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PKG-I inhibition attenuates vascular endothelial growth factor-stimulated angiogenesis

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ABSTRACT

Vascular endothelial growth factor (VEGF) stimulates nitric oxide (NO) production, which mediates many of its angiogenic actions. However, the angiogenic pathways that operate downstream of NO following VEGF treatment are not well characterized. Herein, we used DT-2 and DT-3, two highly selective cGMP-dependent protein kinase I peptide inhibitors to determine the contribution of PKG-I in VEGF-stimulated angiogenesis. Incubation of chicken chorioallantoic membranes (CAM) with PKG-I peptide inhibitors decreased vascular length in a dose-dependent manner, with DT-3 being more effective than DT-2. Moreover, inhibition of PKG-I with DT-3 abolished the angiogenic response elicited by VEGF in the rabbit eye cornea. PKG-I inhibition also blocked VEGF-stimulated vascular leakage. In vitro, treatment of cells with VEGF stimulated phosphorylation of the PKG substrate VASP through VEGFR2 activation; the VEGF-stimulated VASP phosphorylation was reduced by DT-2. Pre-treatment of cells with DT-2 or DT-3 inhibited VEGF-stimulated mitogen-activated protein kinase cascades (ERK1/2 and p38), growth, migration and sprouting of endothelial cells. The above observations taken together identify PKG-I as a downstream effector of VEGFR2 in EC and provide a rational basis for the use of PKG-I inhibitors in disease states characterized by excessive neovascularization.

1. Introduction

Angiogenesis, the formation of blood vessels from pre-existing structures, is a tightly regulated process that is essential both during embryonic life, as well as in adulthood (Carmeliet, 2005; Folkman and Shing, 1992). New blood vessel formation is required for wound healing, tissue regeneration and remodelling (Carmeliet, 2003). On the other hand, excessive/abnormal angiogenesis participates in disease progression, such as diabetic retinopathy, psoriasis, rheumatoid arthritis, tumor growth, and metastasis (Carmeliet, 2005; Ferrara and Kerbel, 2005). Although a great number of endogenous substances have been shown to enhance angiogenesis, VEGF has emerged as a crucial angiogenic mediator and pharmacological agents targeting VEGF have made their way into the clinic as anti-angiogenic therapies (Ferrara et al., 2003; Shojaei and Ferrara, 2007).

VEGF receptor activation on EC leads to NO production that in turn activates sGC to generate cGMP (Papapetropoulos et al., 1997; Ziche and Morbidelli, 2000; Ziche et al., 1997). Evidence generated both from genetic and pharmacological experiments has underscored the importance of the NO/cGMP pathway in mediating postnatal VEGF-driven angiogenesis (Fukumura et al., 2001; Murohara et al., 1998a,b; Ziche et al., 1997, 1994). NO synthase (NOS) and sGC inhibitors reduce angiogenesis in several models, while endothelial NOS knockout animals exhibit reduced tumor and ischemia-stimulated neovascularization (Gratton et al., 2003; Murohara et al., 1998a, b; Pyriochou et al., 2006; Yu et al., 2005; Ziche et al., 1994). In spite of the progress made in unraveling the role of cGMP in angiogenesis, the downstream effectors in these responses have remained elusive. It is well accepted that in vascular cells, cGMP exerts its effects by altering the activity of cGMP-regulated protein kinases, phospodiesterases and ion channels (Lucas et al., 2000). Based on its ability to raise cGMP, VEGF should activate PKG in EC; however, the contribution of PKG in VEGF-stimulated blood vessel formation has not been determined.

PKG belongs to the family of serine/threonine kinase and is expressed in a variety of eukaryotes ranging from the unicellular organism *Paramecium* to *Homo sapiens*. Two PKG genes, encoding for the PKG type I (PKG-I) and PKG type II (PKG-II), have been identified in mammals and two isoforms of PKG-I, PKG-Iα and PKG-Iβ, are produced by alternative splicing (Feil et al., 2003; Hofmann, 2005). PKG-I is abundantly expressed in vascular smooth muscle cells and platelets (Pfeifer et al., 1999); we and others have shown it is also present in EC

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(Draijer et al., 1995; Pyriochou et al., 2007a,b). Traditionally, cGMP analogues and KT-5823 have been used to inhibit PKG (Butt, 2009; Taylor et al., 2004). These inhibitors lack specificity towards PKG, as they also inhibit other serine/threonine kinases; moreover, they do not discriminate between PKG-I and PKG-II and they only inhibit cGMP-stimulated, but not basal, PKG activity (Taylor et al., 2004); some of the older PKG inhibitors actually exhibit partial agonist activity on basal PKG enzymatic activity. Recently, peptide inhibitors that are highly selective for PKG-I have been discovered using a phage display library approach (Dostmann et al., 2000). DT-2 and DT-3 are 1000-fold more selective for PKG vs. PKA and exhibit a 100-fold selectivity for PKG-I vs. PKG-II (Dostmann et al., 2000). These inhibitors, thus, represent useful pharmacological tools when the need to determine the contribution of PKG-I in a biological response arises. In the present study, we have utilized DT-2 and DT-3 to determine the role PKG-I in VEGF-stimulated angiogenesis and to determine the potential usefulness of PKG inhibitors as anti-angiogenic agents.

2. Materials and methods

2.1. Materials

Peptides used include W45 (LRKKKKKH), an octapeptide which acts as a specific inhibitor for PKG I but does not cross the cell membrane, AP (RQIKIWFQNRRMKWKK), the *Drosophila* Antennapedia homeo-domain internalization sequence, TAT (YGRKKRRQRRR), the HIV-1 transcriptional transactivation protein tat translocation signal, (D)DT-2 (YGRKKRRQRRRPPLRKKKKKH), which consists of W45 N-terminally fused to a membrane translocation signal from HIV-1 tat protein and is composed of D- amino acids, DT-2 (YGRKKRRQRRRPPLRKKKKKH), which consists of W45 N-terminally fused to a membrane translocation signal from HIV-1 tat protein DT-3 (RQIKIWFQNRRMKWKK LRKKKKKH), which consists of W45 N-terminally fused to the *Drosophila* Antennapedia (AP) homeo-domain internalization sequence.

W45 was kindly provided by Dr. Dostmann (University of Vermont, USA). DT-3 and control AP peptides were obtained from W. M. Keck biotechnology resource center at Yale University School of Medicine (CT, USA). DT-2 and TAT peptides were obtained respectively from Biolog Life Science (Bremen, Germany) and GenScript Corp (NJ, USA). Cell culture media and serum were obtained from Life Technologies GIBCO-BRL (Paisley, UK). All cell culture plasticware was purchased from Corning-Costar Inc. (Corning, NY), the transwells used for the migration experiments carried polycarbonate membranes with 8 μm pores (catalogue number 3422); leghorn fertilized eggs were obtained from Pindos (Iperos, Greece). SuperSignal West Pico chemiluminescent substrate was purchased from Pierce Biotechnology (Rockford, Illinois); *DC* Protein assay kit, Tween 20 and other immunoblotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA); penicillin and streptomycin were purchased from Applichem (Darmstadt, Germany); gentamycin and heparin were purchased from Biochrom AG (Berlin, Germany); the ethinyl–vinyl copolymer (Elvax-40) was obtained from DuPont-De Nemours, Wilmington, DE; ERK1/2, p38, VASP phospho-specific and total antibodies were obtained from Cell Signalling Technology (Beverly, MA); VEGF-A was obtained from Peprotech (London, UK), while the neutralizing antibodies against VEGFR1 (catalogue number AF321) and VEGFR2 (catalogue number AF357) were purchased from R&D (Minneapolis, USA). All other reagents including bovine serum albumin, thrombin, EDTA, MTT and protease inhibitors were purchased from Sigma-Aldrich (St. Luis, MO).

2.2. Methods

2.2.1. CAM angiogenesis assay

Fertilized White Leghorn chicken eggs were placed in an incubator as soon as embryogenesis started and kept under constant humidity at 37 °C. On day 4, a square window was opened in the shell and then sealed with adhesive tape. On day 9, an O-ring (1 cm^2) was placed on the surface of the CAM and PKG-I inhibitors peptides were added inside this restricted area at the indicated dose. After 48 h, CAMs were fixed in Carson's solution (saline-buffered formalin) and angiogenesis was evaluated using image analysis software (NIH Image).

2.2.2. Rabbit cornea in vivo angiogenesis assay

The effect of PKG-I inhibition on the angiogenic activity of VEGF was assayed in vivo using the rabbit cornea assay as described previously(Ziche et al., 1994). Two experimental protocols were applied: 1) DT-3 or AP bearing pellets implanted simultaneously with VEGF, or 2) DT-3 or AP was added after 3 days following VEGF implantation. Daily observations of the implants were made with a slit lamp stereomicroscope by two independent operators in a blinded manner. Data are expressed as angiogenic score, (number of vessels × distance from the limbus), over time (days). All experiments have been performed in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC Law No. 86/609).

2.2.3. Modified Miles assay

One hour prior to the measurement of vascular leakage mice (C57/Bl6) received 1 mg/kg DT-2, the control peptide TAT, or the vehicle i.p. Mice were then injected i.v. with Evans blue (30 mg/kg) under anesthesia (ketamine/xylazine). After the Evans blue administration mice received VEGF (300 ng in 15 μl) or saline intradermally into the dorsal surface of the right and left ears, respectively. Evans blue extravasation into the ear was measured as previously described (Morbidelli et al., 2010).

2.2.4. HUVEC cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from 2 to 4 fresh cords and grown on 100-mm dishes in M199 supplemented with 15 % fetal calf serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin, 50 μg/ml gentamycin, 5 U/ml sodium heparin and 150–200 μg/ml endothelial cell growth supplement. Cells were used at the first or second passage.

2.2.5. Western blotting

Proteins from cells were extracted after homogenization in a lysis buffer containing 1% Triton X-100, 1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1 mM EGTA, and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml pepstatin, and 20 mM phenylmethylsulfonyl fluoride). Samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with the primary and secondary antibodies. Immunoreactive proteins were detected using a chemiluminescent substrate. Bands on autoradiograms were quantified using the Scion Image Release Beta 4.0.2 software.

2.2.6. Cell growth and migration

HUVEC cells were seeded in 24-well plates at 6×10^3 cells/cm^2 and incubated in M199 supplemented with 15% FBS medium for 24 h. To inhibit PKG-I, cells were exposed to DT-3 or DT-2 (1 μM, 20 min), prior to VEGF treatment. Cells were then treated with VEGF (50 ng/ml) in the presence of the inhibitor peptides and allowed to grow for 48 h. Cell number was measured using the MTT method(Buttke et al., 1993). For migration experiments the modified Boyden chamber assay was used(Pyriochou et al., 2006).

2.2.7. Rat aorta ring assay

Microvessel growth from aortic ring explants grown in fibrin gels was monitored(Morbidelli et al., 2010). Quantitative evaluation of newly formed structures was carried out on day 3. At a magnification of