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

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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 generically therapeutic importance [molecular targets (Coultas 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 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.

ATH-sensitives 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 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.
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 (Kir) channels (Flagg et al., 2010). The two subtypes found in $K_{ATP}$, $K_{b}6.1$ and $K_{b}6.2$, are encoded respectively by the $KCNJ8$ 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 $\beta$-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., 2002), and the pituitary (Wu and Chang, 2006). Their regulatory role in insulin secretion and in heart and vascular homeostasis has been extensively studied (Lawson, 2000; Flagg et al., 2010; Kohler and Ruth, 2010; Burley et al., 2014).

The indirect relationship previously put forth between the ability of two vasoactive agents (H$2$S, CNP) to signal through KATP and their proangiogenic effects prompted us to ask whether direct stimulation of KATP 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 $K_{b}6.1$ 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), using two different populations of endothelial cells: mouse bEnd.3 (Suzuki et al., 2011) and human umbilical vein endothelial cells (HUVEC). Collectively, our data suggest that $K_{ATP}$ stimulation, either directly via $K_{ATP}$ openers or indirectly via vascular endothelial growth factor (VEGF)– or CNP-originated signaling, can induce endothelial angiogenic responses that depend on the presence of the $K_{b}6.1$ $K_{ATP}$ subunit. $K_{ATP}$ modulation thus offers a new means to pharmacologically interfere with angiogenesis and constitutes a novel therapeutic target in angiogenesis.

**Materials and Methods**

2-Nicotinamidethyl acetate (SG-209) and glibenclamide were purchased from Tocris (Minneapolis, MN). Cell culture media and sera were obtained from GIBCO-BRL (Paisley, UK). Leghorn fertilized eggs were obtained from Pindos (Epirus, Greece). Super Signal West Pico chemiluminescent substrate was purchased from Thermo Fisher Scientific (Waltham, MA). The DC Protein kit assay, Tween-20, and other immunoblotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA). Penicillin and streptomycin were purchased from Applichem (Darmstadt, Germany). Amphotericin, gentamycin, and heparin were purchased from Biochorm AG (Berlin, Germany). LY-294002 (2-[4-(morpholino)ethyl]-8-phenyl-4H-1-benzopyran-4-one hydrochloride) was purchased from Cell Signaling Technologies (Beverly, MA). The $K_{b}6.1$ antibody (R-14, used at 1:400 dilution) and the β-actin antibody (H-12, used at 1:1000) were both purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Two small-interfering RNA (siRNA) pairs (control siRNA and $K_{b}6.1$-specific siRNA) were purchased from Santa Cruz Biotechnology and Ambion (Carlsbad, CA). All other reagents including bovine serum albumin, EDTA, gelatin, nicoerandil, 5-hydroxydecanoate (5-HD), tobutamide, SB239063 (4-(4-(4-fluorophenyl)-5-(2-methoxyphosphoryl)-4-yl)imidazol-1-yl)cyclohexan-1-ol), U0126 (22Z32, 2-bis-amino(2-amino phenyl)sulfonylmethylidene)butanedinitrile), protease inhibitors, and endothelial cell growth supplement were purchased from Sigma-Aldrich (St. Louis, MO).

**Endothelial Cell Culture**. The bEnd.3 immortalized mouse brain microvascular endothelial cell line was a gift from Dr. Zongmin Zhou (Thorax Foundation Laboratory, Athens, Greece). The bEnd.3 cells were cultured in a humidified chamber at 37°C, 5% (v/v) CO$_2$, in Dulbecco’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 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin, 2.5 μg/ml amphotericin B, 5 μM endothelial cell growth supplement, 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$^3$) 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 $K_{b}6.1$ $K_{ATP}$ channel subunit from both Santa Cruz Biotechnology and Ambion, referred to as $K_{b}6.1$si#1 and $K_{b}6.1$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 SB239063, 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 0.2%
bovine serum albumin in the presence or absence of CNP (100 μM), SG-209 (1 μM), or nicorandil (10 μM). Cells (HUVECs or bEnd.3) were allowed to migrate for 4 hours, after which 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 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>6.6.1</sub> siRNA-transfected endothelial cells (15,000 cells/well) were plated in 96-well plates precoated with 45 μl of Matrigel per well in the presence or absence of CNP (100 μM), SG-209 (1 μM), 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>ATP</sub> or mitogen-activated protein kinase kinase inhibitor (U0126, 10 μM). 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 using the Rotor Gene 6000 (Corbett Life Science, Concord, NSW, Australia). Each of the PCR amplification reactions was set up in triplicate. Forward and reverse primers were as follows: KCNJ8 gene (F: 5’-CTGGGGCCTTCTGCTATC-3’ and REV 5’-AGAATTCAAAACGTGATGCC-3’), and RPS18: FW 5’-TCGAACTGGGCATGCA-3’ and REV 5’-GAACCTCGACTTTGTC-3’ (used as the endogenous control for expression normalization). The cycle threshold (Ct) values were automatically calculated by the machine’s software and normalized to the endogenous control gene by using the 2<sup>−ΔΔCt</sup> method (Livak and Schmittgen, 2001).

Western Blot Analysis. Protein extracts from HUVECs were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane as previously described elsewhere (Papapetropoulos et al., 2009) and then incubated with K<sub>6.6.1</sub> or β-actin–specific antibodies (both from Santa Cruz Biotechnology) overnight at 4°C. 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>ATP</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>ATP</sub>-opening (Ishibashi et al., 1991). We also included a receptor-mediated K<sub>ATP</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).

In contrast, both parameters (vessel length and branching point number) of basal CAM angiogenesis were reduced in the presence of sulfonylurea-type molecules glibenclamide (1–100 nmol/cm<sup>2</sup>) and tolbutamide (1–100 nmol/cm<sup>2</sup>), which inhibit both membrane and mitochondrial K<sub>ATP</sub> or by the mitochondrial K<sub>ATP</sub>-selective molecule 5-HD (10–1000 nmol/cm<sup>2</sup>) (Fig. 2, A–C).

To test whether the CAM effects of CNP depend on its reported K<sub>ATP</sub> activation, we treated CAMs with glibenclamide before the application of CNP. In a subsequent series of CAM experiments, at the lowest concentration used (1 nmol/cm<sup>2</sup>; Fig. 2D), glibenclamide did not significantly affect basal angiogenesis but was able to suppress the angiogenic effects of

**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>ATP</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.
CNP, indicating that the effects of CNP are sensitive to K<sub>ATP</sub> blockade.

**K<sub>ATP</sub> Activation Is Important for bEnd.3 Cell Proliferation, Migration, and Cord-Like Network Formation.** To further test the link between K<sub>ATP</sub> 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), K<sub>ATP</sub> activation by both SG-209 (1 μM) and nicorandil (10 μM) resulted in elevated cell proliferation (increases of 88.9% ± 11.9% and

![Graph A](image1.png)

**Fig. 3.** Effect of K<sub>ATP</sub> 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 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 reseeded 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 = 30–35 per point. *P < 0.05 versus vehicle; **P < 0.01 versus vehicle; †P < 0.05 versus vehicle control; ‡P < 0.05 versus CNP 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 KATP activation by CNP participates in similar in vitro responses. CNP (1–1000 pM) dose dependently increased cell proliferation (maximal effect: 38.2% ± 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 KATP 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 KATP 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 KATP 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 KATP blockers (Fig. 5B). No significant effect of glibenclamide or 5-HD on basal network morphogenesis was seen (Fig. 5B).

**Knockdown of the Kir6.1 KATP Subunit Expression Using Specific siRNAs.** To further probe the contribution of the pore-forming KATP Kir6.1 subunit, we introduced by transfection either of two different control siRNAs or of two different siRNAs specific for the human sequence of Kir6.1 in HUVECs. Analysis 24 hours later of cell lysates by Western blotting or by qRT-PCR indicated that both siRNAs targeting Kir6.1 reduced the mRNA abundance for this subunit by approximately 60% (Fig. 6) and downregulated Kir6.1 protein content (Fig. 6, insert).

**Requirement of the Kir6.1 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 Kir6.1 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 Kir6.1 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 Kir6.1-specific siRNA (Fig. 7B). This result points to a required role of Kir6.1 in the effects of both angiogenic molecules. Identical results were obtained when a second Kir6.1 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 KATP 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) inhibitors. (A) bEnd.3 cells in 2.5% fetal bovine serum were treated with the indicated concentrations of CNP (100 pM) or VEGF (500 pM) and allowed to proliferate for 48 hours. Cell proliferation was determined by cell counting with a hemocytometer (n = 3). (B) bEnd.3 cells were pretreated with glibenclamide (Glib; 10 μM) or 5-HD (100 μM) before being exposed to VEGF (500 pM). At 48 hours later, cells were trypsinized and counted using a hemocytometer (n = 3). (C) bEnd.3 cells in serum-free medium were pretreated with glibenclamide (10 μM) or 5-HD (100 μM) for 20 minutes, placed in well inserts, and then exposed to either CNP (100 pM) or VEGF (500 pM). Migrated cells were stained and counted 4 hours later. For each condition, n = 5. *P < 0.05 versus vehicle, †P < 0.05 versus VEGF or CNP alone, using Student’s t test.

Fig. 4. Modulation of endothelial cell responses to CNP and VEGF by KATP inhibitors. (A) bEnd.3 cells in 2.5% fetal bovine serum were treated with the indicated concentrations of CNP (100 pM) or VEGF (500 pM) and allowed to proliferate for 48 hours. Cell proliferation was determined by cell counting with a hemocytometer (n = 3). (B) bEnd.3 cells were pretreated with glibenclamide (Glib; 10 μM) or 5-HD (100 μM) before being exposed to VEGF (500 pM). At 48 hours later, cells were trypsinized and counted using a hemocytometer (n = 3). (C) bEnd.3 cells in serum-free medium were pretreated with glibenclamide (10 μM) or 5-HD (100 μM) for 20 minutes, placed in well inserts, and then exposed to either CNP (100 pM) or VEGF (500 pM). Migrated cells were stained and counted 4 hours later. For each condition, n = 5. *P < 0.05 versus vehicle, †P < 0.05 versus VEGF or CNP alone, using Student’s t test.
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. 8A 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 K<sub>ATP</sub> 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 peripheral artery disease or when curbing ectopic or excessive angiogenic growth is desirable, as in solid tumor growth and diabetic retinopathy (Coultas et al., 2005; Ferrara and Kerbel, 2005; Carmeliet and Jain, 2011).

The initial impetus for our work was provided by the observation that two angiogenic molecules, CNP (Yamahara et al., 2003) and H<sub>2</sub>S (Papapetropoulos et al., 2009), can elicit K<sub>ATP</sub> 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 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 K<sub>ATP</sub> activity (Burley et al., 2014), the mechanisms underlying CNP’s angiogenic effects are still poorly characterized.

Our results show that direct or indirect opening of K<sub>ATP</sub>–induced angiogenesis in vivo in the CAM model. In contrast, basal angiogenesis was reduced by all K<sub>ATP</sub> inhibitors (Fig. 2). The lower potency of 5-HD may be attributable to either inadequate effects from mitochondrial-selective K<sub>ATP</sub> inhibition alone, lower affinity in inhibiting mitoK<sub>ATP</sub>, 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 K<sub>ATP</sub> 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 K<sub>ATP</sub> openers (Fig. 3). Nicorandil seemed more effective in...
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 βγ 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 endothelium, 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 of angiogenic sprouts and cords was more abundant after treatment with the K\textsubscript{ATP} activator SG-209 (Fig. 5A) than with the inactive control compound, nicorandil (Fig. 5B), and clearly demonstrated by immunostaining for α-SMA or smooth muscle α-actin (Fig. 5C). These results support the notion that K\textsubscript{ATP} inhibition decreases sprouting and promotes cord formation (Chang et al., 1998; Rubart et al., 2009; Yamaguchi et al., 2010), thereby reducing the average network length and number of the sprouts (Fig. 5D).

Fig. 7. HUVEC responses to SG-209 and to CNP depend on the expression of the K\textsubscript{ATP} subunit Kir6.1. (A) HUVECs were transfected either with a control siRNA or with a siRNA specific for Kir6.1 (both from Santa Cruz Biotechnology). At 24 hours later, the cells were trypsinized, placed in Transwells, and allowed to migrate in the presence or absence of CNP (100 pM) for 4 hours, after which the migrated cells were stained and counted. (B) HUVECs were transfected with a control siRNA or a siRNA specific for Kir6.1 (both from Santa Cruz Biotechnology). At 24 hours later, they were placed in 96-well plates on reduced-growth Matrigel and treated with either SG-209 (1 μM) or CNP (100 pM) for 8 hours. Cord-like network formation was determined and quantified from microphotographs using the Scion image software. For all groups, n = 5. (Similar results were obtained with a second Kir6.1 siRNA; Supplemental Fig. 1.) (C) Representative photomicrographs of cells treated as in (B) (original magnification, 40×). **P < 0.01 versus vehicle control; †P < 0.05 versus CNP or SG-209 alone, using Student’s t test.
endothelial-protective effects of both H2S (Suzuki et al., 2011) and CNP (Lumsden et al., 2010).

The proliferative effects of the direct KATP 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 KATP 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 KATP inhibitors (sulfonylureas) in diabetes. In diabetes, there is an elevated risk of arterial disease (including peripheral artery disease; Laakso and Kuusisto, 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 KATP would logically present a better vascular protective profile in patients with diabetes.

In conclusion, our work has shown that pharmacologic manipulation of KATP in vitro and in vivo, either directly by KATP openers or inhibitors, or indirectly via CNP-cognate receptor interaction, results in modulation of angiogenic responses. It therefore appears that KATP are a novel common mechanism underpinning angiogenesis to various physiologic stimuli including VEGF, H2S, and CNP. KATP 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

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