Differential Expression of Prokineticin Receptors by Endothelial Cells Derived from Different Vascular Beds: a Physiological Basis for Distinct Endothelial Function

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Key Words

Luteal endothelial cells • Bovine aortic endothelial cells
• Endothelial permeability • Brain capillaries • Apoptosis
• Endothelial phenotypic heterogeneity

Abstract

Prokineticins (PKs), multifunctional secreted proteins, activate two endogenous G protein-coupled receptors (R) termed PK-R1 and PK-R2. It was suggested that PK1 acts selectively on the endothelium of endocrine glands, yet PK-Rs were also found in endothelial cells (EC) derived from other tissues. Therefore we examined here the characteristics of PK - system in EC derived from different vascular beds. Corpus luteum (CL)-derived EC (LEC) expressed both PK-R1 and PK-R2. In contrast, EC from the aorta (BAEC) only expressed PK-R1. Interestingly, also EC from brain capillaries (BCEC) expressed only PK-R1. The distinct pattern of PK-R expression may define EC

phenotypic heterogeneity. Regulation of receptor expression also differed in BAEC and LEC: TNF α markedly reduced PK-R1 only in BAEC, but serum removal decreased PK-R1 in both cell types. Therefore, if cells were initially serum-starved, the anti-apoptotic effect of PKs was retained only in LEC. Yet, addition of PKs concomitant with serum removal enhanced the proliferation and survival of both BAEC and LEC. Immunohistochemical staining showed that in CL and aorta PK1 was expressed in smooth muscle cells in vessel walls, suggesting a paracrine mode of action. PK1 enhanced the net paracellular transport (measured by electrical resistance and Mannitol transport) in LEC but not in BAEC or BCEC. Collectively, these findings indicate that PKs serve as mitogens and survival factors for microvascular (LEC) and macrovascular (BAEC) EC. However, the distinct expression and function of PK receptors suggest different physiological roles for these receptors in various EC types.

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Introduction

Prokineticin (PK) - 1 is a newly discovered protein [1, 2]. Together with its close homolog, PK2, these secreted proteins belong to a family of peptides consisting of 80-90 amino acids, including 10 conserved cysteines, which create a five-disulphide-bridged motif (colipase fold) and identical N-termini sequence - AVIT [1-3].

PK1 is widely expressed in steroidogenic glands (such as the ovary, testis, placenta and adrenal gland), as well as in the gastrointestinal tract and the nervous system [2, 4-8].

The two PKs serve as cognate ligands for two closely homologous G protein-coupled receptors (GPCRs) termed PK receptors (PK-R1 and PK-R2). Located in different chromosomes, these two receptors share 85% homology, which is a high value among known GPCRs [9-11]. PK1 and PK2 bind and activate their receptors indiscriminately with similar affinities [9-11]. In cells overexpressing either PK-R1 or R2, PKs mobilized calcium, stimulated phosphoinoside turnover, and activated the p44/p42 MAPK cascade [9-11]. These biological effects were also observed in cells naturally expressing PK - Rs, namely the adrenal cortex - derived endothelial cells (EC) and luteal EC (LEC) [12, 13].

Originally identified as an agent with the ability to contract gastrointestinal smooth muscle [1], PK1 was subsequently shown to also act as a selective angiogenic mitogen: intra-ovarian delivery of PK1 promoted angiogenesis and cyst formation in the rat ovary [2]. Moreover, it induced proliferation, migration and fenestration of EC derived from adrenal capillaries [2, 12]. Therefore, PK1 was also termed endocrine gland-VEGF (EG-VEGF), although structurally it is unrelated to VEGF. We have recently reported that similarly to adrenal gland derived EC, PKs enhanced the proliferation and inhibited apoptosis in bovine luteal EC [13].

Endothelial cells are unique multifunctional cells that have numerous paracrine and endocrine functions (for review see [14]) some of which are to regulate homeostasis, vasomotor tone, permeability, and inflammatory responses. These properties vary both in space and time, giving rise to the phenomenon termed EC heterogeneity [15, 16]. Using DNA microarrays, Chi et al. [17] showed that EC from different blood vessels in various tissues have distinct and characteristic gene expression profiles.

To better understand the role of these novel endothelial regulators, we determined the expression of PK receptors in EC derived from different vascular beds,

an endocrine gland - the corpus luteum (CL), the aorta and brain. We then determined the response of EC from aorta and CL to PKs, explored the cell source of PK1 in these tissues, and finally determined the effects of PK1 on endothelial permeability.

Materials and Methods

Materials

Cell media: Dulbecco's Modified Eagle's Medium - (DMEM-F12 (1:1)), DMEM low (1gr/L glucose), DMEM high (4.5g/L glucose), Hanks balanced salts solution (HBSS), L-Glutamine, Calf Serum (CS), Penicillin - Streptomycin solution, crystalline trypsin solution (0.2g/L trypsin), Trypsin EDTA solution (2.5g/L trypsin, 0.2g/L EDTA), XTT proliferation kit were all obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Vitrogen - type I collagen was purchased from Cohesion Technologies (Palo Alto, CA). Bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), proteases inhibitor cocktail, D-Mannitol-[1-3H(N)] 1mCi/ml, acrylamide/bis-acrylamide electrophoresis reagent 40% solution, paraformaldehyde and horseradish peroxidase-conjugated goat anti-rabbit IgG, and Mounting solution were acquired from Sigma (St. Louis, MO). [methyl-³H] thymidine was purchased from Amersham Biosciences (Buckinghamshire, UK). TRI Reagent was obtained from MRC (Cincinnati, OH). Fetal Bovine Serum (FBS), SuperScriptTM II RNase H- Reverse Transcriptase, and Ultrapure Electrophoresis Agarose were obtained from Invitrogen Corporation (Paisley, Scotland, UK). Random hexamer oligodeoxynucleotides were procured from Fermentas (Vilnius, Lithuania). Deoxynucleotide triphosphates were obtained from Bioline GmbH (Luckenwalde, Germany). Oligo-dT and Oligonucleotide primers were synthesized by MWG Biotech AG (Ebersberg, Germany). The Real-time PCR SYBR Green Master-Mix Kit was purchased from Eurogentec (Seraing, Belgium). Rabbit anti-p-44/42 MAPK was obtained from Cell Signaling Technology (Beverly, MA). Antiserum raised against the human PK1 peptide (residues 84-96: LLCSRFPDGRYRC) was from Covalab (Lyon, France). Recombinant human tumor necrosis factor- α (TNF- α) were obtained from ProSpec - TanyTechnoGene Ltd. (Rehovot, Israel). SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce (Rockford, IL). Type I collagen-coated Transwell polystyrene filters, 1x10⁵/filter (surface area,

Gene	Sequence	Product length (bp)
G3PDH	f: cgtggacagtgggtcataagt r: ggcgtaaccacgagaagtat	141
PK-R1	f: ggtcatggatgagaatgccca r: atacccaccagcgccatgcc	200
PK-R2	f: ataccccaagaacatgcct r: ctgataccacacaagtgccacc	150

Table 1. Real-time PCR primer list

0.33 cm; pore size, 0.4 mm; Corning Costar, Cambridge, MA, USA). X-ray films were from Fuji Photo Film Co., Ltd. (Tokyo, Japan). DAKO anti-rabbit EnVision™ - HRP Labelled Polymer and 3-amino-9-ethylcarbazole chromogen (AEC) were obtained from DakoCytomation (Carpinteria, CA).

Endothelial cell cultures

Luteal Endothelial Cells, isolation and culture: Isolation of LEC was performed as described by Spanel-Borowski et al, [18] with slight changes. Briefly, bovine mid-cycle CLs were collected from a local slaughterhouse and luteal stage was determined by macroscopic examination of the ovaries, according to criteria described by M. Fields [19]. First, the surrounding tissue was removed and CLs were washed several times. Next, the CLs were thinly sliced using a device containing 20 parallel razor blades. The pieces were collected, centrifuged, and resuspended in medium following vigorous pipetting. The tissue was sieved through a 150-µm pore size metal sieve and then through a 20-µm pore size nylon mesh. Cells were centrifuged and resuspended in erythrocyte lysis buffer (0.144mM NH₄Cl/0.017M Tris-HCl, pH=7.4). Finally, the cells were seeded on Vitrogen type I - coated 24-well plates (500 µl/well). The medium was changed every other day. Colonies appeared after 7-14 days; each was collected separately and their identity was verified by vWF staining and CD31 expression. LEC were cultured in DMEM-F12 containing 10% FBS, 2mM glutamine. Preliminary studies had shown that expression of PK-Rs and mitogenic response to PKs remained similar at least until passage 12; therefore, cells from passages 4–12 were used for these experiments. At least three independent cell isolates used in each experiment. Bovine Aortic Endothelial Cells (BAEC) and Brain (Capillary Endothelial Cells (BCEC) were kindly provided by Prof. Israel Vlodavsky (Hadassah-Hebrew Medical Center, Jerusalem, Israel).

BAEC and BCEC (passages 6-13) were cultured in low glucose DMEM containing 10% CS and 2mM glutamine. When indicated, cells were starved in medium containing 0.1% serum and 0.5% BSA.

RNA isolation and Real-time PCR

Total RNA was isolated from the cells with TRI-Reagent according to the manufacturer's instructions. Quantitative real time PCR reactions were performed using PE Biosystems' GeneAmp 5700 sequence detection system, with the SYBR Green I PCR kit used as described by Klipper et al [20]. Briefly, each real-time reaction (18 µl) contained the SYBR Green Master Mix that comprised ROX passive reference, 200 µM dNTPs including dUTP, 5 mM MgCl₂, uracil N-glycosylase, and Amplitaq HotGoldStar DNA polymerase; 0.54 µl of a 1:10,000 dilution of SYBR Green stock solution; 1.5 mM dNTPs; 10 nM of each of the primers and 25-50 ng cDNA. The glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene was used as a standard. Dissociation curve analysis was run following each real-time experiment to confirm the presence of only one product and the absence of the formation of primer dimmers. The threshold cycle number (C_T) for each tested gene X was used to quantify the relative abundance of the gene: $2^{-(C_T \text{ gene X} - C_T \text{ G3PDH})} \times 1000$. For a list of primers, see Table 1.

[³H]-thymidine incorporation

Incorporation of [³H]-thymidine was measured according to Masuda et al. [10]. Briefly, LEC and BAEC were incubated for 32 h in 24-well plates in medium containing 0.5% BSA and 0.1% serum only or medium containing either 50 nM PK1 or PK2. [³H]-thymidine (1 µCi/well) was added during the last 8 h of incubation. The cells were then washed with cold HBSS and incubated with 10% trichloroacetic acid at 4°C for 15 min. The acid-insoluble fraction was dissolved in 0.3N

NaOH (15 min at 37°C) and the radioactivity was evaluated with a scintillation counter.

XTT assay

LEC and BAEC were seeded at 20,000 – 30,000 cells/well in 24 well plates with following addition of PK-1 (50 nM) in medium containing 0.5% BSA and 0.1% serum. After 3 days of incubation the medium was replaced to basal medium (0.5% BSA, 200 µl/well), and 100 µl of activated XTT reagent was added to each well. The cells were then incubated for 4h at 37°C. The absorbance was read at 450 nm. From each value a blank (empty well reading) was subtracted and a number metabolically active cells was evaluated by dividing the mean of treated wells by mean of control (n=3 for each treatment or control).

Immunohistochemistry

Tissues (CLs at the mid cycle and slices of aorta) were fixed in 4% (v/v) paraformaldehyde, embedded in paraffin, and cut into 5 µm sections. The sections were de-waxed in xylene and rehydrated using decreasing ethanol concentrations. Endogenous peroxidase activity was quenched by pretreatment with 3% (v/v) hydrogen peroxide in methanol for 30 min. Antigen retrieval was performed by treating sections for 5 min in a microwave in boiling 0.1% citrate buffer (pH 6.0). Tissue sections were then washed in PBS and incubated with normal horse serum (10%) that served as a blocking agent for non-specific binding for 1h. The serum containing PK1 rabbit polyclonal antibody (1:50 dilution in blocking solution) was added for 18h [6]. After 3 PBS washes, the slides were incubated with DAKO anti-rabbit EnVision™ - HRP labeled Polymer for 30 min. The immunoreactive proteins were visualized after addition of AEC solution for 5 min and then counterstained with hematoxylin.

Immuno (Western) blotting

Detection of p44/42. Proteins were extracted from BAEC with SDS-lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue) and were sonicated for 10 s. Protein extracts were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked in TBST (20 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat milk for 1 h at room temperature, and were incubated overnight with either phosphorylated p42/p44 MAPK or total p42/p44 MAPK antibodies diluted in

TBST-5% BSA (1:10,000 and 1:1000, respectively) at 4°C. The membranes were washed and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted in TBST-5% nonfat milk at room temperature for 2 h. A chemiluminescent signal was generated with SuperSignal and the membranes were exposed to X-ray film.

DAPI staining

LEC and BAEC were grown for 48 h in DMEM-F12 containing 0.1% FBS and 0.5% BSA, alone or with PKs (100 nM), on cover slips that had been precoated with 1% vitrogen up to 70-80% confluence. The cells were fixed with EFA (70% ethanol, 4% paraformaldehyde, 5% glacial acetic acid), permeabilized with 0.25% Triton X-100 in PBS and then stained with 1 µg/ml DAPI reagent [21]. The cover slips were mounted on glass slides and photographed under a fluorescent microscope. Apoptotic cells had condensed and fragmented nuclei [21]. For the analyses, 11-15 fields of view at x 640 magnification were quantified (15–20 cells per field) in each experiment. The percentage of apoptotic cells in each field was evaluated and the average over all fields was determined.

Production of PK1 and PK2

Production, refolding, and purification of recombinant PK1 and PK2 were carried out appropriately as described previously [1]. The activity of PK was further confirmed by calcium mobilization assay in CHO cells that stably expressed human PK-R1 or PK-R2 (9).

Measurement of endothelial monolayer permeability

A) Electrical resistance [22]. Different EC types (LEC, BAEC and BCEC) were seeded onto Vitrogen-coated Transwell polystyrene filters, and grown to confluence for 10-11 days; growth medium was replaced every other day. Twenty-four hours prior to the experiment, confluent endothelial monolayers were serum-starved. Experiments were initiated by washing the upper and lower compartments with warmed (37°C) 20 mM Tris-buffered saline, pH 7.4, supplemented with 25 mM glucose and 0.1% BSA (150 µl/upper and 600µl/lower compartments). The inserts were allowed to equilibrate for 30 min at 37°C before initiating the experiments. After equilibration, the buffer was removed from the inserts and fresh buffer containing various treatments were added to the upper compartment (100U/ml Thrombin, 75nM PK1/PK2). PK1, PK2, and

Fig. 1. PK-R1 and PK-R2 expression in endothelial cells. Numbers (means $\pm$ SEM) represent PK-R1 and PK-R2 mRNA expression determined by Real-Time PCR. Data were from 3 independent cell preparations. Means with no common letters (uppercase for PK-R1 and lowercase for PK-R2) differ significantly ($p < 0.05$).

thrombin-induced changes in the resistance of the endothelial monolayers were measured using MilliCell® ERS (Millipore, MA USA) at 0, 5, 10, 15, 20, 25, and 35 minutes after adding the various treatments. Assays were performed in triplicate and changes in resistance were normalized to their respective controls.

B) [3 H] Mannitol transport (Papp) [23, 24]. Different EC types (LEC, BAEC and BCEC) were grown and treated as described in the previous section. Following the addition of the various treatments, the tracer (3 H] Mannitol, 125 nCi/well) was added. Samples (20 μ l) from the abluminal side were removed at 5, 10, 15, 20, 25, 35, and 45 min and replaced with freshly warmed buffer. The assay was performed in triplicates. Radioactivity was measured using a β counter. Papp (cm/s) was calculated using the following equation: $Papp = dQ/dt \times 1/A \times C_0$, where dQ/dt is the slope of the linear curve describing the cumulative amount of tracer transported (i.e. detected in the abluminal side) vs time [sec], A, the surface area of the filter (cm²), and C_0 (units/ml), the initial concentration in the donor (luminal side).

Statistical analyses

Data are presented as means $\pm$ SEM. All statistical analyses were carried out using JMP 5.1™ (Statistical Software, SAS Institute). One-way ANOVA LSMeans Student's test was used to determine the statistical difference between treatments, as indicated in the results and the legends to the figures. Additionally, CONTRAST-test was used to evaluate the effect of serum withdrawal

on PK-Rs expression in LEC and BAEC. Differences were considered significant at $P < 0.05$.

Results

PK-R mRNA expression and biological responses to PKs in endothelial cell types: BAEC, BCEC and LEC

We initially determined the expression, regulation of expression and biological responses of PK-Rs in two EC types: LEC derived from the corpus luteum that contains mainly microvascular vessels and BAEC - a well characterized model for a macrovessel. Data presented in Fig. 1 show that LEC expressed both PK-R1 and PK-R2, whereas BAEC expressed mainly PK-R1; their levels in BAEC were significantly higher than in LEC. Interestingly, the profile of PK-R expression in another microvascular EC - BCEC, resembled that of BAEC (macrovascular) more than that of LEC (microvascular cells; Fig. 1). In fact, amongst these three cell types, BCEC expressed highest levels of PK-R1 mRNA and were practically devoid of PK-R2.

The effect of the two PKs on the survival of LEC and BAEC in conjunction with serum withdrawal was then evaluated. The addition of PK1 or PK2 to either BAEC or LEC in growth medium containing a low serum concentration (0.1%) significantly increased cell survival as compared to controls ($p < 0.05$; Fig. 2a, 2b "no starvation"). However, if cells were first incubated in starvation medium (without PKs) for 48 hours, and PKs

Fig. 2. Effect of PKs on the survival of LEC (a) and BAEC (b). Cells were either cultured for 48 hours with PKs added concomitantly with serum removal („no starvation“) or cells were initially incubated in starvation medium (without PKs) for 48 hours, and PKs were added only during the following 48-h incubation period („starvation“). The percentage of apoptotic nuclei was evaluated as described in „Materials and Methods“. Data represent means $\pm$ SEM from 3 independent experiments. Means with no common letters differ significantly ($p < 0.05$).

were then added for the following 48h incubation period, different effects were produced in the two cell types. Under these conditions, PKs reduced the number of apoptotic cells only in LEC and not in BAEC (Fig. 2a, 2b „starvation“).

We have previously shown that PKs augment the proliferation of LEC [13]. The data presented in Fig. 3a now show that the two PKs also markedly increased $[^3\text{H}]$ -thymidine incorporation by approximately 1.6-fold in BAEC. Similarly, the XTT assay that determines cell proliferation by the colorimetric oxidative conversion of a tetrazolium salt (XTT) by metabolically active cells, showed that PK1 significantly increased cell numbers by a factor of 1.4.

We then examined whether PKs activated p44/42 MAPK in these cells. The MAPK pathway is a well-recognized signaling mechanism that mediates numerous

Fig. 3. Effects of PKs on $[^3\text{H}]$ -thymidine incorporation (a) and activation of the p42/p44 MAPK pathway (b) in BAEC. For $[^3\text{H}]$ -thymidine incorporation data, cells were incubated with PKs (50 nM each) for 32 hours. $[^3\text{H}]$ -thymidine was added in the last 8 hours of incubation, the results were normalized to the respective control. Data represent means $\pm$ SEM from 5 independent experiments. Means with no common letters (a-b) differ significantly ($p < 0.05$). For PK induction of p42/p44 MAPK cells were treated with PKs (100 nM) for 5 min and then subjected to Western blot analysis with anti-phospho/total-p42/44 MAPK. A representative blot (one of three similar ones) is shown.

cell functions including cell proliferation [25]. We observed that both PKs induced the phosphorylation of p44/42 MAPK in BAEC (Fig. 3 b) and in LEC [13].

Since PKs were shown to act as survival factors, it was interesting to examine whether conditions known to induce apoptosis in EC (serum withdrawal and TNF α exposure) modified the expression of PK-R1 in EC. Withdrawal of serum reduced PK-R1 levels by 50% in

Fig. 4. Regulation of PK-R1 expression by serum withdrawal and $\text{TNF}\alpha$ in LEC and BAEC. Cells were incubated in medium containing different concentrations of serum (10% and 0.1%) with or without $\text{TNF}\alpha$ (10ng/ml) for 48 hours. PK-R1 mRNA expression levels were determined by Real-Time PCR. Data represent the means from 5 independent experiments $\pm$ SEM. Means with no common letters (uppercase for LEC and lowercase for BAEC) differ significantly ($p < 0.05$).

Fig. 5. Immunohistochemical localization of PK1 in bovine mid-cycle CL and aorta. Immunohistochemistry was performed on PFA-fixed sections. Immunoreactivity is observed in smooth muscle cells (SMC) of small arteries present in the CL (C, E) and in SMC bundles of the aorta (A). Faint labeling is observed in steroidogenic luteal cells (SC, panel E). Staining was absent from endothelial cells (EC; panel E). No specific staining was observed when the primary antibody was omitted (B, D). Images were photographed at magnification $\times 100$ –400 as indicated.

both LEC and BAEC (Fig. 4). However, in the presence of serum, $\text{TNF}\alpha$ markedly diminished (by six fold) PK-R1 expression in BAEC only. The addition of $\text{TNF}\alpha$ in the absence of sera did not further reduce PK-R1 in

either BAEC or LEC (Fig. 4).

To determine the cellular source of the PK1 protein for LEC and BAEC, immunohistochemical staining was performed on sections of bovine mid-cycle CL and aorta

Fig. 6. Effects of PKs and thrombin on the transendothelial electrical resistance (TEER) across the LEC, BAEC, and BCEC monolayers. Cells were grown on Transwell polystyrene filters until post-confluent. Twenty-four hours after incubation in medium containing 0.5% BSA, the cells were treated with PKs (each 75 nM) and thrombin (100U/ml). Changes in resistance were measured at the time points 0, 5, 10, 15, 20, 25, and 35 minutes. Data represent the means $\pm$ SEM from 3 independent experiments. The results were normalized to the respective control and are presented as the percent decrease in electrical resistance. Asterices indicate significant difference between treatment and the control group ($p < 0.05$).

(Fig. 5). Intense immunostaining was observed in the smooth muscle cell (SMC) layers of blood vessels in the CL (Fig. 5 C, E). Luteal cells also stained but to a lesser extent (Fig. 5 E). In contrast, EC in all types of vessels remained unstained (Fig. 5 E). As observed in luteal vessels, PK1 protein was localized to SMC clusters in the aortal media and to SMC in vessels of the aortal adventitia (Fig. 5A). Staining was absent when the primary antibody was omitted (Fig. 5 B, D).

Permeability Studies

Maintenance of a semi-permeable barrier by the endothelium is critically important in endothelial cell function. We therefore examined whether PKs affected EC permeability. For these studies we used, together with LEC and BAEC, another EC type, BCEC. These cells represent an additional microvascular bed, different from that of the corpus luteum. As constituents of the blood brain barrier these cells express special features

Fig. 7. Effects of PKs and thrombin on the permeability of endothelial cells to [^3H] Mannitol. Cells were grown on Transwell polystyrene filters until postconfluent. Twenty-four hours after incubation in medium containing 0.5% BSA, the cells were treated with PKs (each 75 nM) and thrombin (100 U/ml). Following the treatments, [^3H] Mannitol was added and samples from the abluminal side were removed at 5, 10, 15, 20, 25, 35, and 45 min. The permeability coefficient was calculated as described in „Materials and Methods“. Data represent the mean $\pm$ SEM of $n=3$ (LEC) or mean $\pm$ range $n=2$ (BAEC, BCEC). Columns with asterisks indicate significant statistical difference from control.

Fig. 8. A representative dQ/dT plot of [^3H] Mannitol accumulation in the luminal chamber of LEC. (For details, see legend to Fig. 7).

in terms of barrier function. The endothelial barrier was evaluated by trans-endothelial electrical resistance (TEER; Fig. 6). Unlike thrombin, which enhanced electrolyte transport (reduction of TEER) in the three cell types, albeit at different rates (Fig. 6a-c), PK1 decreased TEER values only in LEC, reaching a maximum already at 10 minutes after exposure to PK1 (Fig. 6a). At 15 minutes the effect declined (but remained significant) and after 20 minutes there was no further significant decrease. PK2 had a small transient effect on TEER readings only in LEC. In BCEC, PK1 appeared to increase TEER (improved the barrier function), but this effect was not statistically significant (Fig. 6c).

The data summarizing the permeability coefficients for [^3H] Mannitol transport (Papp) in response to thrombin and PK1 are presented in Fig. 7. The basal permeability coefficient (Papp) in LEC was $1.7 \times 10^{-5} \text{ cm/sec}$ (Fig. 7a), whereas the transport rate through BAEC and BCEC was almost twice as high, reaching values of $4.3 \times 10^{-5} \text{ cm/sec}$ and $3.5 \times 10^{-5} \text{ cm/sec}$, respectively (Fig. 7b,c). Thrombin almost doubled [^3H] Mannitol transport through LEC. In contrast, the effect of thrombin on BAEC and BCEC was not statistically significant when compared to non-treated cells. In LEC, in a manner analogous to thrombin, PK1 markedly increased the [^3H] Mannitol transport rate (Fig. 7a). In contrast, PK1 did not influence the transport rates in BAEC and BCEC (Fig. 7b,c). The effects of PK1 and thrombin on mannitol transport in LEC were evident already at the 10 min point and increased with time (Fig. 8).

Discussion

The findings reported here and in our previous study show that PK peptides stimulated the proliferation of EC derived from vessels of either the aorta or an endocrine gland such as the corpus luteum [13]. In the two organs, PK1 was localized to SMC present in blood vessel walls and was absent from EC, suggesting a paracrine mode of action. PK1 mRNA expression was previously demonstrated in these tissues [1, 26], but their specific localization to SMC is revealed here for the first time. The close functional interaction between vascular smooth muscle and endothelial cells control an array of processes such as vascular tone, angiogenesis and is of central importance in the pathogenesis of atherosclerosis and hypertension [27-30]. The particular localization of PK1 adds a new dimension to the SMC-EC interrelationship, suggesting that SMC (via PK1) may regulate the proliferation and survival of the EC lining the vessels.

Distinct expression and function was associated with PK receptors in the different EC types: luteal EC expressed both PK-R1 and PK-R2; whereas EC derived from large vessels such as aorta (BAEC) and umbilical vein EC (data not shown) only expressed receptor subtype 1. Regulation of receptor expression was also different in BAEC and LEC. Although serum removal decreased PK-R1 in both LEC and BAEC, in BAEC it was associated with a reduction of the only receptor type present in these cells, namely PK-R1. In contrast to PK-R1, serum starvation markedly augmented PK-R2 expression in LEC [13]. Consequently, the biological effects induced by PKs were cell-specific: the addition of PKs concomitant with serum removal enhanced cell proliferation and survival of both BAEC and LEC, suggesting the involvement of PK-R1 in these functions. The mitogenic and pro-survival effects of PKs on these cells correlated with the ability of PK1 to induce phosphorylation of the p42/44 MAP kinase. However, if cells were serum-starved prior to the addition of PK1, it retained its pro-survival effect only in LEC and not in BAEC. The reason for the difference between the cells' response may be attributed to the presence of elevated PK-R2 levels in serum-starved LEC, suggesting that PK-R2 can also mediate PK-induced cell survival. However, in addition to these functions, the presence of PK-R2 appears to be indispensable for PK1-induced endothelial permeability. These effects were only observed in PK-R2 expressing LEC and not in BAEC or BCEC which only express PK-R1. The case of BCEC is particularly

interesting: in contrast to other microvascular EC, the brain capillary endothelium serves as a protective shield or barrier between blood and the underlying tissue (central nervous system) [31, 32]. Hence, receptor distribution appears to be regulated by local physiological requirements. In fact, up till now, PK-R2 was only identified in fenestrated EC, such as those found in the adrenal cortex, CL, kidney, and liver, in agreement with notion that PK-R2 plays a role in EC permeability [10, 12, 33, 34]. Regarding PK-R1, our data tend to imply that the expression of this receptor type may enhance EC barrier function as its levels were highest in the least permeable BCEC and lowest in LEC. It is also possible that the balance between PK-R1 and R2 influences cell permeability. Indeed, these possibilities are supported by the data from our permeability studies. The decrease in TEER values and the increase in [^3H] Mannitol permeability strongly suggest that PK1 (and thrombin) augment net paracellular transport in LEC. In both assays PK1 enhanced permeability in LEC and not in BAEC or BCEC. Under basal conditions microvascular luteal EC monolayers were half as permeable as monolayers from macrovascular BAEC. This is consistent with other studies that indicated that pulmonary and retinal microvascular EC formed a tighter barrier than macrovascular EC [35-37]. The short duration of PK1 and the thrombin-induced TEER reduction indicate that LEC may possess a unique capacity to repair intercellular gaps, even in the continued presence of these activators [38].

Endothelial cell structure and functional integrity are important in the maintenance of the vessel wall and circulatory function. Proinflammatory mediators such as thrombin and histamine increase vascular permeability by activating [Ca^{2+}] sensitive signaling pathways [36, 39]. This change in [Ca^{2+}] is thought to be essential for the generation of EC paracellular gaps, thus facilitating permeability [38-40]. Activation of PK receptors leads to mobilization of calcium and stimulation of the turnover of phosphoinositide [9-11], which is consistent with the augmentation of EC permeability by PKs. Yet PKs were shown to stimulate [Ca^{2+}] mobilization in PK-R2 as well as in PK-R1-expressing cells, presumably through a receptor/Gq protein interaction [9, 10, 41, 42]. Thus, the reason why PK1 failed to induce permeability in BAEC and BCEC expressing PK-R1 is seemingly unclear. But as PK-Rs are capable of coupling to multiple G proteins [42], it cannot be ruled out that different EC expressing PK-Rs may have different G protein repertoire and hence respond to PKs differently. Thus, these cells may perform

distinct physiological functions in response to the same ligand stimulation. Furthermore, we reported that the two receptor types are regulated differently by stress conditions such as TNF α , hypoxia, and serum starvation further supporting the notion that these receptors may have distinct functions [13].

As suggested by our findings, PKs act as angiogenic mitogens and survival factors for both LEC and BAEC (this study and [13]). However, this appears to contradict previous reports suggesting that PK1 acts selectively on the endothelium of endocrine glands [2]. The discrepancy might be explained by the fact that the data in the LeCouter et al. [2] study were obtained using cells that were serum-starved before adding PK1. Under these conditions, PKs maintains their effects only in LEC as was also shown in the present study. These findings highlight the importance of PK action on stressed LEC, suggesting that the presence of PK-R2 provides LEC with an additional tool to resist stress-induced apoptosis. Indeed, due to their tissue microenvironment, LEC are more prone than BAEC to experience extreme conditions such as low oxygen tension and a shortage of nutrients. This suggests that PK-induced survival is of physiological significance.

Although all vascular EC share certain common functions, it has become increasingly clear that considerable structural and functional heterogeneity exists along the length of the vascular tree and in the

microvascular beds of various organs [15, 16, 35]. The molecular diversity of EC has now been established, but the origin of this heterogeneity remains poorly understood [17]. Although some genes such as E-selectin, eNOS, endothelin -1 (ET-1), and vWF are considered pan-endothelial, many others are expressed in a temporal and spatial manner [17]. Endothelial heterogeneity arises from cues within the tissue microenvironment [17, 43], through soluble factors and/or cell-cell contacts. For instance, we reported that freshly isolated LEC have a characteristic gene expression profile of the ET-1 system that changes upon removal of the corpus luteum microenvironment [20]. Thus EC gradually lose some of their selective characteristics. However, besides microenvironment signals that may be reversible, during development certain site-specific properties become intrinsic to the cells and are mitotically stable. Therefore, the typical expression of PK receptors in cells of different segments of the vascular tree could be an important novel element in defining the EC phenotypic heterogeneity. Collectively, these data show that both PK-R1 and PK-R2 mediate PK-induced endothelial cell proliferation and survival. In addition, PK-R2 may be the receptor mediating selective microvascular responses such as increased permeability and ability to withstand stress. The full spectrum of the specific roles of PK-Rs in EC requires further research.

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