ATP-Sensitive Potassium Channel Activation Induces Angiogenesis In Vitro and In Vivo

Bukar Umaru, Anastasia Pyriochou, Vasileios Kotsikoris, Andreas Papapetropoulos, and Stavros Topouzis

Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Rio-Patras, Greece (B.U., A.Py., V.K., S.T.); and Department of Pharmacy, National and Kapodistrian University of Athens, Athens, Greece (A.Pa.)

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ABSTRACT

Intense research is conducted to identify new molecular mechanisms of angiogenesis. Previous studies have shown that the angiogenic effects of hydrogen sulfide (H2S) depend on the activation of ATP-sensitive potassium channels (KATP) and that C-type natriuretic peptide (CNP), which can act through KATP, promotes endothelial cell growth. We therefore investigated whether direct KATP activation induces angiogenic responses and whether it is required for the endothelial responses to CNP or vascular endothelial growth factor (VEGF). Chick chorioallantoic membrane (CAM) angiogenesis was similarly enhanced by the direct KATP channel activator 2-nicotinamidoethyl acetate (SG-209) and by CNP or VEGF. The KATP inhibitors glibenclamide and 5-hydroxydecanoate (5-HD) reduced basal and abolished CNP-induced CAM angiogenesis. In vitro, the direct KATP openers nicorandil and SG-209 and the polypeptides VEGF and CNP increased proliferation and migration in bEnd.3 mouse endothelial cells. In addition, VEGF and CNP induced cord-like formation on Matrigel by human umbilical vein endothelial cells (HUVECs). All these in vitro endothelial responses were effectively abrogated by glibenclamide or 5-HD. In HUVECs, a small-interfering RNA–mediated decrease in the expression of the inwardly rectifying potassium channel (Kir) 6.1 subunit impaired cell migration and network morphogenesis in response to either SG-209 or CNP. We conclude that 1) direct pharmacologic activation of KATP induces angiogenic effects in vitro and in vivo, 2) angiogenic responses to CNP and VEGF depend on KATP activation and require the expression of the Kir6.1 KATP subunit, and 3) KATP activation may underpin angiogenesis to a variety of vasoactive stimuli, including H2S, VEGF, and CNP.

Introduction

The mechanisms underlying angiogenesis have been extensively studied for the past 40 years, resulting in an enhanced understanding of the complex cellular processes that together initiate and sustain angiogenesis and generating therapeutically important molecular targets (Coultas et al., 2005; Ferrara and Kerbel, 2005; Carmeliet and Jain, 2011). However, the contribution of additional, as yet unknown, participating mechanisms is the subject of intense ongoing research. It has been reported that the endogenous gasotransmitter H2S, which signals in part through ATP-sensitive potassium channels (KATP), can induce angiogenic responses that are abrogated by KATP inhibition (Papapetropoulos et al., 2009). In addition, the polypeptide CNP (C-type natriuretic peptide), which also mediates some of its effects via KATP activation, has been shown to elicit endothelial cell growth responses in vitro and collateral angiogenesis in vivo (Yamahara et al., 2003; Khambata et al., 2011). Combined, these findings raise the possibility that KATP activation, a relatively little-studied common mechanism, triggers angiogenesis in response to various physiologic stimuli.

ATP-sensitive potassium channels, which were originally discovered in the heart (Noma, 1983), are regulated by a variety of physiologic factors such as hypoxia and ischemia and by hormone levels (Jahangir and Terzic, 2005; Tinker et al., 2014), thus functionally coupling cellular metabolism and membrane excitability to maintain homeostasis by matching cellular and systemic metabolic demands. KATP are membrane-spanning hetero-octameric proteins that

ABBREVIATIONS: Akt, protein kinase B; CAM, chick chorioallantoic membrane; CNP, C-type natriuretic peptide; ERK, extracellular signal-regulated kinases; 5-HD, 5-hydroxydecanoate; HUVEC, human umbilical vein endothelial cells; Kir, inwardly rectifying potassium channel (KIR) 6.1 subunit interfering RNA; KATP, ATP-sensitive potassium channel; LY-294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride; NO, nitric oxide; NPR-C, natriuretic peptide clearance receptor; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SB239063, 4-[4-(4-fluorophenyl)-5-(2-methoxy)pyrimidin-4-yl]imidazo[1,2-a]pyridine-1-ol; SG-209, 2-nicotinamidoethy acetate; siRNA, small-interfering RNA; VEGF, vascular endothelial growth factor; U0126, (2Z,3Z)-2,3-bis[amino-(2-aminophenyl)sulfanyl]methylidene)butanedinitrile.
selectively allow efflux of K\(^+\) ions across the plasma membrane and the mitochondria through a permeation pathway (Billman, 2008; Kohler et al., 2010). The pore-forming ion channel is established by four subunits that belong to the family of inwardly rectifying potassium (K\(_r\)) channels (Flagg et al., 2010). The two subtypes found in K\(_{ATP}\), K\(_{6.1}\) and K\(_{6.2}\), are encoded respectively by the KCNJ18 and KCNJ11 genes and are thought to be inhibited by rises in cytosolic ATP (Flagg et al., 2010). K\(_{ATP}\) are widely distributed in many tissues and cell types, including pancreatic β-cells (Tarasov et al., 2004), the heart (Kohler and Ruth, 2010), neurons and brain (Zhou et al., 2002), skeletal muscle (Miki et al., 1999), smooth muscle (Teramoto, 2006), the kidney (Zhou et al., 2006), brain (Zhou et al., 2002), and endothelial cells (Suzuki et al., 2011) and human umbilical vein (bEnd.3; Suzuki et al., 2011) and endothelial cell line bEnd.3 (Suzuki et al., 2011) and human umbilical vessel (Lawson, 2000; Flagg et al., 2010; Kohler and Ruth, 2010; Burley et al., 2014). K\(_{ATP}\)-modulating molecules, exemplified by the inhibitory sulfonylureas glibenclamide and tolbutamide, have been used therapeutically for decades to augment insulin secretion by pancreatic β-cells in type-2 diabetes (Lawson, 2000; Wallia and Molitch, 2014). However, the involvement of K\(_{ATP}\) in angiogenesis remains largely unknown.

The indirect relationship previously put forth between the ability of two vasoactive agents (H\(_2\)S, CNP) to signal through K\(_{ATP}\) and their proangiogenic effects prompted us to ask whether direct stimulation of K\(_{ATP}\) can indeed induce endothelial growth, migration, and cord-like structure formation, processes that are critical during new vessel growth. In parallel, we aimed to better characterize the angiogenesis-related endothelial effects of CNP, which have been up to now poorly documented. Last, we wanted to know whether the pore-forming K\(_{ATP}\) subunit K\(_{6.1}\) is present in endothelial cells and whether its expression is required in their responses to CNP.

To evaluate the angiogenic effects of K\(_{ATP}\) modulation and assess the endothelial effects of CNP, we used both a classic in vivo angiogenesis model (chick embryo chorioallantoic membrane (CAM)), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vitro cell-based assays (proliferation, migration, cord-like network formation on Matrigel), together with in vivo chick chorioallantoic membrane assay. White Leghorn chicken eggs were placed in an incubator as soon as embryogenesis started (day 0) 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 the various treatments were added inside this restricted area as previously described elsewhere (Papapetropoulos et al., 2009). After 48 hours, CAMs were fixed in Carson’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum, 2 mM glutamine, 1 g/l glucose, 100 IU/ml penicillin, and 100 μg/ml streptomycin. HUVECs were isolated from cords obtained according to clinical consent agreement protocols and grown on gelatinized dishes in M199 supplemented with 15% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 5 U/ml bovine pituitary extract, and 150–200 μg/ml endothelial cell growth supplement. Cells were used between passages 1 and 3. Each experiment shown derives from three independent repeats, each time using different pools (isolates) and/or passages of cells.

In Vivo Chick Chorioallantoic Membrane Assay. White Leghorn chicken eggs were placed in an incubator as soon as embryogenesis started (day 0) 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 the various treatments were added inside this restricted area as previously described elsewhere (Papapetropoulos et al., 2009). After 48 hours, CAMs were fixed in Carson’s solution (saline-buffered formalin), and angiogenesis was evaluated using image analysis software. For the CAM experiments, 30 to 35 eggs were used per group, distributed in three independent experiments. Handling and use of chick embryos was performed according to University of Patras institutional animal welfare protocols.

Cell Transfection with siRNA. HUVECs were treated either with vehicle (vehicle control) or were transfected with siRNAs. The siRNA final concentrations used were 14 nM for the Ambion siRNAs and 40 nM for the Santa Cruz Biotechnology siRNAs. We used two different control siRNAs, one from Santa Cruz Biotechnology, referred to as CTLSi#1, and one from Ambion, referred to as CTLSi#2, and two different siRNAs targeting specifically the K\(_{6.1}\) K\(_{ATP}\) subunit from both Santa Cruz Biotechnology and Ambion, referred to as K\(_{6.1}\)si#1 and K\(_{6.1}\)si#2, respectively. Four hours later, the transfection medium was replaced by fresh medium, and cells were allowed to grow for another 20 hours. At the end of this incubation time, the cells were washed twice with phosphate-buffered saline, trypsinized, and used in migration and Matrigel assays. RNA was also collected for quantitative reverse-transcription polymerase chain reaction (qRT-PCR), and cell lysates were collected for Western blotting experiments.

Transwell Migration Assay. The capacity of endothelial cells to migrate through a pore-bearing membrane was assessed using 6.5 mm diameter Transwell chambers with polycarbonate membrane inserts (8 μm pore size). Control or siRNA-transfected endothelial cells (HUVECs or bEnd.3) were serum starved overnight. Some of the cells were pretreated with 5-HD (100 μM), glibenclamide (10 μM), or kinase inhibitors (10 μM U0126, 10 μM SB2039063, and 5 μM LY-294002) for 20 minutes before the end of the starvation time. Subsequently, cells were trypsinized, and 1 × 10\(^5\) cells were added to each Transwell in 100 μl of serum-free medium containing 2.0% growth supplement.
bovine serum albumin in the presence or absence of CNP (100 pM), SG-209 (1 uM), or nicorandil (10 uM). Cells (HUVECs or bEnd.3) were allowed to migrate for 4 hours, or the nonmigrated cells at the top of the Transwell filter were removed with a cotton swab. The migrated cells on the bottom side of the filter were fixed in Carson's solution for 30 minutes at room temperature and then were stained with toluidine blue. Migrated cells were scored and averaged from eight random fields per Transwell as previously described elsewhere (Pyriochou et al., 2006).

Matrigel Cord-Like Morphogenesis Assay. The formation of cord-like structures by endothelial cells (HUVECs or bEnd.3) was assessed in growth factor-reduced Matrigel. Untransfected (vehicle control), control siRNA-transfected or K<sub>K<sub>6.1</sub></sub> siRNA-transfected endothelial cells (15,000 cells/well) were plated in 96-well plates precoated with 45 uM of Matrigel per well in the presence or absence of CNP (100 pM), SG-209 (1 uM), or vehicle. After 8 hours of incubation, cord-like structure formation was quantified using Scion image software. One image per well was analyzed and used for the statistical analysis (Pyriochou et al., 2006; Papapetropoulos et al., 2009).

Endothelial Cell Proliferation Assay. The bEnd.3 cells were seeded in 24-well plates at a density of 6,000 cells/cm<sup>2</sup> and incubated overnight in Dulbecco’s modified Eagle’s medium. Cells were then pretreated for 20 minutes with vehicle or with K<sub>K<sub>ATP</sub></sub> or mitogen-activated protein kinase kinase inhibitor (U0126, 10 uM). Cells were then exposed to different concentrations of CNP, SG-209, or vehicle and allowed to proliferate for 48 hours. At the end of this incubation time, the cells were trypsinized, and their number was determined using a Neubauer hemocytometer.

Quantitative Reverse-Transcription Polymerase Chain Reaction. The expression of mRNA was evaluated using real-time qRT-PCR. Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified by a NanoDrop 2000 (Thermo Fisher Scientific). We subjected 250 ng of total RNA to reverse transcription and real-time PCR amplification using the KAPA SYBR Fast One-step qRT-PCR protocol (KapaBiosystems, Wilmington, MA). Amplification and real-time fluorescence detection was performed according to the manufacturer’s instructions (Pierce chemiluminescent horseradish peroxidase substrate kit; Thermo Fisher Scientific). After incubation with the appropriate secondary antibodies, the immunoreactive proteins were detected using a chemiluminescent substrate according to the manufacturer’s instructions (Pierce chemiluminescent horseradish peroxidase substrate kit; Thermo Fisher Scientific).

Statistical Analysis. Data are expressed as the mean ± S.E.M. of the given number of observations. Results were compared between groups using Student’s t tests using SPSS 10.0 software (IBM, Armonk, NY) under Windows XP (Microsoft, Redmond, WA). P < 0.05 was considered statistically significant.

Results

SG-209 and CNP Promote In Vivo CAM Angiogenesis. To determine whether K<sub>K<sub>ATP</sub></sub> activation elicits angiogenic responses, we first tested in the CAM angiogenesis model a direct channel opener, the nicorandil derivative SG-209. SG-209 lacks nicorandil’s nitric oxide (NO)–donor properties because nicorandil’s nitrate moiety has been replaced with acetate in SG-209. Thus, SG-209 is thought to act only through K<sub>K<sub>ATP</sub></sub>-opening (Ishibashi et al., 1991). We also included a receptor-mediated K<sub>K<sub>ATP</sub></sub> activator, CNP. We compared their maximal responses with those of a well accepted angiogenic factor VEGF, used at a maximal (for this assay) concentration of 500 pM. Treatment of CAMs with SG-209 (0.1–10 nmol/cm<sup>2</sup>) or with CNP (300 and 3000 nmol/cm<sup>2</sup>) promoted angiogenesis, measured as total vascular length and number of branching points (Fig. 1, A and B). The maximal effects of SG-209 and CNP were comparable with those of 500 nmol/cm<sup>2</sup> VEGF (Fig. 1B).

Fig. 1. Effect of SG-209 and polypeptide growth factors on CAM angiogenesis. CAMs were treated with the indicated concentrations of either (A) the K<sub>K<sub>ATP</sub></sub> opener SG-209 or (B) CNP or VEGF. Vessel length and branching point number were determined via National Institutes of Health image analysis software 48 hours after treatment. Inserts are representative photomicrographs (original magnification, 2.5×). Data are expressed as mean ± S.E.M.; n = 30–35 per point. *P < 0.05 versus vehicle; **P < 0.01 versus vehicle, using Student’s t test.
KATP modulation is sensitive to KATP blockade. CNP, indicating that the effects of CNP are sensitive to KATP blockade.

**KATP Activation Is Important for bEnd.3 Cell Proliferation, Migration, and Cord-Like Network Formation.** To further test the link between KATP activation and triggering of angiogenic responses at the cellular level, we moved to endothelial cell–based in vitro assays because endothelial activation or mobilization is paramount in bona fide angiogenic responses. In bEnd.3 mouse brain endothelial cells in vitro (Papapetropoulos et al., 2009), KATP activation by both SG-209 (1 μM) and nicorandil (10 μM) resulted in elevated cell proliferation (increases of 88.9% ± 11.9% and

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**Fig. 2.** Effect of KATP inhibitors on CAM angiogenesis. (A) Gilbenclamide (Glib; 1–100 nmol/cm²), (B) 5-HD (10–1000 nmol/cm²), or (C) tolbutamide (1–100 nmol/cm²) were applied on CAMs in vehicle, and vessel length and branching point number were determined 48 hours later. (D) CAMs were pretreated with glibenclamide (1 nmol/cm²) for 30 minutes before application of CNP (300 nmol/cm²), and vessel length and branching point number were determined 48 hours later. Inserts are representative photomicrographs (original magnification, 2.5×). Data are expressed as mean ± S.E.M.; n = 30–35 per point. *P < 0.05 versus vehicle; **P < 0.01 versus vehicle; †P < 0.05 versus CNP alone, using Student’s t test.

**Fig. 3.** Effect of KATP modulation on bEnd.3 endothelial cell proliferation and migration in vitro. (A) bEnd.3 cells were maintained in Dulbecco’s modified Eagle’s medium + 2.5% fetal bovine serum in 24-well plates and pretreated with either glibenclamide (Glib; 10 μM) or with 5-HD (100 μM) for 20 minutes before addition of SG-209 (1 μM) or nicorandil (10 μM). At 48 hours later, cells were trypsinized and counted using a hemocytometer. (B) bEnd.3 cells were resuspended in serum-free medium and pretreated with either glibenclamide (10 μM) or 5-HD (100 μM) for 20 minutes before being placed in Transwells and allowed to migrate for 4 hours in response to SG-209 (1 μM) or nicorandil (10 μM), after which the migrated cells were stained and counted. Data are expressed as mean ± S.E.M., n = 5. *P < 0.05 or **P < 0.01 versus vehicle control; †P < 0.05 versus nicorandil or SG-209 alone, using Student’s t test.
105.0% ± 9.33%, respectively; Fig. 3A). In addition, SG-209 and nicorandil also increased cell motility through a Transwell compartment to 2.5- and 3.5-fold of vehicle control, respectively (Fig. 3B). Pretreatment of bEnd.3 cells with either glibenclamide (10 μM) or 5-HD (100 μM), while not significantly affecting basal responses (Fig. 3, A and B), abolished the growth and motility effects of both SG-209 and of nicorandil (Fig. 3, A and B). The increase in bEnd.3 motility was more robust in cells treated with nicorandil than with SG-209, reflecting either possible use of a submaximal concentration of SG-209 or the contribution of the NO-releasing effects of nicorandil in motility but not in cell growth (Fig. 3B).

We next tested whether receptor-mediated K_{ATP} activation by CNP participates in similar in vitro responses. CNP (1–1000 pM) dose dependently increased cell proliferation (maximal effect: 39.2% ± 5.9% increase at 1000 pM) (Fig. 4A), an effect comparable to that elicited in parallel by 500 pM VEGF (increase of 49.9% ± 7.5%; Fig. 4A). The proliferative effects of VEGF were almost entirely suppressed by the K_{ATP} blockers glibenclamide and 5-HD (Fig. 4B). CNP (100 pM) and VEGF (500 pM) also induced comparable increases in bEnd.3 cell motility through Transwells by approximately 2.5-fold, which were abolished by either glibenclamide (both VEGF and CNP responses) or 5-HD (VEGF responses; Fig. 4C), indicating dependence of critical endothelial angiogenesis processes on K_{ATP} activation by these agents.

To further assess this dependence, we used a well characterized in vitro angiogenesis assay, cord morphogenesis in reduced-growth Matrigel. In addition, to address any concerns for cell type-selective responses, we also incorporated HUVECs in this assay for comparison. As can be seen in Fig. 5A, the direct K_{ATP} channel opener SG-209 (1 μM) elicited cord-like formation in bEnd.3 cells (increase by 72% ± 4.4%), which was effectively blocked by either glibenclamide or by 5-HD. Similarly, when HUVECs were used in this assay, the effect of CNP (100 pM, 131.7% ± 10.9% increase) was also abrogated by the two K_{ATP} blockers (Fig. 5B). No significant effect of glibenclamide or 5-HD on basal network morphogenesis was seen (Fig. 5B).

**Knockdown of the K_{ir6.1} K_{ATP} Subunit Expression Using Specific siRNAs.** To further probe the contribution of the pore-forming K_ATP K_{ir6.1} subunit, we introduced by transfection either of two different control siRNAs or of two different siRNAs specific for the human sequence of K_{ir6.1} in HUVECs. Analysis 24 hours later of cell lysates by Western blotting or by qRT-PCR indicated that both siRNAs targeting K_{ir6.1} reduced the mRNA abundance for this subunit by approximately 60% (Fig. 6) and downregulated K_{ir6.1} protein content (Fig. 6, insert).

**Requirement of the K_{ir6.1} Subunit in HUVEC Responses to SG-209 and CNP.** In subsequent experiments, we transfected HUVECs with either a control siRNA or a siRNA specific for K_{ir6.1} for 24 hours and determined their responses in Transwell migration and Matrigel assays. CNP (100 pM) alone produced 3-fold increases above basal in cell migration (Fig. 7A). These increases were markedly suppressed (83% reduction of CNP’s effect) by K_{ir6.1} siRNA, while the control siRNA had only a small, although significant, effect (Fig. 7A). Similarly, in the Matrigel assay, cord network formation induced by SG-209 (1 μM, 134% ± 3.5% increase above vehicle control) or by CNP (100 pM, 158% ± 2.4% increase above basal) was reduced to 18% ± 3.8% and 17.3% ± 4.2% above control, respectively, in cells transfected with the K_{ir6.1}-specific siRNA (Fig. 7B). This result points to a required role of K_{ir6.1} in the effects of both angiogenic molecules. Identical results were obtained when a second K_{ir6.1}siRNA was used (Supplemental Fig. 1).

**Dependence of the Effects of SG-209 on Erk1/2, p38, and Akt Kinases.** To characterize the downstream effectors of K_{ATP} activation by a direct activator in endothelial cells, we pretreated bEnd.3 cells with either the p38 inhibitor SB239063 (10 μM), the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway inhibitor U0126 (10 μM) or the protein kinase B (Akt)
inhibitor LY294002 (5 μM). Pretreatment of bEnd.3 cells with U0126, while not affecting basal responses, significantly reduced SG-209–induced motility and growth, bringing both responses down to basal levels (Fig. 8, A and B). Similarly, both the Akt (LY249002) and the p38 (SB239063) inhibitors abrogated the Transwell migratory responses of bEnd.3 to SG-209 without significantly affecting basal migration/motility (Fig. 8B). These results indicate that typical angiogenic responses to direct KATP activation depend on the function of Akt, p38, and Erk1/2 kinases.

Discussion

The identification of new basic molecular mechanisms in angiogenesis, especially if they are amenable to pharmacologic targeting, has important therapeutic implications in situations where its upregulation is beneficial, such as in alleviating the sequelae of ischemic heart disease and peripheral artery disease or when curbing ectopic or excessive angiogenic growth is desirable, as in solid tumor growth and angiogenesis, especially if they are amenable to pharmaco-

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Fig. 5. Cord-like network morphogenesis in vitro is affected by KATP modulation. The bEnd.3 cells (A) and HUVECs (B) were placed in 96-well plates in reduced-growth Matrigel and were treated with either SG-209 (1 μM) or CNP (100 pM) in the presence or absence of glibenclamide (Glib; 10 μM) or 5-HD (100 μM) for 8 hours. The cord-like network length was determined from microphotographs using the Scion image software. For each condition, n = 5. Inserts are representative photomicrographs of cells treated with vehicle, SG-209, or CNP (original magnification, 40×). *P < 0.05 versus vehicle; †P < 0.05 versus SG-209 or CNP alone, using Student’s t-test.

Our results show that direct or indirect opening of KATP–induced angiogenesis in vivo in the CAM model. In contrast, basal angiogenesis was reduced by all KATP inhibitors (Fig. 2). The lower potency of 5-HD may be attributable to either inadequate effects from mitochondrial-selective KATP inhibition alone, lower affinity in inhibiting mitoKATP, or suboptimal tissue and cell penetration of 5-HD. Our results do not allow us to weigh in favor of a particular possibility.

Vasodilatation, an action shared by SG-209 and CNP (Ishibashi et al., 1991; O’Rourke, 1996; Andrade et al., 2014), could indirectly promote angiogenesis. Our in vitro results, however, indicate that the CAM effects of both reagents can be attributed to direct endothelial cell–targeted activity. CAM responses to CNP were blocked by glibenclamide, revealing for the first time a critical involvement of KATP in CNP’s endothelial effects. These actions of CNP are likely mediated via activation of the NPR-C receptor (also referred as natriuretic peptide clearance receptor), whose signaling is required for CNP-dependent hyperpolarization, vasorelaxation, and endothelial growth (Villar et al., 2007; Kun et al., 2008; Khambata et al., 2011).

In bEnd.3 cells, proliferation and migration were promoted by direct KATP openers (Fig. 3). Nicorandil seemed more effective in

Fig. 6. Downregulation of the Kir6.1 subunit expression by siRNAs. HUVECs were transfected with small interfering (si) control RNA or siRNA specific for Kir6.1. After 24 hours, cell lysates were collected for protein and mRNA analysis. qRT-PCR analysis of Kir6.1 expression at the mRNA level, normalized for expression of the RPS18 ribosomal protein. Data represent n = 3 independent experiments. **P < 0.01 versus control siRNA, using Student’s t test. Insert is a representative photomicrograph of a Western blot probed with Kir6.1 and β-actin antibodies.

CNP is thought to exert an overall vascular protective role: it can fine-tune vascular cell growth (Khambata et al., 2011), establish tone and flow in resistance arteries (Villar et al., 2007; Lumsden et al., 2010), influence vessel wall remodeling (Itoh et al., 2004; Moyes et al., 2014), accelerate re-endothelialization (Ohno et al., 2002), or reduce inflammation (Itoh et al., 2004; Lumsden et al., 2010; Moyes et al., 2014). In contrast to CNP–triggered vasodilatation, directly linked to its stimulation of KATP activity (Burley et al., 2014), the mechanisms underlying CNP’s angiogenic effects are still poorly characterized.

The initial impetus for our work was provided by the observation that two angiogenic molecules, CNP (Yamahara et al., 2003) and H2S (Papapetropoulos et al., 2009), can elicit KATP activation via incompletely understood mechanisms. CNP is a member of the natriuretic peptide family (Margulies and Burnett, 2006; Lumsden et al., 2010; Baliga et al., 2012) and is widely expressed in various tissues, including the vasculature, especially by the endothelial cells (Baliga et al., 2012; Moyes et al., 2014). CNP is thought to exert an overall vascular protective role: it can fine-tune vascular cell growth (Khambata et al., 2011), establish tone and flow in resistance
promoting migration/motility than its derivative, SG-209 (Fig. 3B). This is unlikely due to the additional ability of nicorandil to donate NO, because K\textsubscript{ATP} inhibitors reduced equally the effects of both agents (Fig. 3B), but may be related to stability differences apparent in this assay. The reported growth-related effects of both CNP (Khambata et al., 2011) and of H\textsubscript{2}S (Papapetropoulos et al., 2009) on endothelial cells as well as our present results (Figs. 3 and 4) are indeed compatible with the reported ability of K\textsubscript{ATP} activators to induce proliferation in a wide variety of cell types, both normal (Fogal et al., 2010) and tumor (Ru et al., 2014). However, it should be noted that the activators' proliferative effect depends on the specific cell type under study (Zuo et al., 2011).

Endothelial proliferation and motility were comparably increased by CNP and VEGF (Fig. 4) and abrogated by K\textsubscript{ATP} inhibition (Fig. 4, B and C). These results establish CNP as the second angiogenic polypeptide, next to VEGF (Papapetropoulos et al., 2009), whose effects are regulated by K\textsubscript{ATP} function. Papapetropoulos et al. (2009) attributed part of VEGF’s dependence to synergy with endogenously produced H\textsubscript{2}S and the ensuing K\textsubscript{ATP} activation by H\textsubscript{2}S. It is unknown whether such a mechanism is used by CNP. The molecular pathway linking CNP-receptor stimulation to K\textsubscript{ATP} modulation is not entirely elucidated, but data in smooth muscle, cardiac, and endothelial cells support a role of the NPR-C-associated \( \beta \gamma \) subunits of the G\textsubscript{i} (Chauhan et al., 2003; Rose and Giles, 2008; Khambata et al., 2011), upregulation of cGMP levels, and implication of calcium-activated K\textsuperscript{+} channels (Simon et al., 2009). The involvement of cGMP (possibly via the NPR-B receptor), the dependence on NPR-C and the role of H\textsubscript{2}S in the angiogenic responses of CNP are the subject of ongoing investigations in our laboratory.

Our present observations establish unequivocally CNP as an angiogenic molecule, in agreement with preliminary evidence by Khambata et al. (2011) and Yamahara et al. (2003), but in contrast with the report by Del Ry et al. (2013) that showed inhibition of HUVEC responses on Matrigel by CNP. The discrepancy is probably explained by the high (10–1000 nM) CNP concentrations used by Del Ry et al., while those used by us and by Khambata et al. (picomolar range) are closer to circulating levels in human and mice (Kalra et al., 2003; Moyes et al., 2014) and arguably more relevant physiologically.

Taken together, our data support a bona fide regulatory role for K\textsubscript{ATP} in angiogenesis, which opens to investigation their molecular composition and their cellular localization. K\textsubscript{ATP} composition varies, based on the specific expression of the regulatory (sulfonylurea-binding; SUR) and the pore-forming (inwardly rectifier, Kir) subunits (Ashcroft, 1988; Flagg et al., 2010), resulting in different nucleotide sensitivities and pharmacologic sensitivity. Cardiomyocyte K\textsubscript{ATP} are composed of SUR2A and Kir6.2, while in smooth muscle cells they are formed by SUR2B and Kir6.1 (Seino and Miki, 2003). In the coronary and other endothelia, where ATP-sensitive potassium channels are known to be present (Janigro et al., 1993; Mederos y Schnitzler et al., 2000), SUR2B is combined with both Kir6.1 and Kir6.2 subunits (Yoshida et al., 2004). In agreement with Yoshida et al. (2004) we have been able to detect both Kir6.1 (this study) and Kir6.2 (B. Umaru, unpublished observations) subunits in HUVECs by qRT-PCR and Western blotting analysis. The cord-like network

![Fig. 7.](Image)
endothelial-protective effects of both H₂S (Suzuki et al., 2011) and CNP (Lumsden et al., 2010).

The proliferative effects of the direct K_{ATP} activator SG-209 were almost abolished by ERK1/2 pathway inhibition whereas the increase in motility was abrogated by inhibition of either ERK1/2, Akt, or p38 kinase (Fig. 8) at concentrations that block kinase phosphorylation/activation (B. Umaru, unpublished results; Papapetropoulos et al., 2009). These findings overall agree with reports (Papapetropoulos et al., 2009; Khambata et al., 2011) examining kinase-dependence of endothelial responses to H₂S or CNP. However, the inability of Akt inhibition to block H₂S-induced migration (Papapetropoulos et al., 2009) suggests an only partial overlap between H₂S and K_{ATP} mechanisms.

The present results also imply that the benefit of nicorandil’s use in the treatment of ischemic heart disease (Andreadou et al., 2008; Horinaka, 2011) and its effects in ischemic preconditioning in humans (Matsubara et al., 2000) may also be attributable, in addition to vasodilatation, to endothelial protective effects. Furthermore, our results present some intriguing implications regarding the use of K_{ATP} inhibitors (sulfonylureas) in diabetes. In diabetes, there is an elevated risk of arterial disease (including peripheral artery disease; Laakso and Kuusiisto, 2014), initiated by or coexisting with endothelial impairment. In peripheral artery disease, physiologic collateral angiogenesis is deficient, so its preservation is important therapeutically. Given that sulfonylureas inhibit endothelial cell survival and function (e.g., proliferation in response to angiogenic agents), it is not surprising that treatment with sulfonylureas is associated with worse outcomes of heart ischemic events in diabetic patients (Riddle, 2003) and results in loss of ischemic preconditioning during vascular surgery in patients with diabetes (Kottenberg et al., 2014). Furthermore, sulfonylureas have a varying profile regarding inhibition of endothelial-mediated ischemic preconditioning in humans (Okorie et al., 2011). This means that in choosing the sulfonylurea-type drug to treat diabetes one should consider its negative vascular (endothelial) effects. New compounds that inhibit even more selectively the pancreatic but spare the endothelial K_{ATP} would logically present a better vascular protective profile in patients with diabetes.

In conclusion, our work has shown that pharmacologic manipulation of K_{ATP} in vitro and in vivo, either directly by K_{ATP} openers or inhibitors, or indirectly via CNP-cognate receptor interaction, results in modulation of angiogenic responses. It therefore appears that K_{ATP} are a novel common mechanism underpinning angiogenesis to various physiologic stimuli including VEGF, H₂S, and CNP. K_{ATP} should therefore be considered as a valid therapeutic target in angiogenesis, and molecules that modulate their activity, especially if already in clinical use, should be re-examined under this new light.

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Authorship Contributions

Participated in research design: Papapetropoulos, Topouzis.
Conducted experiments: Umaru, Pyriochou, Kotsikoris.
Performed data analysis: Umaru, Kotsikoris, Topouzis.
Wrote or contributed to the writing of the manuscript: Umuru, Pyriochou, Kotsikoris, Papapetropoulos, Topouzis.

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Address correspondence to: Dr. Stavros Topouzis, Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, 26504 Rio-Patras, Greece. E-mail: stto@upatras.gr

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