introduced in PIK peptides in order to increase their stability in a biological milieu, did not reduce the inhibitory activity of these peptides toward MLCK. It is concluded that PIK2 and PIK4 appear to be the molecular candidates for development of novel drugs intended to attenuate the hyperpermeability of vascular endothelium in various clinical situations associated with acute disturbances of microvascular endothelial barrier function.

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