ATP-Sensitive Potassium Channel Activation Induces Angiogenesis

in vitro and in vivo

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\textit{Nonstandard abbreviations}: \( \text{K}_{\text{ATP}} \): ATP-sensitive potassium channel; CAM: chick chorioallantoic membrane; VEGF: Vascular Endothelial Growth Factor; CNP: C-type Natriuretic Peptide; HUVEC: Human Umbilical Vein Endothelial cells; bEnd.3: mouse brain transformed endothelial cell line; qRT-PCR, quantitative reverse transcription – polymerase chain reaction; siRNA, small interfering RNA
ABSTRACT

Intense research is conducted to identify new molecular mechanisms of angiogenesis. Previous studies have shown that the angiogenic effects of Hydrogen Sulfide (H$_2$S) depend on the activation of ATP-sensitive potassium channels (K$_{ATP}$) and that C-type Natriuretic Peptide (CNP), which can act through K$_{ATP}$, promotes endothelial cell growth. We therefore investigated whether direct K$_{ATP}$ 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 K$_{ATP}$ channel activator SG-209 and by CNP or VEGF. The K$_{ATP}$ inhibitors Glibenclamide and 5-Hydroxydecanoate (5-HD) reduced basal and abolished CNP-induced CAM angiogenesis. In vitro, the direct K$_{ATP}$ 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, siRNA-mediated decrease in the expression of the inwardly rectifying potassium channel (K$_{ir}$) 6.1 subunit impaired cell migration and network morphogenesis in response to either SG-209 or CNP. We conclude that: a) direct pharmacological activation of K$_{ATP}$ induces angiogenic effects in vitro and in vivo, b) angiogenic responses to CNP and VEGF depend on K$_{ATP}$ activation and require the expression of the K$_{ir}$6.1 K$_{ATP}$ subunit, and c) K$_{ATP}$ activation may underpin angiogenesis to a variety of vasoactive stimuli, including H$_2$S, VEGF and CNP.
INTRODUCTION

The mechanisms underlying angiogenesis have been extensively studied for the past forty years, resulting in an enhanced understanding of the complex cellular processes that together initiate and sustain angiogenesis and generating therapeutically important molecular targets (Carmeliet and Jain, 2011; Coultas et al, 2005; Ferrara and Kerbel, 2005). However, the contribution of additional, as yet unknown, participating mechanisms is the subject of intense ongoing research. Recently, it was reported that the endogenous gasotransmitter, H$_2$S, which signals in part through ATP-sensitive potassium channels (K$_{ATP}$), can induce angiogenic responses which are abrogated by K$_{ATP}$ inhibition (Papapetropoulos et al., 2009). In addition, the polypeptide CNP, which also mediates some of its effects via K$_{ATP}$ activation, has been shown to elicit endothelial cell growth responses in vitro and collateral angiogenesis in vivo (Khambata et al., 2011; Yamahara et al., 2003). Combined, these findings raise the possibility that K$_{ATP}$ activation is a relatively little-studied common mechanism triggering angiogenesis in response to various physiological stimuli.

ATP-sensitive potassium channels were originally discovered in the heart (Noma A, 1983) and are regulated by a variety of physiological 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. K$_{ATP}$ are membrane-spanning hetero-octameric proteins that 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 Kᵦᵢᵣ, Kᵢᵣ6.1 and Kᵢᵣ6.2, are encoded respectively by the KCNJ8 and KCNJ11 genes and are thought to be inhibited by rises in cytosolic ATP (Flagg et al., 2010). Kᵦᵢᵣ 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., 2007) and the pituitary (Wu and Chang, 2006). Their regulatory role in insulin secretion and in heart and vascular homeostasis has been extensively studied (Burley et al., 2014; Flagg et al., 2010; Kohler and Ruth, 2010; Lawson, 2000). Kᵦᵢᵣ-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 II diabetes (Lawson, 2000; Wallia and Molitch, 2014). However, the involvement of Kᵦᵢᵣ in angiogenesis remains largely unknown.

The indirect relationship previously put forth between the ability of two vasoactive agents (H₂S, CNP) to signal through Kᵦᵢᵣ and their pro-angiogenic effects, prompted us to ask whether, indeed, direct stimulation of Kᵦᵢᵣ can 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 Kᵦᵢᵣ subunit Kᵢᵣ6.1 is present in endothelial cells and whether its expression is required in their responses to CNP.
To evaluate the angiogenic effects of K\textsubscript{ATP} modulation and assess the endothelial effects of CNP, we used both a classic \textit{in vivo} angiogenesis model (chick embryo chorioallantoic membrane, CAM), together with \textit{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 HUVECs. Collectively, our data suggest that K\textsubscript{ATP} stimulation, either directly, using K\textsubscript{ATP} openers, or indirectly, via VEGF- or CNP-originated signaling, can induce endothelial angiogenic responses which depend on the presence of the K\textsubscript{ir}6.1 K\textsubscript{ATP} subunit. K\textsubscript{ATP} modulation, therefore, offers a new means to pharmacologically interfere with angiogenesis and constitutes a novel therapeutic target in angiogenesis.
METHODS

Materials

2-Nicotinamidoethyl acetate (SG-209) and Glibenclamide were purchased from Tocris (Minneapolis, MN, USA). 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 Scientific (USA); 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 Biochrom AG (Berlin, Germany). LY242009 was purchased from Cell Signalling. The Kᵢ₆.1 antibody (R-14, used at 1:400 dilution) and the β-actin antibody (H-12, used at 1:1,000) were both purchased from Santa Cruz Biotechnology, Heidelberg, Germany. Two siRNA pairs (Control siRNA and Kᵢ₆.1-specific siRNA) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and Ambion (Carsbad, USA). All other reagents including bovine serum albumin, EDTA, Gelatin, Nicorandil, 5-hydroxydecanoate (5-HD), Tolbutamide, SB239063, U0126, protease inhibitors and endothelial cell growth supplement (ECGS) 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). bEnd.3 were cultured in a humidified chamber at 37°C, 5 % (v/v) CO₂, in DMEM containing 10% (v/v) FBS, 2 mM glutamine, 1 g/l glucose, 100 IU/mL penicillin and 100 μg/mL streptomycin.
Human umbilical endothelial cells (HUVEC) 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 gentamycin, 2.5 μg/ml amphotericin B, 5 U/ml heparin and 150-200 μg/ml endothelial cell growth supplement. Cells were used between passages 1 and 3. Each experiment shown derives from 3 independent repeats, each time using different pools (isolates) and/or passages of cells.

**In vivo chick chorioallantoic membrane assay (CAM)**

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 cm²) was placed on the surface of the CAM and the various treatments were added inside this restricted area as previously described (Papapetropoulos et al., 2009). After 48 h, CAMs were fixed in Carson’s solution (saline-buffered formalin) and angiogenesis was evaluated using image analysis software. For the CAM experiments, 30-35 eggs were used per group distributed in 3 independent experiments. Handling and use of chick embryos was performed according to University of Patras institutional animal welfare protocols.

**Cell transfection with small interfering RNAs (siRNAs)**

HUVECs were treated either with Vehicle (Vehicle CTL) or were transfected with siRNAs. The siRNA final concentrations used were 14nM for the Ambion siRNAs and 40nM for the Santa Cruz siRNAs. We used two different Control siRNAs: Cat#sc-37007 from Santa Cruz (Heidelberg, Germany), referred to as CTLsi#1 and Cat#4390843 from
Ambion (Carlsbad, USA), referred to as CTLsi#2 and two different siRNAs targeting specifically the Kir6.1 KATP channel subunit: Cat#sc-35752 from Santa Cruz, referred to as Kir6.1si#1 and Cat#4392420 from Ambion, referred to as Kir6.1si#2. 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, cells were washed twice with PBS, trypsinized and used in migration and Matrigel assays. RNA was also collected for 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 pre-treated with 5-HD (100 µM), Glibenclamide (10 µM) or kinase inhibitors (U0126, 10 µM, SB230963 10 µM, and LY-242009 5 µM) for 20 minutes before the end of the starvation time. Subsequently, cells were trypsinized and 1×10⁵ cells were added to each Transwell in 100 µL of serum-free medium containing 0.2 % BSA in the presence or absence of CNP (100 pM), SG-209 (1 µM) or Nicorandil (10 µM). Cells (HUVECs or bEnd.3) were allowed to migrate for 4 h after which the non-migrated 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 min at room temperature and then stained with toluidine blue. Migrated cells were scored and averaged from eight random fields per Transwell as previously described (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 si-transfected or Kir6.1 siRNA-transfected endothelial cells (15,000 cells/well) were plated in 96-well plates pre-coated with 45µL of Matrigel / well in the presence or absence of CNP (100 pM), 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**

bEnd.3 cells were seeded in 24-well plates at a density of 6,000 cells/cm² and incubated overnight in DMEM medium. Cells were then pre-treated for 20 minutes with vehicle or with K<sub>ATP</sub> or MEK kinase inhibitor (U0126, 10 µM). Cells were then exposed to different concentrations of CNP, SG-209 or vehicle and allowed to proliferate for 48 h. At the end of this incubation time, cells were trypsinized and their number was determined using a Neubauer hemocytometer.

**Quantitative reverse-transcription – polymerase chain reaction (qRT-PCR)**

mRNA expression was evaluated using Real-Time Quantitative RT-PCR (qRT-PCR). Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) and quantified by a NanoDrop 2000 (Thermo Scientific, USA). 250 ng of total RNA were subjected to reverse transcription and real time PCR amplification using the KAPA SYBR Fast One-step qRT-PCR protocol (KapaBiosystems). Amplification and Real-Time fluorescence detection was performed using the Rotor Gene 6000 (Corbett, USA). Each of the PCR amplification reactions was set up in triplicate. Forward and reverse
primers were as follows: KCNJ8 gene (Kir6.1): FW 5’- CTGGCTGCTCTTCGCTATC-3’ and REV 5’- AGAATCAAAACCGTGATGGC-3’, and RPS18: FW 5’- TCGGAACTGAGGCCATGA-3’ and REV 5’-GAACCTCCGACTTTTCGTTT-3’, (used as the endogenous Control for expression normalization). Ct values were automatically calculated by the machine’s software and normalized to the endogenous control gene by using the 2- ΔΔCT method (Livak and Schmittgen, 2001).

Western blotting

Protein extracts from HUVECs were separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane as previously described (Papapetropoulos et al., 2009) and then incubated with Kir6.1- or β-actin-specific antibodies (both from SantaCruz, Heidelberg, Germany) overnight at 4°C. Following incubation with the appropriate secondary antibodies, the immunoreactive proteins were detected using a chemiluminescent substrate according to the manufacturer’s instructions (Pierce Chemiluminescent HRP substrate kit, Thermo Scientific).

Statistical Analysis

Data are expressed as the mean ± SEM 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. A p value of <0.05 was considered to be significant.
RESULTS

SG-209 and CNP promote *in vivo* chick CAM angiogenesis

To determine whether K$_{ATP}$ 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 NO-donor properties, since Nicorandil’s nitrate moiety has been replaced with acetate in SG-209. Thus, SG-209 is thought to act only through K$_{ATP}$-opening (Ishibashi et al., 1991). We also included a receptor-mediated K$_{ATP}$ activator, CNP. We compared their maximal responses to those of a well-accepted angiogenic factor, VEGF, used at a maximal (for this assay) concentration of 500pM. Treatment of CAMs with SG-209 (0.1-10 nmole/cm$^2$) or with CNP (300 and 3000 nmole/cm$^2$) promoted angiogenesis, measured as total vascular length and number of branching points (Fig. 1A and 1B). The maximal effects of SG-209 and CNP were comparable with those of 500 nmol/cm$^2$ 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 nmole/cm$^2$) and Tolbutamide (1-100nmole/cm$^2$), which inhibit both membrane and mitochondrial K$_{ATP}$ or by the mitochondrial K$_{ATP}$-selective molecule, HD-5 (10-1000 nmol/cm$^2$) (Fig. 2A-C).

To test whether the CAM effects of CNP depend on its reported K$_{ATP}$ activation, we treated CAMs with Glibenclamide prior to the application of CNP. In a subsequent series of CAM experiments, at the lowest concentration used (1 nmole/cm$^2$, Fig 2D), Glibenclamide did not significantly affect basal angiogenesis, but was able to suppress the angiogenic effects of CNP, indicating that the effects of CNP are sensitive to K$_{ATP}$ blockade.
K$_{\text{ATP}}$ activation is important for bEnd.3 cell proliferation, migration and cord-like network formation

To further test the link between K$_{\text{ATP}}$ activation and triggering of angiogenic responses at the cellular level, we moved to endothelial cell-based in vitro assays, since endothelial “activation” or “mobilization” is paramount in bona fide angiogenic responses. In bEnd.3 mouse brain endothelial cells (Papapetropoulos et al., 2009) in vitro, K$_{\text{ATP}}$ activation by both SG-209 (1µM) and Nicorandil (10µM) resulted in elevated cell proliferation (increases of 88.9±11.9% and 105.0±9.33% respectively, Figure 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 (10µM) or 5-HD (100µM), while not significantly affecting basal responses (Fig. 3A and 3B), abolished the growth and motility effects of both SG-209 and of Nicorandil (Fig. 3A and 3B). The increase in bEnd.3 motility was more robust in cells treated with Nicorandil than with SG-209, reflecting either possible use of a sub-maximal 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$_{\text{ATP}}$ activation by CNP participates in similar in vitro responses. CNP (1-1000pM) dose-dependently increased cell proliferation (maximal effect: 38.2±5.9% increase at 1000pM, Fig 4A), an effect comparable to that elicited in parallel by 500pM VEGF (increase of 49.9±7.5%, Fig. 4A). The proliferative effects of VEGF were almost entirely suppressed by the K$_{\text{ATP}}$ blockers Glibenclamide and 5-HD (Fig 4B). CNP (100pM) and VEGF (500pM) 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, in order 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$\mu$M) 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 (100pM, 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).

Knock-down of the $K_{ir}6.1$ $K_{ATP}$ subunit expression using specific siRNAs

To further probe the contribution of the pore-forming $K_{ATP}$ $K_{ir}6.1$ subunit, we introduced by transfection either of 2 different Control siRNAs or of 2 different siRNAs specific for the human sequence of $K_{ir}6.1$ in HUVECs. 24 hours later, analysis of cell lysates by western blotting or by qRT-PCR indicated that both siRNAs targeting $K_{ir}6.1$ reduced the mRNA abundance for this subunit by approximately 60% (Fig. 6) and down-regulated $K_{ir}6.1$ protein content (Fig. 6, insert).

Requirement of the $K_{ir}6.1$ 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 24h and determined their responses in Transwell migration and Matrigel assays. CNP (100pM) alone produced 3-fold increases above basal in cell migration (Fig. 7A). These increases were markedly suppressed (83% reduction of CNP’s effect) by Kir6.1siRNA, 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 (1µM, 134±3.5% increase above Vehicle Control) or by CNP (100pM, 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 Kir6.1siRNA was used (Supplementary Figure 1).

Dependence of the effects of SG-209 on Erk1/2, p38 and Akt kinases

To characterize the downstream effectors of K\textsubscript{ATP} activation by a direct activator in endothelial cells, we pretreated bEnd.3 cells with either the p38 inhibitor SB239063 (10µM), the Erk1/2 pathway inhibitor U0126 (10µM) or the Akt inhibitor LY249002 (5µM). 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 8B). Similarly, both the Akt (LY249002) and the p38 (SB 239063) 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\textsubscript{ATP} 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 pharmacological targeting, has important therapeutic implications in situations where its up-regulation is beneficial, e.g. in alleviating the sequellae of ischemic heart disease and peripheral artery disease, or when curbing ectopic or excessive angiogenic growth is desirable, e.g. in solid tumor growth and in diabetic retinopathy (Carmeliet and Jain, 2011; Coultas et al., 2005; Ferrara and Kerbel, 2005).

The initial impetus for our work was provided by the observation that two angiogenic molecules, CNP (Yamahara et al., 2003) and H$_2$S (Papapetropoulos et al., 2009) can elicit K$_{ATP}$ activation, via incompletely-understood mechanisms. CNP is a member of the natriuretic peptide family (Baliga et al., 2012; Margulies and Burnett, 2006; Lumsden et al., 2010) 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 (Lumsden et al., 2010; Villar et al., 2007), influence vessel wall remodeling (Itoh et al., 2004; Moyes et al., 2014), accelerate re-endothelialization (Komeda and Nakao, 2002; Ohno et al., 2002) or reduce inflammation (Lumsden et al., 2010; Itoh et al., 2004; Moyes et al., 2014). In contrast to CNP-triggered vasodilatation, directly linked to its stimulation of K$_{ATP}$ 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$_{ATP}$ induced angiogenesis in vivo in the CAM model. In contrast, basal angiogenesis was reduced by all K$_{ATP}$ inhibitors (Fig.
2). The lower potency of 5-HD may be attributable to either inadequate effects from mitochondrial-selective $K_{ATP}$ inhibition alone, lower affinity in inhibiting mito$K_{ATP}$ or suboptimal tissue and cell penetration of HD-5. Our results do not allow us to weigh in favor of a particular possibility.

Vasodilatation, an action shared by SG-209 and CNP (Andrade et al., 2014; O’Rourke, 1996; Ishibashi et al., 1991), 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 $K_{ATP}$ 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 (Khambata et al., 2011; Kun et al., 2008; Villar et al., 2007).

In bEnd.3 cells, proliferation and migration were promoted by direct $K_{ATP}$ 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, since $K_{ATP}$ 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., 2010) and of $H_2S$ (Papapetropoulos et al., 2009) on endothelial cells, as well as our present results (Fig. 3 and 4) are indeed compatible with the reported ability of $K_{ATP}$ 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 $K_{\text{ATP}}$ inhibition (Fig. 4B and C). These results establish CNP as the second angiogenic polypeptide, next to VEGF (Papapetropoulos et al., 2009), whose effects are regulated by $K_{\text{ATP}}$ function. Papapetropoulos et al. (2009) attributed part of VEGF’s dependence to synergy with endogenously-produced H$_2$S and the ensuing $K_{\text{ATP}}$ activation by H$_2$S. It is unknown if such a mechanism is used by CNP. The molecular pathway linking CNP-receptor stimulation to $K_{\text{ATP}}$ 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 Gi (Chauhan et al., 2003, Rose and Giles, 2008; Khambata et al., 2011), upregulation of cGMP levels and implication of calcium-activated K$^+$ channels (Simon et al., 2009). The involvement of cGMP (possibly via the NRP-B receptor), the dependence on NPR-C and the role of H$_2$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 contrast with the report by Del Ry et al., (2013), which showed inhibition of HUVEC responses on Matrigel by CNP. The discrepancy is probably explained by the high (10-1000nM) CNP concentrations used by Del Ry et al., while those used by us and by Khambata et al. (pM range) are closer to circulating levels in human and mice (Karla 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, begging the question of 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, K\textsubscript{ir}) subunits (Aschcroft, 1988; Flagg et al., 2010), resulting in different nucleotide sensitivities and pharmacological sensitivity. Cardiomyocyte K\textsubscript{ATP} are composed of SUR2A and K\textsubscript{ir}6.2, while in smooth muscle cells they are formed by SUR2B and K\textsubscript{ir}6.1 (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 K\textsubscript{ir}6.1 and K\textsubscript{ir}6.2 subunits (Yoshida et al., 2004). In agreement with Yoshida et al., we have been able to detect both K\textsubscript{ir}6.1 (this study) and K\textsubscript{ir}6.2 (B. Umaru, unpublished observations) subunits in HUVECs by qPCR and western blotting analysis.

The cord-like network formation elicited by SG-209 or CNP (Fig. 7) in HUVECs required unperturbed expression of the K\textsubscript{ir}6.1 subunit (Fig. 6, Fig. 7), suggesting a) participation of K\textsubscript{ir}6.1 in these responses and b) existence of a K\textsubscript{ir}6.1 "expression-dosage"-sensitive effect, not compensated by K\textsubscript{ir}6.2. As a consequence, molecules modulating the activity of K\textsubscript{ir}6.1-containing K\textsubscript{ATP} are expected to impact endothelial function quite effectively.

The function and pathophysiology of mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) has been extensively characterized, especially in the heart (O’Rourke et al., 2004). However, the presence of K\textsubscript{ir}6.1 in mitoK\textsubscript{ATP} is unclear. While K\textsubscript{ir}6.1 has been detected in mitochondrial membranes by antibodies and its inclusion is supported by pharmacological approaches (O’Rourke, 2004; Suzuki et al., 1997), genetic deletion of K\textsubscript{ir}6.1 in mice fails to disrupt...
mitoK\textsubscript{ATP} opening (Miki et al., 2002; Flagg et al., 2010). The ability of 5-HD, a mitoK\textsubscript{ATP} inhibitor (O’Rourke et al., 2004; Sato et al., 1998), to antagonize the functional responses of direct or indirect K\textsubscript{ATP} activation as effectively as the non-selective inhibitor Glibenclamide (Fig. 3-6), combined with the dependence of these responses on the expression of Kir6.1 (Fig. 7), suggest that mitoK\textsubscript{ATP}, comprising Kir6.1 subunits, are key mediators of angiogenesis and may underpin the endothelial-protective effects of both H\textsubscript{2}S (Suzuki et al., 2011) and of CNP (Lumsden et al., 2010).

The proliferative effects of the direct K\textsubscript{ATP} activator, SG-209, were almost abolished by Erk1/2 pathway inhibition, while the increase in motility was abrogated by inhibition of either the Erk1/2, the Akt or the 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 (Khambata et al., 2011; Papapetropoulos et al., 2009) examining kinase-dependence of endothelial responses to H\textsubscript{2}S or CNP. However, the inability of Akt inhibition to block H\textsubscript{2}S-induced migration (Papapetropoulos et al., 2009) suggests an only partial overlap between H\textsubscript{2}S and K\textsubscript{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\textsubscript{ATP} inhibitors (sulfonylureas) in diabetes. In diabetes, there is elevated risk of arterial disease (including peripheral artery disease, PAD, Laakso and Kuusisto, 2014), initiated by or coexisting with
endothelial impairment. In PAD, physiological collateral angiogenesis is deficient and therefore 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 outcome of heart ischemic events in diabetic patients (Riddle, 2003) and results in loss of ischemic preconditioning during vascular surgery in diabetics (Kottenberg et al., 2014). Furthermore, sulfonylureas have a varying profile regarding inhibition of endothelial-mediated ischemic post-conditioning 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, which inhibit even more selectively the pancreatic but spare the endothelial $K_{\text{ATP}}$ would logically present a better vasculoprotective profile in diabetics.

In conclusion, our work has shown that pharmacological manipulation of $K_{\text{ATP}}$ in vitro and in vivo, either directly, by $K_{\text{ATP}}$ openers or inhibitors, or indirectly, via CNP-cognate receptor interaction, results in modulation of angiogenic responses. It therefore appears that $K_{\text{ATP}}$ are a novel common mechanism underpinning angiogenesis to various physiological stimuli including VEGF, $H_2S$ and CNP. $K_{\text{ATP}}$ 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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FINANCIAL DISCLOSURE

None declared

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FOOTNOTES

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LEGENDS FOR FIGURES

FIGURE 1:

Effect of SG-209 and polypeptide growth factors on CAM angiogenesis. CAMs were treated with the indicated concentrations of either (A) the K_{ATP} opener SG-209 or (B) CNP or VEGF. Vessel length and branching point number were determined via NIH image analysis software 48h post-treatment. Inserts are representative photomicrographs (2.5×magnification). Data are expressed as means ± S.E.M; n=30-35 per point. *: P<0.05 versus Vehicle; **: P<0.01 versus Vehicle, using Student’s t-test.

FIGURE 2:

Effect of K_{ATP} inhibitors on CAM angiogenesis. (A) Gilbenclamide (1-100 nmole/cm²), (B) 5-HD (10-1000 nmole/cm²) or (C) Tolbutamide (1-100 nmole/cm²) were applied on CAMs in Vehicle and vessel length and branching point number were determined 48 h later. (D) CAMs were pre-treated with Gilbenclamide (1 nmole/cm²) for 30 min before application of CNP (300 nmole/cm²) and vessel length and branching point number were determined 48 h later. Inserts are representative photomicrographs (2.5×magnification). Data are expressed as means ± 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.

FIGURE 3:
Effect of \(K_{ATP}\) modulation on bEnd.3 endothelial cell proliferation and migration in vitro. (A): bEnd.3 cells were maintained in DMEM + 2.5% FBS in 24-well plates and pretreated with either Glibenclamide (10 \(\mu M\)) or with 5-HD (100 \(\mu M\)) for 20 min before addition of SG-209 (1 \(\mu M\)) or Nicorandil (10 \(\mu M\)). 48h later, cells were trypsinized and counted using a hemocytometer. (B): bEnd.3 cells were resuspended in serum-free medium and pre-treated with either Glibenclamide (10 \(\mu M\)) or 5-HD (100 \(\mu M\)) for 20min, before being placed in Transwells and allowed to migrate for 4h in response to SG-209 (1 \(\mu M\)) or Nicorandil (10 \(\mu M\)), after which the migrated cells were stained and counted. Data are expressed as means ± 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.

FIGURE 4:

Modulation of endothelial cell responses to CNP and VEGF by \(K_{ATP}\) inhibitors. (A) bEnd.3 cells in 2.5% FBS 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 (10 \(\mu M\)) or 5-HD (100 \(\mu M\)) before being exposed to VEGF (500 pM). 48h later cells were trypsinized and counted using a hemocytometer (n=3). (C): bEnd.3 cells in serum-free medium were pretreated with Glibenclamide (10 \(\mu M\)) or 5-HD (100 \(\mu M\)) for 20min, placed in well inserts and then exposed to either CNP (100 pM) or VEGF (500 pM). Migrated cells were stained and counted 4h later. n=5 for each condition. *: \(P<0.05\) versus Vehicle, †: \(P<0.05\) versus VEGF or CNP alone, using Student’s t-test.
FIGURE 5:

Cord-like network morphogenesis in vitro is affected by $K_{ATP}$ modulation. bEnd.3 cells (A) and HUVECs (B) were placed in 96-well plates in reduced-growth Matrigel and were treated with either SG-209 (1µM) or CNP (100 pM) in the presence or absence of Glibenclamide (10 µM) or 5-HD (100 µM) for 8 hours. The cord-like network length was determined from microphotographs using the Scion image software. n=5 for each condition. Inserts are representative photomicrographs of Vehicle-, SG-209- or CNP-treated cells (40x magnification); *: P<0.05 versus. Vehicle, **: P<0.01 versus. Vehicle, †: P<0.05 versus. SG-209 or CNP alone, using Student’s t-test.

FIGURE 6:

Downregulation of the $K_{ir}6.1$ subunit expression by siRNAs. HUVECS were transfected with small interfering (si) Control RNA or siRNA specific for $K_{ir}6.1$. After 24h, cell lysates were collected for protein and mRNA analysis. (A) qRT-PCR analysis of $K_{ir}6.1$ expression at the mRNA level, normalized for expression of the RPS18 ribosomal protein. 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 $K_{ir}6.1$ and β-actin antibodies.

FIGURE 7:

HUVEC responses to SG-209 and to CNP depend on the expression of the $K_{ATP}$ subunit $K_{ir}6.1$. A) HUVECs were transfected either with a Control siRNA or with a
siRNA specific for Kir6.1 (both from Santa Cruz). 24h later, cells were trypsinized, placed in Transwells and allowed to migrate in the presence or absence of CNP (100 pM) for 4h, after which 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). 24h 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 h. Cord-like network formation was determined and quantified from microphotographs using the Scion image software. n=5 for all groups. (Similar results were obtained with a second Kir6.1siRNA, Supplementary Fig. 1). (C): Representative photomicrographs of cells treated as in (B) (40× magnification); **: P<0.01 versus Vehicle control, †: P<0.05 versus CNP or SG-209 alone, using Student’s t-test.

**FIGURE 8:**

The effects of the K<sub>ATP</sub> opener SG-209 depend on kinase activation. *In vitro* endothelial responses to SG-209 depend on functional cell kinases Erk1/2, Akt and p38. (A) bEnd.3 cells were treated with the ERK1/2 inhibitor U0126 (10 μM) and/or SG-209 (1 μM) for 48h, then cells were trypsinized and counted using a hemocytometer. (B) bEnd.3 cells were serum-starved and pretreated for 20 min with the ERK1/2 inhibitor U0126 (10 μM), the p38 inhibitor SB230963 (10 μM) or the AKT inhibitor LY-242009 (5 μM). The treated cells were placed in Transwells in the presence or absence of SG-209 (1 μM). 4h later, migrated cells were stained and counted. n=5 for all groups. *: P<0.05 versus Vehicle, †: P<0.05 versus SG-209, using Student’s t-test.
FIGURE 1
**FIGURE 2**

A bar graph showing the effects of Glibenclamide on vessel length and branching points. The graph includes data for vehicle, 1, 10, and 100 nmol/cm² of Glibenclamide. The y-axis represents CAM Angiogenesis (% of control) and the x-axis shows Glibenclamide concentration.

A second bar graph shows the effects of 5-HD on vessel length and branching points. The graph includes data for vehicle, 10, 100, and 1000 nmol/cm² of 5-HD.

A third bar graph shows the effects of Tolbutamide on vessel length and branching points. The graph includes data for vehicle, 1, 10, and 100 nmol/cm² of Tolbutamide.

A fourth bar graph shows the effects of CNP on vessel length and branching points. The graph includes data for vehicle, Glib, CNP, and CNP+Glib.
FIGURE 3

A

B

Proliferation (% of Vehicle)

Migrated cells (% of Vehicle)

Vehicle, Glb, S-HD, SG-268, SG-268+Glbb, Nicorandil, Nicorandil+HD

** •

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FIGURE 4

A

B

C

Proliferation (% of Vehicle)

Proliferation (% of Vehicle)

Migration (cells, % of Vehicle)

Vehicle 1 10 100 1000 VEGF-500

Vehicle Glb. S-AHD VEGF VEGF+Glub. VEGF+S-AHD

Vehicle Glb. S-AHD CNP CNP+Glub. VEGF VEGF+Glub. VEGF+S-AHD

Vehicle Glb. S-AHD CNP CNP+Glub. VEGF VEGF+Glub. VEGF+S-AHD

* † † †
FIGURE 5

A. bEnd.3

Network length (% of Vehicle)

- Vehicle
- Gilb.
- S-HD
- SG-209
- SG-209/Gilb.
- SC-209/S-HD

B. HUVEC

Network length (% of Vehicle)

- Vehicle
- Gilb.
- S-HD
- CNP
- CNP/Gilb.
- CNP/S-HD

* Significantly different from control (Vehicle).
† Significantly different from SG-209.
FIGURE 6

Kir6.1 mRNA expression, normalized to RPS18 (as % of CTL)

CTL
CTLsi#1
Kir6.1si#1
CTLsi#2
Kir6.1si#2

**

β-actin

Kir6.1

CTL
CTLsi#1
Kir6.1si#1

**
FIGURE 7

A

B

C

Vehicle

SG-209

CNP

**

†

**

**

**

**

**

†
FIGURE 8

A. Proliferation (% of Vehicle)

B. Migrated cells (% of Vehicle)