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ROLE OF $K_{ATP}$ CHANNELS
IN ANGIOGENESIS

by

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ΠΕΡΙΛΗΨΗ

Σε πολλά εργαστήρια, σε παγκόσμιο επίπεδο, συντελείται σημαντική ερευνητική προσπάθεια για την ταυτοποίηση νέων μοριακών μηχανισμών στο πεδίο της αγγειογένεσης ως στόχων ανάπτυξης φαρμάκων, καθώς η θεραπευτική ρύθμιση της αγγειογένεσης μπορεί να επηρεάσει σημαντικά την εξέλιξη πολλών ασθενειών. Η διέγερση της αγγειογένεσης είναι επιθυμητή σε καταστάσεις ισχαιμίας όπου η παροχή αίματος μειώνεται σοβαρά, με αποτέλεσμα δυσλειτουργία ή νέκρωση ιστών, όπως το συμβάνει κατά την εμφάνιση αρτηριοπάθειας στο διαβητικό κάτω άκρο ή μετά από έμφραγμα του μυοκαρδίου. Σε αντιδιαστολή, η αναστολή υπερβολικής ή έκτοπης αγγειογένεσης μπορεί να είναι επωφελητική σε καταστάσεις όπως κατά την ανάπτυξη συμπαγούς άγκου ή σε νεαγγειακή εκφύλιση της ωχράς κηλίδας σχετίζομενη με την ηλικία.

Έχει δειχθεί ότι το Υδρόθειο (H2S), ένας νέος αγγειοδραστικός αέριος διαβιβαστής (gasotransmitter), μπορεί να εκκινήσει αγγειογόνες αποκρίσεις που εξαρτώνται από την ενεργοποίηση των ευαίσθητων στο ATP διαύλων καλίου (KATP). Μας κίνησε επίσης το ενδιαφέρον μια αναφορά του το C-νατριουρητικό πεπτίδιο (CNP), που είναι γνωστό ότι ενεργοποιεί τους ιδίους διαύλους καλίου, δύναται να προάγει τον πολλαπλασιασμό των ενδοθηλιακών κυττάρων. Ωστόσο, οι αγγειογενετικές ιδιότητες του CNP δεν έχουν διερευνηθεί σε βάθος. Αυτή η σύγκλιση μας ύποπτη στη διερεύνηση του κατά πόσον η άμεση ενεργοποίηση των KATP διαύλων καλίου προκαλεί αγγειογενετική απόκριση και επίσης εάν η απόκριση των ενδοθηλιακών κυττάρων στο CNP ή στον αγγειακούς ενδοθηλιακούς αυξητικούς παράγοντα (VEGF) απαιτούν πράγματι ενεργοποίηση των διαύλων KATP.

Η πειραματική προσέγγιση σχεδιάστηκε με βάση τον in vivo, όσο και in vitro μεθόδους. In vivo, η φυσιολογικά συντελεύτησε αγγειογένεση στην χοριοαλανταρακή μεμβράνη εμβρύου όρνιθας (CAM) ενισχύθηκε από τον άμοσο ενεργοποιητή των διαύλων KATP, το μόριο SG-209, καθώς και από τα πολυπεπτίδια CNP και VEGF. Δύο αναστολές των διαύλων KATP, η γλιβενκλαμίδη (glibenclamide) και το 5-αδρενετικό σεξ (5-HD), ανέστειλαν τόσο την αγγειογένεση υποβάθρου (basal) της χοριοαλανταρακής μεμβράνης, όσο και την επαγόμενη από το CNP αγγειογένεση το σύστημα αυτό. In vitro, άμεσοι ενεργοποιητές των διαύλων KATP όπως η νικορανδίλη (nicorandil) και το SG-209, καθώς και έμμεσοι ενεργοποιητές, μέσω αρχικής αγωνιστικής δράσης τους σε ειδικούς υποδοχέας, όπως ο VEGF και το CNP, αύξησαν τον πολλαπλασιασμό και τη μετανάστευση γεγεφαλικών ενδοθηλιακών κυττάρων πολτικού της κυτταρικής σειράς bEnd.3. Επιπλέον, o VEGF και to CNP επάγουν με παρόμοιο τρόπο το σχηματισμό τριχοειδών αγγείων από ενδοθηλιακά κύτταρα απομονωμένα από φλέβα.
ανθρώπινου ομφάλου λώρου (HUVEC) που δημιουργούνται σε Matrigel με χαμηλή περιεκτικότητα σε ανθρωπονομότομες. Όλες αυτές οι in vitro θετικές αγγειογενετικές ενδοθηλιακές αποκρίσεις αναστέλλονται παρουσία της γλιβενκλαμίδης και του 5-HD.

Διαμόλυνση κυττάρων HUVECs με siRNA που στοχεύει ειδικά την υπομονάδα του διαύλου καλίου $K_\text{Ca}$ 6.1 μείωσε αποτελεσματικά την έκφραση αυτής της υπομονάδας τόσο σε επίπεδο mRNA όσο και σε πρωτεϊνικό επίπεδο. Η μείωση στην έκφραση της υπομονάδας $K_\text{Ca}$6.1 των διαύλων καλίου προκάλεσε καταστολή της μετανάστευσης των κυττάρων HUVECs σε βαθμός τύπου φρέατος (transwells) καθώς και σημαντική απομείωση στο σχηματισμό δικτύου τριχοειδών αγγείων σε Matrigel, όταν αυτές οι αποκρίσεις επάγονται είτε από τον άμεσο ενεργοποιητή των διαύλων $K_\text{ATP}$, το μόριο SG-209, είτε από τον έμμεσο ενεργοποιητή των διαύλων $K_\text{ATP}$ (μέσω δράσης σε ειδικούς υποδοχείς), το πεπτίδιο CNP.

Επιπλέον, ο πολλαπλασιασμός και η μετανάστευση των ενδοθηλιακών κυττάρων bEnd.3 που διεγείρονται ως απόκριση στο μόριο SG-209 απαιτούν κινητοποίηση των "κλασσικών" ενδοθηλιακών προ-αγγειογενετικών κινασών Erk1 / 2, p38 και Akt, δεδομένου ότι οι αποκρίσεις αυτές στον SG-209 καταστάλθηκαν από κάθε ένα από τους αντίστοιχους αναστολείς των κινασών.

Η παρούσα μελέτη μας επιτρέπει να συμπεράνουμε ότι:

α) άμεση ψευδοκαρδιακή τροποποίηση της λειτουργίας των διαύλων $K_\text{ATP}$ επηρεάζει σημαντικά τις αγγειογόνες αποκρίσεις τόσο in vitro όσο και in vivo, β) το CNP είναι όντως ένας αγγειογενετικός παράγοντας, τόσο υπομονέας και αποτελεσματικός για την κινητοποίηση ενδοθηλιακών κυττάρων όσο και ο VEGF, γ) οι αγγειογόνες επιδράσεις που CNP και το VEGF εξαρτώνται από την ενεργοποίηση των ενδοθηλιακών διαύλων $K_\text{ATP}$ και συγκεκριμένα απαιτούν την έκφραση της υπομονάδας $K_6.1$ του διαύλου $K_\text{ATP}$, και τέλος δ) η ενεργοποίηση των διαύλων $K_\text{ATP}$ φαίνεται ότι είναι ένας κοινός μοριακός μηχανισμός που υποστηρίζει την αγγειογένεση που διεγείρεται από ένα ευρύ φάσμα ενδογενών αγγειοδραστικών χημικών ερεθισμάτων, που περιλαμβάνουν το H2S, τον VEGF και το CNP.

Οι επιπτώσεις της μελέτης αυτής στην θεραπευτική πράξη είναι πιθανώς σημαντικές: Οι τύποι σουλφουνολυδιας αναστολείς διαύλων των διαύλων $K_\text{ATP}$, που στοχεύουν μη-επαρκώς επιλεκτικά σε β-παγκρεατική κύτταρα, χρησιμοποιούνται ευρέως ως φάρμακα για τη θεραπεία του διαβήτη τύπου II, μια ασθένεια που χαρακτηρίζεται από γενικευμένη δυσλειτουργία του αρτηριακού συστήματος, με συνέπεια υψηλή επίπτωση εμφράγματος του μυοκαρδίου και ισχαιμίας των κάτω άκρων. Η εξέλιξη ενός επεισοδίου μυοκαρδιακής ισχαιμίας σε διαβητικός
ασθενείς είναι χειρότερη εάν αυτοί ακολουθούσαν φαρμακολογική θεραπεία με σουλφονυλουρίες, υποδεικνύοντας κάποια, μέχρι τώρα σε σημαντικό βαθμό ανεξήγητη, επιβλαβή καρδιαγγειακή επίδραση αυτής της κατηγορίας ενώσεων. Η παρούσα μελέτη αποδεικνύει ότι η ενεργοποίηση ενδοθηλιακών διαύλων $K_{ATP}$ είναι ένας κοινός προ-αγγειογενετικός μηχανισμός, που μπορεί εν μέρει να εξηγήσει αυτή την αρνητική έκβαση της πρότερης θεραπείας με σουλφονυλουρίες σε διαβητικούς. Επιπλέον, η έρευνα αυτή υποστηρίζει την ανάγκη για το σχεδιασμό νέων μορίων, τα οποία, ενώ θα αναστέλλουν τους διαύλους $K_{ATP}$ στο πάγκρεας, δεν θα επηρεάζουν τους ενδοθηλιακούς διαύλους $K_{ATP}$ και την εξαρτώμενη από αυτούς αγγειογένεση, και έτσι θα παρουσιάζουν, βάσει των ανωτέρω, βελτιωμένη θεραπευτική δράση.
SUMMARY

Worldwide research devotes significant effort to identify new, targetable molecular mechanisms in the field of angiogenesis, since therapeutic modulation of angiogenesis can critically alter the progression of a number of diseases. Stimulation of angiogenesis is desirable in situations characterized by tissue-damaging ischemia where blood supply is severely reduced, such as lower limb diabetic arteriopathy or following myocardial infarct. In contradistinction, stemming excessive or ectopic angiogenesis can be beneficial in situations such as solid tumor growth or in neovascular age-related macular degeneration.

It has been previously shown that Hydrogen Sulfide (H$_2$S), a new vasoactive gasotransmitter, can initiate angiogenic responses which depend on the activation of ATP-sensitive potassium channels (K$_{ATP}$). Intriguingly, C-type Natriuretic Peptide (CNP), which is also known to activate K$_{ATP}$, has been reported to promote endothelial cell growth; however, its angiogenic properties have not been explored at any depth. This pattern prompted us to investigate whether direct K$_{ATP}$ activation induces angiogenic responses and whether endothelial responses to CNP or Vascular Endothelial Growth Factor (VEGF) indeed require K$_{ATP}$ activation.

We undertook a dual-pronged approach, based on both in vivo and in vitro experimental approaches. In vivo, chick embryo chorioallantoic membrane (CAM) angiogenesis was similarly enhanced by the direct K$_{ATP}$ channel activator SG-209 and by the polypeptides CNP or VEGF. Two K$_{ATP}$ inhibitors, Glibenclamide and 5-Hydroxydecanoate (5-HD), abrogated both basal and CNP-induced CAM angiogenesis. In vitro, direct activators of K$_{ATP}$ such as Nicorandil and SG-209 and receptor-acting agonists such as VEGF and CNP, increased proliferation and migration in the mouse brain endothelial cell line bEnd.3. In addition, VEGF and CNP induced comparable capillary tube-like formation by Human Umbilical Vein Endothelial cells (HUVECs) in low growth factor Matrigel. All these in vitro pro-angiogenic endothelial responses were effectively abrogated by Glibenclamide or by 5-HD. Transfection of HUVECs with a siRNA specifically targeting the inwardly rectifying potassium channel (Kir) 6.1 subunit decreased the expression of this subunit at both the mRNA and the protein level. The resulting knock-down of the Kir6.1 K$_{ATP}$ subunit impaired HUVEC migration through transwells in vitro and substantially decreased tubular network formation in Matrigel in response to either the direct K$_{ATP}$ activator SG-209 or the receptor-operating K$_{ATP}$ activator CNP. Furthermore, the bEnd.3 endothelial cell proliferation and migration responses to SG-209 required mobilization of the “classic”
endothelial pro-angiogenesis kinases Erk1/2, p38 and Akt, since the responses to SG-209 were all abolished by each of the respective kinase inhibitors.

This work allows us to firmly conclude that:

a) direct pharmacological modulation of K$_{\text{ATP}}$ channels affects angiogenic responses in vitro and in vivo, b) CNP is a bona fide angiogenic factor, as potent and efficient to mobilize endothelial cells as VEGF, c) the angiogenic effects of CNP and VEGF depend on the activation of endothelial K$_{\text{ATP}}$ channels and specifically require the expression of the K$_{ir6.1}$ pore-forming K$_{\text{ATP}}$ subunit, and finally d) K$_{\text{ATP}}$ activation may be a common molecular mechanism that underpins angiogenesis to a wide variety of endogenous vasoactive stimuli that includes H$_2$S, VEGF and CNP.

The therapeutic implications of this work are significant: Sulfonylurea-type K$_{\text{ATP}}$ channel inhibitors, with questionable selectivity for pancreatic β-cells, are widely used drugs to treat type II diabetes, a disease characterized by arterial dysfunction and higher incidence of myocardial and lower limb ischemia. The outcome of cardiac ischemia in diabetic patients is worse if they have been treated with sulfonylureas, indicating some, until now unresolved, deleterious cardiovascular activity of this class of compounds. The present demonstration that endothelial K$_{\text{ATP}}$ channel activation is a common pro-angiogenic mechanism, may in part explain this unfavorable outcome of sulfonylurea treatment in diabetics. Furthermore, it raises the need to design new molecules which, while inhibiting the pancreatic K$_{\text{ATP}}$ channels, should spare the endothelial K$_{\text{ATP}}$ channels and the ensuing angiogenesis, thus exhibiting increased therapeutic benefit.
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DISCUSSION

REFERENCES
1 Angiogenesis

The present thesis focuses on the effects on angiogenesis via modulation of $K_{\text{ATP}}$ channels.

The mechanisms underlying angiogenesis have been extensively studied for the past forty years, allowing an enhanced understanding of the molecular events that underpin the various cellular processes that together initiate and sustain angiogenesis and generating therapeutically important molecular targets (Carmeliet and Jain, 2011). The term “angiogenesis” is commonly used to refer to the process of vessel growth but in the strictest sense denotes vessel sprouting from pre-existing, differentiated vascular endothelial cells (ECs), in order to deliver nutrients and oxygen to various organs and tissues. These studies have provided immense insights into most of the fundamental aspects of angiogenesis, and have led to a mechanistic model of vessel branching; this model considers and is in agreement with the processes that regulate branching morphogenesis in other organs, for example in the tracheal tree (Herbert and Stainier; 2011; Potente et al; 2011).

In physiological conditions, angiogenesis plays a critical, health-promoting role in embryo implantation and embryonic development, during wound healing, in recovery after ischemic heart disease or cerebrovascular disease, and in alleviating peripheral arterial disease (Carmeliet, 2000; Zhou et al., 2007). However, inhibition of angiogenesis can also provide therapeutic benefit, for example in solid cancer growth, in stemming diabetic retinopathy and in alleviating the effects of rheumatoid arthritis (Carmeliet and Jain; 2011; Lai et al; 2012). As a case in point, anti-angiogenic approaches targeting Vascular Endothelial Growth Factor (VEGF) have been validated therapeutically and are in current use in oncology and in ophthalmic angioproliferative disease (Bagri et al., 2011, Loong and Ma; 2012).

Physiologically, the body controls angiogenesis through a series of “on” and “off” regulatory switches: The main “on” switches are known as angiogenesis growth factors (cytokines); the main “off switches” are known as endogenous angiogenesis inhibitors (Pandya et al; 2006). Pathological or ectopic angiogenesis may give rise to abnormally rapid proliferation of blood vessels, thus contribute to the pathogenesis of the disease or tissue repair processes gone awry, and is usually characterized by abnormal vessel structure and growth (Oklu et al; 2010; Yin et al; 2012; Lai et al; 2012).
Angiogenesis depends on endothelial cell adhesion and proteolytic mechanisms that aid cell migration, which globally involve the coordinated activity of growth factors, extracellular matrix proteins, proteases and adhesion molecules (Goodwin; 2007; Myers et al; 2011; Shin et al; 2011). Blood vessels form mainly through angiogenesis, where vascular endothelial cells proliferate and migrate into the extracellular matrix (Bentley et al., 2009; Staton et al; 2009), in which the transcapillary pillars are formed and partition the vessel lumen or by looping angiogenesis, where vessels translocate by biomechanical forces (Kilarski et al; 2009; Ribatti; 2010).

1.1 Sprouting angiogenesis

In adulthood, in the absence of injury or other pathological influence (e.g. trauma), most blood vessels remain quiescent, with the exception of the vasculature in the female reproductive tract that undergoes cyclic modulation according to the female reproductive cycle (Carmeliet; 2005). The vascular endothelial cells, however quiescent, retain their capability to divide if placed under a physiologic stimulus. A cascade of events must take place in order for vascular sprouting to occur. These events include: release of growth factors, degradation of basement membrane, activation of tip cells (Ribatti and Crivellato; 2012) which leads to sprouting towards the growth factor gradient, endothelial stalk cell proliferation, formation of solid sprouts of endothelial cells and connecting of sprouts to vascular loops (Cimpean et al., 2010; Frontini et al; 2011; Tengood et al; 2011), shown in figure 1. For sprouting to occur, precise spatial and temporal regulation of extracellular proteolytic activity mediated by matrix degrading enzymes is important (Cimpean; 2011; Ribatti and Crivellato; 2012). Matrix metalloproteinases (MMPs) can act together or in cooperation with other enzymes to degrade most components of the extracellular matrix (ECM) and play an important role in the regulation of angiogenesis (Ebrahem et al; 2010; Ribatti and Crivellato; 2012). VEGF-A, the most important and well-studied regulator of sprouting angiogenesis, especially in hypoxia, induces a signal cascade which activates an endothelial cell to become a tip cell, consequently navigating the growing sprout towards a growth factor gradient (Gerhardt et al; 2003; Phng and Gerhardt; 2009). The tip cell of the sprout leads the way for the growing vessel, guided by fine cellular extensions called filopodia. The stalk cells, however, trail behind the tip cell, proliferate and elongate the stalk of the sprout and form a lumen (Ribatti and Crivellato; 2012). To form a functional loop, tip cell filopodia from different sprouts connect subsequently and establish perfused vessels (figure 1).
Figure 1: An illustrative diagram indicating processes involved during angiogenesis (1) Tumors produce and release angiogenic growth factors (proteins) that diffuse into the nearby tissues. (2) The angiogenic growth factors bind to specific receptors located on the endothelial cells (EC) of nearby pre-existing blood vessels. (3) Once growth factors bind to their receptors, the endothelial cells become activated. Signals are sent from the cell's surface to the nucleus. The endothelial cells' machinery begins to produce new molecules including enzymes. (4) Enzymes dissolve tiny holes in the sheath-like covering (basement membrane) surrounding all existing blood vessels. (5) The endothelial cells begin to divide (proliferate), and they migrate out through the dissolved holes of the existing vessel towards the diseased tissue (tumor). (6) Specialized molecules called adhesion molecules, or integrins (αvβ3, αvβ5) serve as grappling hooks to help pull the sprouting new blood vessel sprout forward. (7) Additional enzymes (matrix metalloproteinases or MMP) are produced to dissolve the tissue in front of the sprouting vessel tip in order to accommodate it. As the vessel extends, the tissue is remodeled around the vessel. (8) Sprouting endothelial cells roll up to form a blood vessel tube. (9) Individual blood vessel tubes connect to form blood vessel loops that can circulate blood. (10) Finally, newly formed blood vessel tubes are stabilized by specialized muscle cells (smooth muscle cells, pericytes) that provide structural support. Blood flow then begins. (From: Pandya et al; 2006).

1.2 Intussusceptive angiogenesis

Blood vessel growth can also take place by non-sprouting, intussusceptive angiogenesis. This is a well-characterized morphogenetic process which can be observed during growth and remodeling of pre-existing networks (Ackermann et al; 2014). Intussusception was late to be recognized as a process by which angiogenesis occurs. It has received considerable attention as an important mode for subsequent growth and remodelling of the developing vasculature (Burri et al; 2004, Makanaya et al; 2009).

Intussusceptive angiogenesis involves the maturation of vascular networks through the formation of transluminal pillars within capillaries, consequently splitting one vessel into two
parallel vessels without vascular endothelial cell proliferation (Makanya et al; 2009) as in figure 2. The pillars are then invaded by pericytes and myofibroblasts that provide stabilizing collagen to the vessel wall (Burri et al; 2004; Djnov and Makanya; 2005). In general, intussusceptive microvascular growth results in enlargement of the capillary plexuses with a resultant large endothelial exchange surface, without requiring much energy. In contrast to sprouting, intussusception is fast, occurring within hours or even minutes, and is energetically more economic as it does not require cell proliferation or substantial degradation of basement membrane. Whether sprouting or intussusception shall occur depends mainly on metabolic and hemodynamic factors (Makanya et al; 2009).

Sprouting is normally driven by a number of angiogenic factors, which include VEGF, FGF, Angiopoietins and PDGFs (Saharinen et al; 2011). Intussusception is normally stimulated by higher levels of shear stress (mechanical forces from blood flow) (Alameddine et al; 2014; Styp-Rekowska et al; 2011) or by a drop in the local concentration of angiogenic factors (Hlushchuk et al; 2008). So far, intussusceptive angiogenesis has been identified in many developing organs, such as the retina, but also as a vascular defense mechanism to escape anti-angiogenic treatment in tumors (Makanya; 2009). Until now, intussusception has not been observed as a mode of blood vessel formation in the cornea.

Below is the short sketch of primary vascular growth that schematically explains the two angiogenic processes.

![Figure 2: An illustrative sketch of primary vascular growth that briefly explains the angiogenic processes. (From: Carmeliet and Collen; 2000).](image-url)
The precursor cells (angioblast) initially form a primitive network that undergoes further
development by either sprouting or intussusceptive angiogenesis. The latter process postulates
that the capillary network increases its complexity and the vascular surface by insertion of a
multitude of transcapillary pillars (intussusception). This results in the maturation of vascular
networks and consequently splitting one vessel into two parallel vessels without notable
vascular endothelial cell proliferation (figure 2). Subsequently, as described above, there is
pericytes and myofibroblast contribution (Burri et al., 2004; Makanya, 2009). The multitude of
growth factors that have been shown to be required for normal vascular development include
TGF-ß, PDGF, FGF e.t.c, which perform numerous overlapping but also unique functions within
the complex angiogenic cascade.

Some of the soluble growth factors, membrane bound molecules and mechanical forces that
participate or modulate angiogenic processes, whether it is sprouting or intussusception, are
summarized below (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th>Activators</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF family members</td>
<td>Stimulate vasculogenesis / angiogenesis, permeability, leukocyte adhesion</td>
</tr>
<tr>
<td>Angiopoietin1, Tie2</td>
<td>Stabilize vessels, inhibit vessel permeability</td>
</tr>
<tr>
<td>PDGF</td>
<td>Recruit pericytes, smooth muscle cells</td>
</tr>
<tr>
<td>TGF-ß1, endoglin, TGF-ß-receptors</td>
<td>Stimulate ECM production, recruit Macrophages</td>
</tr>
<tr>
<td>FGF, HGF, MCP1</td>
<td>Stimulate angiogenesis/arteriogenesis</td>
</tr>
<tr>
<td>Integrons</td>
<td>Receptor for matrix macromolecules, Proteinases</td>
</tr>
<tr>
<td>VE-cadherin, PECAM (CD31)</td>
<td>Endothelial junctional molecules</td>
</tr>
<tr>
<td>Pasminogen activators, MMPs</td>
<td>Remodel ECM, release and activate growth factors</td>
</tr>
<tr>
<td>NOS-COX2</td>
<td>Stimulate angiogenesis and vasodilation</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Activate expression of VEGF and other pro-angiogenic factors</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-1, solubleVEGFR-1, VEGFR-3</td>
<td>Sink for VEGF, PIGF</td>
</tr>
</tbody>
</table>
### Table 1

<table>
<thead>
<tr>
<th>Activator/Inhibitor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiopoietin 2</td>
<td>Antagonist for Angiopoietin1 (?)</td>
</tr>
<tr>
<td>Trombospondin 1,-2</td>
<td>Inhibit endothelial migration, growth, adhesion and survival</td>
</tr>
<tr>
<td>Angiostatin</td>
<td>Suppress tumor angiogenesis</td>
</tr>
<tr>
<td>Endostatin (collagen XVIII fragment)</td>
<td>Inhibit endothelial survival and migration</td>
</tr>
<tr>
<td>TIMP</td>
<td>Inhibits MMP</td>
</tr>
<tr>
<td>IL-4, IL-12, IL-8, IFN-α,-β,-γ</td>
<td>Inhibit endothelial migration, downregulate bFGF expression and signaling</td>
</tr>
</tbody>
</table>

**1.3 Vascular Endothelial growth factor-A (VEGF-A), a major regulator of angiogenesis**

One of the best-studied, most cell-specific and critical regulators of angiogenesis is vascular endothelial growth factor-A (VEGF-A), which regulates endothelial proliferation, permeability, and survival. It belongs to a family of cysteine-knot polypeptide factors that are closely related and share receptors for activity, that includes VEGF-B, -C and –D (Shibuya; 2013). However, VEGF-A is by far the best-studied and characterized among them in terms of blood vessel angiogenesis (in contrast to lymphatic angiogenesis). VEGF-A is a mitogen for vascular endothelial cells derived from arteries, veins, and lymphatics (Hoeben et al; 2004 Pandya et al; 2006). VEGF-A is a unique, potent angiogenic factor that stimulates capillary formation in vivo and has direct mitogenic actions that are mostly restricted to the endothelial cells, at least in low concentrations. Human VEGF-A has at least four structurally related isoforms, VEGF121, VEGF165, VEGF189, and VEGF206 (Benjamin et al., 1999; Ferrera; 2001; Takahashi and Shibuya; 2005). Of these, VEGF165 has the most potent biological activity and is the most abundant subtype in vivo. VEGF-A is secreted by many cell types, and its expression and secretion is regulated by a number of growth factors and cytokines. For example, interleukin 1-β, platelet-derived growth factor (PDGF), and transforming growth factor β stimulate VEGF production by smooth muscle cells. VEGF-A also elicits a pronounced angiogenic response in a wide variety of in vivo and in vitro models, providing strong evidence that VEGF is a requisite survival factor for endothelial cells, both *in vitro* and *in vivo*.

In contradistinction, VEGF-A withdrawal has been shown to result in regression or malformation of vasculature in several physiological and pathological circumstances (Karkkainen and...
Petrova; 2000). For instance, there is considerable evidence that VEGF-A is a major tumor angiogenic factor. There is an extensive body of data documenting that inhibition of VEGF activity results in suppression of growth of a wide variety of tumor cell lines in murine models (Hoeben et al; 2004; Tammela et al; 2005). Validating this preclinical evidence, clinical trials in cancer patients have placed anti-VEGF therapy in the clinic, while there are still quite a few ongoing trials with several VEGF inhibitors, including a humanized monoclonal antibody (Presta et al., 1997; Nowak et al; 2009), and various small molecules inhibiting signal transduction (Wood et al., 2000; Olsson et al; 2006; Mattuella et al; 2007), with the aim to optimize and expand the medical indications for which anti-VEGF-A therapy is approved.

1.4 Experimental Angiogenesis

Numerous experimental models are available that can be used to visualize, analyze and characterize angiogenesis (McDonald and Choyke; 2003). When evaluating new experimental angiogenesis treatments and targets, there is a need for comprehensive models, where different contributing factors to vessel growth and remodeling can be observed. Moreover, their characteristics should be tailored to the therapeutic modality where they are aimed to be ultimately used, so that reliable in vitro, cell-based, or in vivo models mimicking a clinical setting have been developed. Despite the imaginative, large number of in vivo and in vitro assay models currently available, a gold-standard angiogenesis model that includes most features of angiogenesis has yet to be developed. Instead, a combination of models, each with its strengths and weaknesses, has been used to address this problem (Staton et al; 2004b).

1.4.1 In vitro Models

The first widely used approach to study angiogenesis mechanisms is to measure the ability of endothelial cells to form vessels in vitro. These models have also been extensively used to test the effect of pro and anti-angiogenic factors (Staton et al; 2004b). Below, we mainly focus on the assays we have used in the course of this thesis.

1.4.1.1 Proliferation, Migration and Matrigel assays

Endothelial proliferation and ability to migrate are characteristics necessary for the extension of a vascular network by angiogenesis, and the ability of endothelial cells to increase their growth and migratory behavior denotes their ability to participate in angiogenic responses. Proliferation and migration properties of endothelial cells treated with pro- and anti-angiogenic factors can be measured with classical in vitro assays. These assays are easy to perform; however, they are...
not suitable to measure the ability of endothelial cells to form tubes. Therefore, a specific in vitro angiogenesis assay has been developed, the sprouting assay. This assay is one of the most specific in vitro tests for angiogenesis studies because it measures the ability of endothelial cells to form a tube network when plated on reduced growth factor Matrigel, a laminin-rich basement membrane matrix formed by cancer cells in culture (Grant et al 1992; Staton et al; 2009).

Several additional in vitro models have been developed, which include culture of different origin, tissue-specific vascular endothelial cells on collagen for two-dimensional growth studies, as opposed to Matrigel, which allows for three-dimensional network formation (Poulaki; 2011). Endothelial cells have been cultured alone or in co-culture with fibroblasts or pericytes for studying various cell-cell interactions, mimicking, as best they can, the in vivo cell-cell interactions. By far the most commonly used type of endothelial cell type is Human Umbilical Vein Endothelial Cells (HUVECs), which are suitable for such assays at low passage (<4), while at higher passages they tend to lose their differentiated characteristics and functional capacities. Nevertheless, over 19 different types of endothelial cells (of cardiac, pulmonary, uterine, and dermal origin, amongst others) are now available to investigate potentially origin-specific differences among them in responding to external chemical cues (Staton et al; 2009). The main advantage of the in vitro models is that one can easily alter endothelial cell growth and proliferation by using a multitude of pharmacological agents, growth factors or media (mimicking for example, hyperglycaemia) and thus perform a very detailed study of the molecular mechanisms regulating their functional responses. The main disadvantage of these models is, naturally, that the surrounding environment is artificial; lack of blood flow and mechanical forces will hinder the study of more complex physiological process taking place in vivo. In addition, long-term cell-cell interactions, reflecting the quiescent nature of the native environment, are also not reflected by these models. Therefore, caution should be taken when interpreting the results and multiple assays are required to be used, to be followed-up by an in vivo assay.

Matrigel is a solubilized basement membrane extract from Engleberth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in ECM proteins such as laminin, collagen IV and heparan sulfate proteoglycans. Matrigel is liquid at 4°C but polymerizes at body temperature. The Matrigel plug angiogenesis assay involves subcutaneous injection of Matrigel containing cells or substances, which turns into solidified matrigel plugs (Passaniti et al., 1992). These plugs can be recovered 1–3 weeks after implantation and examined histologically to determine the extent of blood vessel formation within the Matrigel. In contrast to the corneal angiogenesis assay, the Matrigel
plug assay does not require extensive technical skill and is relatively easy to administer, although frequently the vessels formed are not well-formed and there is some variability between Matrigel lots.

1.4.2 Organ Culture (Ex vivo) models

This category includes foetal mouse metartasal assay, vena cava assay and rat aortic ring assay. The aortic ring assay was introduced by Nicosia and Ottinetti in 1990 and currently is the most commonly used organ culture. In the ex vivo models, segments, discs or section of the specific tissue are cultured in three dimensional matrices in vitro and monitored for microvascular outgrowth for 10-14 days. An advantage with the aortic ring model is the presence of non-endothelial cells, pericytes and fibroblasts, and potentially other supporting cell types providing a model more closely related to the in vivo situation. Although, it is cost effective and easy to perform, but it lacks sufficient blood flow to the tissues which is the physiological natural environment as in vivo (Staton et al; 2009).

1.4.3 In vivo Models

When using the wide range of in vitro and ex vivo assays discussed above, care must be taken when interpreting results, especially when comparisons between different assays published by different groups are attempted, because of differences in the origin of endothelial cells, passage number, substrates and media composition used. A compound which has shown angiogenic efficacy in vitro, may not show any activity in vivo (Liekens et al. 2001) and vice versa, with some compounds showing little efficacy in vitro while exhibiting strong activity in vivo. Therefore, the in vivo evaluation of agents is a vital step in drug development and the current in vivo assays are described below:

1.4.3.1 The Chick Embryo Chorioallantoic Membrane (CAM) Model

The chick embryo chorioallantoic membrane (CAM) has been used as an experimental technique for the study of angiogenesis and in the development of anti-angiogenic molecules, since it can respond to treatment with soluble factors and proximal engraftment of cells in vivo (Rabbati; 2010). Because the CAM is a living system, it provides a more complex biological model for in vivo analysis of active, but also potentially toxic, pharmacological substances. The CAM is readily accessible since it is an extraembryonic tissue and provides a technically simple way of studying a complex biological system. The chick is relatively immunotolerant, which enables the study of cross-species xenografts including tumor fractions (Vogel & Berry 1975), cultured human cancer cells (Auerbach et al. 1975) and mammalian tissue explants (Ausprunk
& Folkman 1976) for extended periods of time. Alternatively, a test substance can be placed on the CAM, through a window cut carefully in the egg shell, in either slow release polymer pellets, in gelatin sponges or air-dried onto filter discs. There are two main methods of accessing the CAM: the first one allows the embryo to develop in the shell and then cutting a window in the shell, while the second one entails culturing the embryo in a Petri dish, without the shell (Zijlstra et al; 2006). Both techniques are minimally invasive to the embryo, and while the ‘in shell’ technique requires less maintenance and embryos can be maintained to later stages of development, the ‘ex ovo’ method provides improved access to the test site, it improves the ability to repetitively treat or to have multiple test sites on one CAM, and allows us to image a wider area of the CAM. Angiogenesis is typically allowed to proceed for 2-3 days post-treatment, although longer time periods have been used. The advantages for the CAM assay include the fact that it is relatively simple and inexpensive and therefore suitable for large-scale screening. The CAM is also amenable to biochemical analysis and extracts of CAM have been used to demonstrate signalling events in vivo (Alavi et al. 2003). Furthermore, it is possible to apply test substances directly or via intravenous or intra-allantoic injections, and the lack of active substance excretion permits maintenance of the test reagents in circulation for an extended period.

Additionally, accessibility of the CAM permits serial application to be easily performed. However, there are also important limitations that must be taken into account. The CAM itself is well vascularized; hence, to distinguish new capillaries from the existing ones can prove difficult. Moreover, as developmental angiogenesis occurs within the CAM up to 11 days, results obtained from embryos younger than this have the additional complication that unspecified interactions with endogenous factors may be affecting angiogenesis. Therefore, in most cases it is preferable to wait until day 11. Furthermore, chemical or physical irritation of the CAM, including shell dust generated when cutting the window in the shell, can lead to an inflammatory response, which in itself may induce angiogenesis, thereby hampering identification of the specific responses to the test substance. It is often necessary to wait 3 days after making the window before adding the test substance to check for an immune response or alternatively add an anti-inflammatory agent to the filter discs prior to their use. However, overt suppression of inflammation may not always be desirable, especially as some angiogenic stimuli work in concert with the inflammatory response (Leibovich et al. 1987). Finally, the membrane is sensitive to changes in oxygen tension, making the sealing of the window a vital part of the procedure (Auerbach et al; 2000).
For the sake of completeness, we briefly include below a few more widely-used in vivo models:

1.4.3.2 The Zebrafish Model

Zebrafish (*Danio rerio*) is a small tropical freshwater fish that under optimal conditions yield hundreds of embryos per mating. These large numbers and the optical clarity of the developing embryos facilitate the study of development and angiogenesis (Gore et al; 2012). The zebrafish share many genes and mechanisms of angiogenesis regulation with mammals, thus making this organism a useful model organism in which one can analyze the development and function of the vasculature (Rubinstein; 2003). The formation of the intersegmental vessels and the subintestinal veins in early embryos is well characterized and easily monitored (Isogai et al; 2001) making them suitable for the study of angiogenesis inhibitors. However, there has been some debate as to whether angiogenesis or vasculogenesis is being measured, as these processes are not clearly separated spatially or temporally in the developing embryo: the dorsal aorta and posterior cardinal vein are formed by vasculogenesis (Zhong et al; 2001; Gore et al; 2012) while the intersegmental vessels are thought to be formed by angiogenesis.

1.4.3.3. Corneal angiogenesis assay model

The cornea is the only avascular transparent tissue in the body, so any vessels penetrating from the limbus into the corneal stroma are newly formed, readily visible and can be quantified (Gimbrone et al; 1974; Muthukkaruppan & Auerbach; 1979). As almost all types of corneal injury induce neovascularization, many experimental techniques including chemical cauterization, intrastromal injection, mechanical scraping of the limbal epithelium, intracorneal suture, surgical grafts and, most commonly, micropockets with implantation of slow release pellets, have all been employed in the development of corneal angiogenesis assays (Shan and Dewhirst; 2006). The original experiments investigating tumor-induced neovascularization in a corneal pocket used solid tumor fragments implanted in the rabbit corneal pocket (Gimbrone et al. 1974), which has since been adapted for use in guinea pigs, rats and mice (Muthukkaruppan & Auerbach; 1979; Fournier et al; 1981; Hori; 1990). Materials placed within the pocket have included tumor tissue, tumor cells, tumor cell extracts, other tissues and cells, concentrated conditioned medium, and more recently purified/recombinant cytokines and/or growth factors incorporated into slow release pellets (Conn et al; 1980; Fournier et al; 1981; Gross et al; 1981; D’Amato et al; 1994; Lingen et al; 1996). Thus, any preclinical model that comprises systemic administration is more relevant clinically (Volpert et al. 1998; Shaw et al. 2003).

1.4.3.4 Tumor models
Subcutaneous implantation of tumor cells is one of the easiest and most frequently used approaches for investigating tumor growth and angiogenesis in vivo (Steimberg et al; 2014; Staton et al. 2004b). Despite the well-documented differences in microenvironment of subcutaneously grown tumors with the tumors grown at an orthotopic site (Keyes et al; 2003; Steimberg et al; 2014), this is a valuable model as tumor growth can be easily accessed by measuring tumor dimensions regularly and weighing excised tumors at the end of the study (Stoeltzing et al; 2004; Staton et al; 2007a).

Although angiogenesis cannot be visualized daily, if the groups are sufficiently powered, subgroups of randomized animals may be killed sequentially for imaging angiogenesis (Bruns et al; 2000). It is generally advised that if subcutaneous tumor experiments have promising results then these results should be confirmed in an orthotopic tumor model, where feasible. A major disadvantage of all the tumor models described so far is that tumors are established within a few days or weeks after cell implantation, whereas human cancer develops over a period of several months or years. However, there are now transgenic mouse models of tumor angiogenesis where animals overexpress targeted oncogenes leading to spontaneous but predictable and reproducible tumor formation over a longer time period. These include the RIP-Tag mice where overexpression of the SV40 Tag oncogene is under control of the insulin regulatory region, giving rise to pancreatic tumors (Hanahan; 1985).

In brief, in order to fully interpret the effects of a test substance on the process of angiogenesis, it is still necessary to use multiple in vitro assays designed to investigate the different steps in the angiogenesis pathway with either the relevant endothelial cell or multiple endothelial cell types, followed by more than one in vivo assay, (Steimberg et al; 2014), where the microenvironment will influence angiogenesis and the effect of the test substance. This will enhance the likely success of preclinical studies translating into clinical modalities.

Below is a summary on the in vitro, in vivo and tumor experimental angiogenic models which highlights the advantages and disadvantages of the procedures.
Table 2: Comparison summary of in vitro, in vivo and Mouse tumor models in experimental angiogenesis (From: Doumont et al; 2008).

<table>
<thead>
<tr>
<th></th>
<th>In vitro models</th>
<th>In vivo angiogenesis models</th>
<th>Mouse tumor models</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Applications</strong></td>
<td>• In vitro testing of potential angiogenesis inhibitors and pre-angiogenic mediators</td>
<td>• In vitro testing of potential angiogenesis inhibitors and pre-angiogenic mediators</td>
<td>• Analysis of angiogenic switch in de novo tumor development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• In vivo testing of potential angiogenesis inhibitors and pre-angiogenic mediators</td>
<td>• In vivo testing of anti-tumor efficacy of potential angiogenesis inhibitors</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Analysis of tumor dormancy</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>• Relatively fast assays to test pre- or anti-angiogenic factors</td>
<td>• Closer to in vivo situation than in vitro models;</td>
<td>• Mouse tumor models recapitulate human cancer phenotypes</td>
</tr>
<tr>
<td></td>
<td>• Results are obtained in a short time period</td>
<td>• Adequate modeling of extracellular environment</td>
<td>• Highly relevant results</td>
</tr>
<tr>
<td></td>
<td>• Useful for characterization of cells from in vivo models</td>
<td></td>
<td>Results may be directly translated to humans</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>• Artificial systems</td>
<td>• Potential variability in results, depending on starting material</td>
<td>Results are obtained after a long time period</td>
</tr>
<tr>
<td></td>
<td>• Observations need to be validated in in vivo systems</td>
<td>• Possibly difficult to perform</td>
<td></td>
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</table>

2 $K_{\text{ATP}}$ CHANNELS

ATP-sensitive $K^+$ channels ($K_{\text{ATP}}$ channels) were first described in the cardiac muscle by Noma A. (1983). They are widely distributed and characteristically activated by declining intracellular ATP levels and elevated nucleotide-diphosphate (ADP) concentrations, thus providing a link between cellular metabolic status and membrane excitability (Yoshida et al; 2004 Matsuo; 2005). Subsequently, they were found in many other tissues, including pancreatic $\beta$-cells (Tarasov et al; 2004; Sanchez-Duarte et al; 2012). The $K_{\text{ATP}}$ channel of the pancreatic $\beta$-cell is the target of sulfonylureas such as glibenclamide and tolbutamide (Miki and Seino; 2005), widely used in the treatment of type 2 (non-insulin dependent) diabetes mellitus (NIDDM), in which they are used because they can stimulate insulin secretion by closing the $\beta$-cell membrane $K_{\text{ATP}}$ channels. In pancreatic $\beta$-cells, the increase in ATP/ADP ratio generated by exposure to high circulating glucose levels closes the $K_{\text{ATP}}$ channels to elicit the secretion of insulin while, a decrease in glucose increases the open possibility (Miki et al; 1999). $K_{\text{ATP}}$ channels in cardiac and skeletal muscle have been implicated in cell excitability, cytoprotection and the cellular loss of $K^+$ during ischemia, hypoxia, or other metabolic insults (McCulley and
Levitsky; 2003). In brain, \( K_{\text{ATP}} \) channels are involved in neuronal excitability, appetite control and in cytoprotection in ischemia and hypoxia. \( K_{\text{ATP}} \) channels also have been implicated in other functions, including smooth muscle relaxation and vasodilatation, secretion of pituitary hormone, \( K^+ \) recycling in renal epithelia, and oocyte maturation (Yamada and Inagaki; 2002; Ballanyi et al; 2004). \( K_{\text{ATP}} \) channels with distinct electrophysiological and pharmacological properties correspond to the various \( K_{\text{ATP}} \) channels found in native tissues, offering the opportunity for tissue- or cell-selective pharmacological intervention. For example, Kir6.2 and SUR1 constitute the pancreatic \( \beta \)-cell type \( K_{\text{ATP}} \) channel (Miki and Seino; 2005); Kir6.2 and SUR2A constitute the cardiac type \( K_{\text{ATP}} \) channel (Nichols et al; 2013). Kir6.1 and SUR2B constitute the vascular smooth muscle type \( K_{\text{ATP}} \) channel (Suzuki et al; 2001), which are activated by nucleoside diphosphates (NDPs) and inhibited by glibenclamide.

2.1 Molecular structure of \( K_{\text{ATP}} \)-channel

\( K_{\text{ATP}} \)-channels are hetero-octameric complexes consisting of four pore-forming subunits (Miki et al; 1999; Yoshida; 2004; Teramoto; 2006; McTaggart et al; 2010) which are inwardly rectifying \( K^+ \) channel subunits (termed Kirs) and four sulphonylurea receptor regulatory subunits (SURs), which are members of the family of ATP-binding cassette (ABC) transporter proteins. Two different Kir6 subunit genes have been described, Kir6.1 and Kir6.2 (Miki et al; 1999; Mikhailov et al; 2005). Two closely related genes encoding the sulphonylurea-binding receptors SUR1 (high affinity receptor) and SUR2 (low affinity receptor) have also been cloned (Inagaki et al., 1996; Isomoto et al., 1996). Additional complexity is provided by the existence of splice variants: three different splice variants of SUR2 have been identified: SUR2A (Inagaki et al., 1996; Yoshida et al., 2004), SUR2B (Isomoto et al., 1996) and SUR2C (Chutkow et al., 1996). Each of the Kir-channel subunit has two putative transmembrane segments and a \( K^+ \) ion pore-forming region (H5) (Aguilar-Bryan and Clement; 1999; Miki et al; 1999; Yoshida et al; 2004). Both Kir6.1 and Kir6.2 contain several potential protein kinase A and protein kinase C-phosphorylation sites (Inagaki et al., 1995a; Inagaki et al., 1995b).

Although the regulatory SUR subunits were originally thought to have 13 transmembrane segments, a 17-transmembrane-segment model (figure 3B) was more recently proposed by (Hibino et al; 2010). The SUR subunit is thought to have three distinct regions, each consisting of five, six and six transmembrane segments, respectively (figure 3B). It has been proposed that each subunit contains N-linked glycosylation sites, protein kinase A- and protein kinase C-dependent phosphorylation sites, as well as two nucleotide binding folds (NBFs). Each NBF
contains the Walker A and B motifs (Gros et al; 2002; Hibino et al; 2010), which are thought to be important for nucleotide regulation of the functional activity of ABC proteins (Yoshida et al; 2004). $K_{ATP}$ channels are the first example of a heteromultimeric complex of an ion channel and a receptor that are structurally unrelated to each other.

The various Kir and SUR subunits combine to form functional $K_{ATP}$-channels with different sensitivities to nucleotides and $K_{ATP}$ channel drugs, depending on the final composition of the complex. In addition, the composition of the channel is, to a significant degree, characteristic of the cell type or tissue type, examined, thus providing the basis for some tissue selectivity of pharmacological agents.

Comparison of the properties of cloned and wild-type $K_{ATP}$-channels suggests that the pancreatic ß-cells and neuronal $K_{ATP}$-channels are composed of Kir6.2 and SUR1, the cardiac type of Kir6.2 and SUR2A, while two smooth muscle–like combinations were identified, SUR2B/Kir6.2 and SUR2B/Kir6.1. (Isomoto et al., 1996; Chutkow et al., 1996; Yamada et al., 1997; Cui et al; 2002; Miki and Seino; 2005). An ATP-insensitive, but MgGDP-activated, channel is observed when Kir6.1 and SUR2B are co-expressed; such a channel might correspond to the nucleotide-activated channel found in some smooth muscle cells (Yamada et al., 1997). As depicted in (figure 3A) both subunits are required to co-assemble in a 4:4 stoichiometry in order to form a functional channel (Miki et al; 1999; Yoshida et al; 2004).

Figure 3: Molecular structure of $K_{ATP}$ channel. (A) Assembly of $K_{ATP}$ channel. The $K_{ATP}$ channel comprises of two subunits: Kir6.x (Kir6.1 or Kir6.2) and the regulatory subunit sulphonylurea receptor SUR (SUR1 or SUR2). (B) Membrane topology of SUR and Kir6.x. The sulphonylurea receptor has been proposed to have seventeen transmembrane domains. Kir6.x has two transmembrane domains (M1 and M2) linked by an extracellular pore-forming region (H5) and cytoplasmic amino (NH2) - and carboxy (COOH) - terminal domains. Abbreviations: NBD1 and NBD2, nucleotide binding domains 1 and 2; TMD, transmembrane domains). (From: Gros et al; 2002).
2.2 Role of ATP-Sensitive K⁺ Channels as Metabolic Sensors

ATP-Sensitive K⁺ channels are present in various tissues including pancreatic β-cells, brain and heart. Analysis of Kir6.2 null mice has shown that Kir6.2/SUR1 channels in pancreatic β-cells and the hypothalamus are essential in glucose-induced insulin secretion and hypoglycemia-induced glucagon secretion (Minami et al; 2004). In pancreatic β-cells, the increased ATP concentration due to increased glucose metabolism closes the K⁺ATP channels, depolarizing the plasma membrane, leading to opening of the voltage-dependent calcium channels (VDCCs), which in turn allows calcium influx (figure 4). The resultant rise in the intracellular calcium concentration (Ca²⁺) triggers exocytosis of the insulin-containing granules. Sulfonylureas, such as glibenclamide, widely used in treatment of type 2 diabetes, stimulate insulin release by closing the K⁺ATP channels (Minami et al; 2004; Alexandro et al; 2009; Quan et al; 2011). Thus, the K⁺ATP channels in pancreatic β- cells have been known to be critical in the regulation of insulin secretion. In the brain, K⁺ATP channels with different properties in various cell types have been reported, including hippocampus neurons (Fujimura et al; 1997; Sun and Feng; 2013), glial cells (Zawar et al; 1999), dorsal vagal neurons (Trapp et al; 1995), hypothalamic neurons (Sun and Feng; 2013), and substantia nigra (Liss et al; 1999), but their physiological roles remains incompletely understood. In the cardiovascular system, K⁺ATP channels play a protective role in metabolic stress, such as ischemia and hypoxia, which decreases the intracellular ATP concentration (Fujita and Kurachi, 2000; Lang and light; 2010). In heart, K⁺ATP channels are involved in the shortening of action potential duration and the loss of cellular K⁺, which occur during metabolic inhibition (Terzic et al; 1995; Nakaya; 2014). In the vascular system, K⁺ATP channels are thought to regulate tonus of smooth muscles (Suzuki et al; 2001; Teramoto; 2006). These beneficial effects in the cardiovascular system are exemplified by the clinical use of Nicorandil to treat stable angina (Siama et al; 2013).

2.2.1 Glucose Sensing and Insulin Secretion

The best-described role of K⁺ATP channels is by far in insulin secretion, where they are targets of drugs acting at the SUR subunit. K⁺ATP channels serve as glucose sensors and effectively initiate the glucose stimulated insulin secretion (GSIS) pathway figure 4, (Minami et al; 2004; Koster et al; 2005; Nichols et al; 2007; Sanchez-Duarte et al; 2012). Glucose enters β-cells via the glucose transporter GLUT2 and is broken down through cytoplasmic glycolytic pathways. Pyruvate then proceeds to the citric acid cycle, leading to the generation of ATP and fall of the concentration of ADP, a change that in turn deactivates K⁺ATP channels. As K⁺ATP channels close,
the cell membrane tends to depolarize, leading to opening of voltage gated calcium channels, influx of calcium, and Ca\(^{2+}\)-dependent secretion of insulin granules (Dunne et al; 2004; Minami et al; 2004; McTaggart et al; 2010). Hence, pancreatic K\(_{\text{ATP}}\) channels are biological sensors of blood sugars, linking glucose levels to insulin secretion by modulating membrane excitability.

![Figure 4: The glucose stimulated insulin secretion (GSIS) pathway in the pancreatic β-cell. An increase in the intracellular ratio of (ATP) to (ADP) will inhibit K\(_{\text{ATP}}\) channels, leading to membrane depolarization. The increase in membrane potential activates voltage dependent calcium channels (VDCC) and the resulting influx of Ca\(^{2+}\) triggers insulin secretion. (From: Alexandro et al; 2009).](image)

**2.3 Pharmacological Modulation of K\(_{\text{ATP}}\) Channels**

Modulation of K\(_{\text{ATP}}\) channel activity is an important therapeutic target in the treatment of type-2 diabetes and the resulting cardiovascular complications, but also, independently of diabetes, to treat cardiovascular dysfunction. Drugs have been developed that more or less selectively inhibit K\(_{\text{ATP}}\) channels in pancreatic β-cells, or activate K\(_{\text{ATP}}\) channels in vascular smooth muscle cells and cardiomyocytes. Thus the K\(_{\text{ATP}}\) channels can be pharmacologically modulated by a family of structurally diverse agents collectively known as potassium channel openers or potassium channel blockers (Gribble and Riemann; 2002). The potassium channel openers are thought to act by interaction and/or active competition at regulatory ATP binding sites within a conserved amino acid motif of the K\(_{\text{ATP}}\) channel, thus effecting a conformational stabilization (Garlid et al; 1997; Sato et al; 2000). In contrast, potassium channel blockers act in a way which results in inhibition of the conformational stabilization of the channel. In general, most potassium channel openers are non-selective and induce channel activation with potency that may vary quite dramatically, mainly as a consequence of the characteristics of their binding to the regulatory SUR subunits of the K\(_{\text{ATP}}\) channels (Gribble and Riemann; 2002).
2.3.1 $K_{ATP}$ channel activators

$K_{ATP}$ channel openers (KCOs), such as nicorandil, diazoxide, cromakalim and pinacidil are a group of compounds that vary a lot in their chemical structure, and which activate $K_{ATP}$ channels via binding to the SUR subunit (Ashcroft and Gribble, 2000a; Tricarico et al; 2012; Virgili et al; 2013). Nicorandil has been used as an anti-anginal drug for many years, and achieves a better overall outcome of stable angina by decreasing major coronary events (IONA Study Group, 2002). Different SUR subunits display distinct sensitivities to KCOs. For example, diazoxide stimulates all $K_{ATP}$ channel containing SUR1, SUR2A (in the presence of Mg$^{2+}$ADP), and SUR2B. However, Kir6.2/SUR2A channels are only weakly activated by diazoxide in the absence of ADP (D'Hahan et al., 1999; Lee et al; 2008). Pinacidil and cromakalim preferentially activate SUR2A and SUR2B, instead of SUR1 (Seino and Miki, 2003). A number of studies have shown that KCOs such as pinacidil stimulate ATP hydrolysis at nucleotide binding domain 2 (NBD2) and increase channel opening by stabilizing channels in a Mg$^{2+}$-nucleotide bound state (Bienengraeber et al., 2000; Zingman et al., 2001). This activity gains more importance when dealing with a channel comprising Kir6.1/SUR2B (Satoh et al., 1998). Therefore, the stimulatory effect of KCOs depends on the interaction with SUR subunit as well as the cellular nucleotide level (Mannhold et al; 2004).

Below (figure 5) are some selected $K_{ATP}$ channel donor and their chemical structures.

![Chemical structure of some selected potassium ATP-sensitive K$^+$ channel openers](From Mannhold; 2004).
The selectivity of channel openers and blockers for membrane- or mitoK\textsubscript{ATP} is primarily dependent upon the “intrinsic” SUR phenotype (SUR1 vs SUR 2) and is also controlled by the SUR subtype sensitivity to the specific compound (Yamada, 2010; Sato et al; 2000). Pinacidil, cromakalim, nicorandil, and related analogues are potassium channel openers that have been shown to act preferentially on SUR2A/B subunit-K\textsubscript{ATP} channels and have relatively little effect on SUR1 subunit-containing K\textsubscript{ATP} channels.

2.3.2 K\textsubscript{ATP} channel inhibitors

Sulfonylureas, such as tolbutamide and glibenclamide (glyburide), have been widely used in the management of type II diabetes (Babenko et al; 1999; Proks et al; 2002). Thus, tolbutamide and other first generation sulfonylurea such as gliclazide block channels containing SUR1 (β-cell type), but not SUR2 (cardiac, smooth muscle types), whereas glibenclamide, glimepiride, repaglinide, and meglitinide block channels that are made with either regulatory SUR subunit. Their activity is essentially voltage-regulate, but is also dependent on the concentration of intracellular nucleotides, especially ATP (which inhibits channel activity) and Mg\textsuperscript{2+}ADP (which stimulates channel activity). Consequently, the K\textsubscript{ATP} channel activity is regulated by cell metabolism and provides a means of linking the electrical activity of the cell to its energy and metabolic rate (Ashcroft and Gribble; 2000a; Mikhailov et al; 2001; Quan et al; 2011).

The therapeutic effect of potassium channel blockers used in the clinic is due to stimulation of insulin release by functional blockade of pancreatic β-cell K\textsubscript{ATP} channels. Sulfonylurea binds to the transmembrane domain 2 (TMD2) of the SUR subunit, especially the region between transmembrane segment (TM) 15 and 16 (Ashfield et al., 1999; Hosy and Vivaudou; 2014). Ser1237, which is located in the intracellular loop between TM15 and TM16, is a critical site for tolbutamide, responsible for high-affinity inhibition. Glibenclamide has both sulfonylurea and benzamido groups. It binds to SUR1 at two regions: a sulfonylurea binding site that is the same as the one where tolbutamide binds and at an additional benzoamido binding site (Ashcroft and Gribble, 2000b; Moreau et al; 2000; Loffler-walz et al; 2002). SUR2 lacks the sulfonylurea binding site; however, tolbutamide still inhibits SUR2B-containing channels (Isomoto et al., 1996; Babenko et al; 1999), suggesting that the C-terminal 42 amino acids of SUR2B comprise additional sulfonylurea-binding regions. The best-studied potassium channel blocker is glibenclamide (glyburide), which binds at the interface between a high-affinity site localized on the SUR subunit and a low affinity site on the adjacent Kir subunit of the K\textsubscript{ATP} channel. Glibenclamide is a K\textsubscript{ATP} channel blocker that is thought to act non-selectively on both sarcolemmal and mitochondrial K\textsubscript{ATP} channels (mitoK\textsubscript{ATP}).
Since sulfonylurea has some nonspecific effects (Cocks et al., 1990), efforts have been underway to develop another type of inhibitor, with emphasis on molecules blocking the pore region of Kir6 subunit. One example is PNU-37883A, which displays more potent inhibitory effect on channels that are made up from Kir6.1/SUR2B than those that are formed by Kir6.2/SUR2B subunits (Kovalev et al., 2004).

An additional level of complexity is contributed by the variable ability of these compounds to modulate the activity of mitochondrial channels, membrane channels or both. In vitro studies have shown that the mitoK$_{\text{ATP}}$ channels can be selectively blocked by 5-hydroxy decanoate (Garlid et al; 1997). Garlid et al. (2000) and Lee et al, (2008) have shown that KR-31762, a newly synthesized sarcK$_{\text{ATP}}$ channel opener, exerts potent cardioprotective activity through opening of sarcK$_{\text{ATP}}$ in cardiac myocytes but displays much weaker vasodilating activity, compared with BM-180448 (a putative cardio selective mitoK$_{\text{ATP}}$ opener). MitoK$_{\text{ATP}}$ channels can be selectively opened with diazoxide (7-chloro-3-methyl-1, 2,4-benzothiadiazine-1,1-dioxide), a non diuretic benzothiadiazine analogue shown to be 2,000-fold more selective for cardiac mitoK$_{\text{ATP}}$ channels as compared with cardiac sarcK$_{\text{ATP}}$ channels (Garlid et al; 2001). Although progress has been made in delineating the mechanisms involved in mitoKATP-mediated protection, the absence of a clear molecular definition of the protein has hampered structure-function studies of this intracellular channel. Nevertheless, the recent development of novel, more specific, mitochondrial K channel openers and inhibitors lends support to the general paradigm that increased mitochondrial K influx contributes to the cell’s defense against ischemic injury (O’Rourke; 2004).

Below is general formula of sulfonylurea and few selected sulfonylureas.

![General Formula of Sulfonylurea](image)

Chlorpropamide  Glibenclamide  Tolbutamide
Figure 6: Chemical structure of some selected sulfonylurea's Compound, the general formula above show sulfonylurea backbone itself in red and the side chains that distinguish each compound in blue.

3 C-type Natriuretic Peptide (CNP)

CNP is a hormone that is highly expressed by the vascular endothelium, with a demonstrated hyperpolarizing effect on vascular smooth muscle cells (Baliga et al., 2012; Izumiya et al; 2012). CNP is the third member of the natriuretic peptide family and consists of 22 amino acid residues. It contains, similarly to atrial natriuretic peptide ANP and BNP, a 17 amino-acid disulfide ring structure. It is highly conserved among species and is a known regulator of vascular tone and blood pressure (Simon et al; 2008). The current thinking agrees that the hyperpolarization is calcium-dependent and is mediated by signaling through NPR-B, while CNP’s overall actions involve also rises in intracellular cGMP (through NPR-B), activation of BK$_{ca}$ channels. (Simon et al., 2008; Baliga et al., 2012; Margulies and Burnett, 2006; Nishikimi et al., 2011) and of the NPR-C « clearance » receptor. The involvement and requirement of these receptors in the diverse functions of CNP has only recently started to be unravelled (Baliga et al., 2012).

It is now established that the CNP-induced hyperpolarization of rat mesenteric arteries depends upon the activation of inwardly-rectifying potassium channels and is mediated exclusively by NPR-C and the β-γ subunits of the Gi-protein (Simon et al; 2008). CNP is widely expressed in various tissues, among which the vascular system, where it is produced and secreted by the endothelial cells (Izimiya et al; 2012; Baliga et al., 2012; Moyes et al., 2014) and where it is thought to act in a paracrine manner. It has been recently reported that CNP regulates differentially endothelial and smooth muscle growth, by increasing cell proliferation of the former
and inhibiting growth of the latter (Khambata et al., 2011). It is also capable of influencing vascular tone and flow, especially in resistance arteries (Villar et al., 2007; Lumsden et al., 2010), and has been shown that it can modulate vessel wall remodeling in several disease models (Itoh et al., 2004; Moyes et al., 2014). Last, CNP can display anti-inflammatory effects (Lumsden et al., 2010; Itoh et al., 2004; Moyes et al., 2014). Due to its characteristic actions in resistance vessels, CNP has been proposed to be in large part responsible for the flow and tone effects of the hypothetical, and still elusive, smooth muscle-relaxing factor, referred to as Endothelium-Derived Hyperpolarizing Factor (EDHF) (Chauhan et al., 2003; Villar et al., 2007). EDHF is distinct from two other endothelium-released relaxing molecules, namely nitric oxide (NO) and prostacyclin, and for this reason it is referred as “non-NO, non-prostanoid EDHF”. CNP has been suggested to partly account for the endothelial-dependent relaxation in certain vascular beds (Simon et al., 2008), where in addition to releasing NO, endothelial cells can alter vascular tone by modulating ion channel activity. Also, the hypothetical “EDHF” is associated with endothelial hyperpolarization (autocrine effect) and this hyperpolarizing effect of CNP is directly linked to its established ability to stimulate $K_{ATP}$ channels (Burley et al., 2014).

Despite the well-characterized and studied pharmacodynamic effects of exogenous CNP, until very recently the cardiovascular effects of the CNP that is produced endogenously remained in large part unexplored. Thanks to the development and use of genetically modified mouse models, we now know that endogenous CNP plays a significant role in heart and vascular homeostasis. A recent report, for example, has shown that deletion of CNP expression specifically in the vascular endothelium leads to increased atherosclerosis, increased incidence of arterial aneurysms and elevated inflammatory responses (Moyes et al., 2014). These effects are in overall agreement with the ability of exogenous CNP, given by systemic infusion, to prevent intimal thickening in experimental pulmonary hypertension in vivo (Itoh et al., 2004) and the reported inhibition of vascular smooth muscle proliferation in vitro (Khambata et al., 2011). Taken together, these observations suggest that CNP has, potentially, a strong vasculoprotective role. Whether CNP can affect angiogenesis is not explored in any detail, and is only briefly mentioned in a report by Yahamara et al (Yahamara et al., 2003), where exogenous CNP has been noted to improve hindlimb ischemia (Yamahara et al., 2003). This effect correlated with the reported endothelial effects of CNP in vitro via the NPR-C receptor (also referred as “natriuretic peptide clearance receptor”; Kambata et al., 2011). However, whether CNP induces these responses via $K_{ATP}$ channels and how important angiogenesis-related processes are affected by CNP (e.g. migratory behavior and capillary-like formation) has not been investigated before.
4. HYDROGEN SULPHIDE (H₂S)

H₂S, a gasotransmitter molecule that is produced endogenously, shares critical features with CNP: it can also stimulate K<sub>ATP</sub> channels, induce vasodilatation, protect the heart from ischemic damage and attenuate the damage inflicted to the arteries by inflammation (reviewed in Elsey et al., 2010; Szabo 2007; Wang; 2012). H₂S synergizes with NO by potentiating cGMP responses, via inhibition of the activity of phosphodiesterase 5 (PDE5). It can also, though poorly understood molecular mechanisms, induce K<sub>ATP</sub> channel opening in the plasma membrane and in mitochondria (Papapetropoulos et al., 2009). Last but not least, H₂S can induce bona fide angiogenic effects (Papapetropoulos et al., 2009), which depend on K<sub>ATP</sub> channel activation. However, the involvement of specific Kir K<sub>ATP</sub> channel subunits in the endothelial effects of either endogenous vasoactive molecule, CNP or H₂S, is currently unknown.
QUESTIONS

The intriguing similarities between CNP and H₂S, especially the ability to concommittantly activate $K_{ATP}$ channels and induce endothelial responses, prompted us to ask the following questions:

1) Is *direct* pharmacological modulation of $K_{ATP}$ channels capable of affecting angiogenesis?

2) Can C-type Natriuretic Peptide (CNP) trigger *bona fide*, endothelial-based angiogenic responses *in vitro* and *in vivo*?

3) Assuming that CNP does indeed induce endothelial mobilization, do these responses require $K_{ATP}$ channel activation?

4) Which kir $K_{ATP}$ channel subunit underpins these responses?

AIM OF THE STUDY

We undertook this doctoral work in an attempt to test whether modulation of the $K_{ATP}$ channel is a common, until now unexplored, mechanism of angiogenesis and thus could be a target of pharmacological efforts to regulate angiogenesis.
MATERIALS AND METHODS

1. IN VIVO CHICKEN CHORIOALLANTOIC MEMBRANE ASSAY (CAM)

The chicken chorioallantoic membrane (CAM) assay has been a model for studying neovascularization (Folkman et al; 1971; 1985). The assay has been developed over a decade with little variations. Folkman et al have detected the proteins and unraveled the processes that regulate angiogenesis. Meanwhile, a new generation of angiogenesis research has emerged widening the field into new areas.

The CAM assay was initially used in the study of embryonic development and later modified to study tumor angiogenesis (Folkman; 1985). The assay works on the same principle that a putative stimulator is placed in a site where subsequent blood vessel growth can be easily quantified. For initial widespread screening, the CAM assay was probably more appropriate than either rats or rabbits in vivo plug assays. The CAM assay involves incubation of fertilized chicken eggs for 9 days during which the CAM is developing. The assay was performed by implanting an O-ring (rubber) on the membrane with the molecule or compound of interest placed on the chicken chorioallantoic membrane (CAM) inside the O-ring for either 24 - 48h. Finally, the CAM was fixed, removed from the egg and analyzed, as previously reported by: (Folkman; 1985; Papadimitriou et al; 2001; Papapetropoulos et al; 2009).

Materials:

- Eggs (for all CAM experiments leghorn fertilized eggs were purchased from Pindos (Iperos, Greece).
- An Incubator with temperature maintained at 37°C.
- Adhesive sole tape/or parafilm
- Sterile syringes and needles (25/26 hypodermic gauge needles)
- Fine forceps and scissors
- Micro-pipettes and tips.
- Phosphate buffered saline and 70% ethanol.
• Beakers and glass slides.

The items listed above were kept sterile at room temperature or disinfected with 70% ethanol before use.

Methods

• Day 0 Fertilized leghorn eggs were clean, arranged on crate and left overnight at room temperature.

• Day 1 Cleaned fertilized leghorn eggs were placed in an incubator at which temperature was maintained at 37°C.

• Day 3 Eggs were taken from the incubator. A small opening was created and 5ml of albumin was removed from each egg to create more space.

• Day 4 A window/opening of about 2×2cm² was created on each egg and covered with adhesive tape/or parafilm. The windows on the CAM were enlarged with a forceps. The egg shell was carefully removed. The eggs were then returned to the incubator at 37°C and 95% humidity for the next 5 days.

Note: Care should be taken when windows were open to avoid the fall of egg shell parts inside the opening. Fallen shells should be carefully removed with fine forceps; fallen egg dust/shells could cause inflammations and could lead to false result.

• Day 9 Eggs were checked for mortality. 8-10 eggs were used per experimental group and at least 30-35 eggs per data point. The experiment was repeated three times The dead embryos were separated before addition of the test substance, to restricted area on the CAM and subsequent treatment.

Note: When introducing the O-ring to the CAM, large vessels were avoided, as this will affect subsequent analysis.

Preparation of agents/ molecules

2-Nicotinamidoethyl acetate (SG-209): 0.1, 1 and 10 nmoles were prepared from 10 mM stock solution and 40 µl final volume applied on each CAM.

All other tested agents were prepared to the indicated concentrations and 40 µl applied on the CAM for 48 hours.

Treatment of the CAM

The test substances used to treat CAM were as follows:
• 2-Nicotinamidoethyl acetate (SG-209) stock solution: 10 mM in DMSO
• Glibenclamide stock solution: 100 µM in DMSO
• Tolbutamide stock solution: 100 mM in DMSO
• 5-Hydroxydecanoate (5-HD): 100 mM in distilled water
• C-type natriuretic peptide (CNP): 1 mM in distilled water.
• Vascular endothelial growth factor (VEGF): 50µg/ml stock solution

Fixation of the CAM

CAM was fixed using carson’s fixative (Buffered formalin solution) containing:

1. NaH$_2$PO$_4$.2H$_2$O = 18.2 g/l
2. NaOH= 4.2 g/l

Both were dissolved in distilled H$_2$O with gentle agitation on rotary and 200 ml of formalin was added to make it 1 liter. Approximately about 5 ml was used on CAM.

Excision of CAM membrane

In each experiment the CAMs were excised a day after fixation. The CAM was excised as close as possible to the shell with fine scissors and forceps and rinsed in a plasticware containing distilled water, to remove excess debris if any, or apply gentle suction with tissue paper to remove the excess fluids. CAMs were then placed on clean labeled glass slides to dry overnight before a photograph was taken. The photograph of the CAM, covering most areas of O-ring, was taken with a Canon camera mounted on a Zeiss stereoscope and transferred to a computer; the analysis was made using Photoshop and scion image software programs.

2. CELL CULTURE

Human umbilical vein endothelial cells (HUVECs):

Human umbilical vein endothelial cells were isolated from cords collected from university hospital in Patras. Isolation of HUVECs was performed as described by Baudin et al; 2007.

The reagents and umbilical cords issued for research purposes, were collected from the Hospital after the appropriate inquiry was made.

Equipment
● Laminar flow (hood) for cell culture equipped with UV light
● Water-bath with temperature control
● Centrifuge
● Cell incubator with temperature maintained at 37°C and 5% CO₂.
● A phase-contrast optical microscope
● Sterilized equipment such as scissors, clamp clips, forceps, scalpel blade, sterilized syringes, safety glasses and gloves.

**Reagent set up**

These include the buffers for collection of the cords:

1×PBS containing 1 ml of 1 million unit Penicillin G (Peni G, Panpharm) and 1 ml of 1 million unit Colistine (Colimycine, Aventis) was prepared. for conservation and transportation of cords from hospital to the laboratory

Collagenase solution (0.5 mg/ml): Dissolve 0.5 g of collagenase from *Clostridium histolyticum* (Roche, cat. no. 103586) in 100 ml of 1×PBS and gently stirred for 10 min at laboratory temperature (20°C) and in the dark (cover with an aluminum foil); the pH was adjust to 7.4 with 1 N NaOH; filtered through 0.22 mm pore size filter; fractionate in 500µl aliquots and froze at -20°C until used.

**3. HUVECs isolation procedure**

**Preparation of medium and buffers**

M199 medium used containing 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin, 2.5 μg/ml amphotericin B, 5 U/ml sodium heparin and collagenase (500 µl) per ml of M199.

0 % ethanol was spread onto the working hood area. Inside the hood, the cord was removed from the container and washed with 1×PBS to remove the excess amount of blood and other debris. Hematic or damaged cords were discarded.

1. The umbilical vein was identified at one end of the cord. The umbilical vein is the widest vessel and must not be confused with the two arteries. Next using a 20 ml syringe the entire vein was flushed with 30-50ml of 1×PBS to clear the excess blood until the effluent buffer
was transparent or slightly pink to make sure that red blood cells were removed from the vein.

2. 0.5% of collagenase solution contained in M199 medium was inserted from one extremity of the vein with 20 ml syringe, until it leaked out from other extremity, the ends were tightly clamped immediately with the surgical clamp, the cord was placed into a sterile container and incubated in a water-bath for 20 minutes.

**Note:** Avoid over distension of the cord with collagenase solution to avoid excess fibrin/debris in cell culture. Also it is preferred to isolate cells from more than one donors each time. These cells should be pooled together after the first split. These decreases the differences between different donors.

3. While incubated in water bath, 100 mm Petri dish was coated with 1% gelatin and labeled zero passage (P₀) and was incubated at 37°C for at least 30 minutes to polymerize.

4. Under the hood and after incubation the cord was gently squeezed to facilitate cells detachment.

5. Sterile 50 ml tube containing 10 ml of “complete M199 medium” was used to collect the cells, the vein was flushed with 30 - 40 ml of 1×PBS and care was taken not to lose cells suspension during manipulation.

6. The closed tubes were centrifuged at 1200 rpm for 10 minutes.

7. Carefully, the supernatant was discarded and the cells pellet was re-suspended in 10 ml of “complete M199 medium”;

8. The cells were placed on the gelatinized culture plate and incubated at 37°C in, 5% CO₂ atmosphere saturated with H₂O.

9. A day after isolation, cells were checked for contamination if any and blood clots or debris. Non-adherent cells were removed by washing with 1×PBS. Fresh 10 ml “complete M199 culture medium” was added after the replacement of the old one. The medium should be changed every 2-3 days, until cells were 80-90% confluent.

10. To harvest or subculture HUVECs, cells were washed with 1×PBS; 0.25% trypsin containing 0.02% EDTA was added and the cells were incubated at 37°C for 5-6 minutes. The trypsin activity was inhibited by harvesting the cells in complete medium contained calcium and proteins in 15% fetal bovine serum (FBS).
4. Murine brain endothelial cell line (bEnd.3)

The bEnd.3 immortalized mouse brain microvascular endothelial cell line was maintained in Dulbecco’s modified eagle’s medium (DMEM) supplied with 10% fetal bovine serum (FBS), 4.5g/L glucose (5.5 mM), 2 mM glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were seeded in 100mm dish, and incubated at 37°C, 5% CO₂, humidified chamber as described previously (Sheibani and Frazier; 1995; Coleta et al; 2012).

5. Cell splitting / trypsinization

The procedure for cells splitting is as follows:

1. The old medium was removed from the cell culture plate/ or flask by gentle aspiration with a sterilized pasteur pipette
2. Cells were washed with 10 ml of 1×PBS
3. 1.5 ml of 0.1% trypsin was added (for 100 mm dish cell culture plate) enough to cover the surface.
4. Cells were incubated for 5-6 minutes (time depending on the cell line).
5. Cell detachment was observed under the microscope and 5 ml of complete medium was added to inactivate the trypsin.
6. The cells were harvested in 15 ml falcon tube and centrifuged at 2800xg relative centrifuge force (RCF) for 5 minutes.
7. The supernatant was removed carefully, trying not to dislodge cell pellet. The cells were re-suspended in complete medium and placed in incubator at 37ºC until confluency was reached.

6. Cell freezing

Cells were frozen following trypsinization as follows:

1. Cells were counted using a hemacytometer. and they were diluted to a concentration of 2 million cells per ml of freezing medium (the freezing media contained DMSO in 1:10 dilution and FBS or medium).
2. Cells were aliquoted 1 ml/cryo-vial. On each vial date, name and passage number was written.

3. The cryo-vials containing cells were placed in a freezing container (Mr frosty) and taken directly to -80°C for freezing. Sometimes cells were taken immediately to -20°C for one hour, followed by transfer at -80°C overnight before long storage in liquid nitrogen.

4. Cells after 24 hours at -80°C were stored in liquid nitrogen.

5. Few days after the freezing procedure, cells were thawed and tested for viability.

7. Cell thawing

Cells were thawed from -80°C or from liquid nitrogen, using the following procedure:

2. Cells thawed from -80°C or from liquid nitrogen for cell culture.

3. The vial was rapidly thawed in 37°C, in a water bath, until only a small ice pellet remains. The vial was sprayed with 70% ethanol, wiped, and placed inside the hood. The content of vial (~ 1 ml) was placed in 15 ml falcon tube.

4. Slowly, 4 ml of culture medium was added at a rate of about 1 drop every 10 seconds, swirled occasionally, and another 5 ml of culture medium was added.

5. The cells were spin down at (~2000 rpm) for 5 minutes to remove dimethylsulfoxide (DMSO) and medium.

6. Cells were re-suspended in fresh pre-warmed culture medium, containing FBS, and the Petri dish was incubated at 37°C. The medium was changed every 2-3 days until 80-90% confluence was reached.

8. Cell counting

Cells were counted using Neubauer haemocytometer as described (Papadimitriou et al; 2001). A hemocytometer is an etched glass chamber with raised and hold a quartz cover slips exactly 0.1mm above the chamber floor.

Cell suspensions were diluted enough, so that the cells do not overlap each other on the grid, and uniformly distributed as it was assumed that the total volume in the chamber represents a
random sampleCells suspensions were thoroughly mixed before the sample was taken for counting. The protocol is as follows:

1. The hemocytometer was prepared; the mirror-like polished surface was carefully cleaned with 70% ethanol and dried out. The cover slip was cleaned and placed over the counting surface prior to addition of cell suspension.

2. The cells were re-suspended and diluted enough to avoid cells overlapping. Enough liquid was introduced so that the mirror surface was just covered.

3. The counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power.

4. The hemacytometer was filled by capillary action. The pipette filled with cells was placed at notch edge of the hemacytometer. Don't over or under fill the chamber.

5. The cells in 4 of the outer "large" squares were counted

6. Add these counts together and divide by 4 to obtain the average.

7. The cell concentration was then calculated as follows:

   Cell concentration (in cells/ml) = average count x 2 x 10,000 x dilution factor of original cells.

Figure 7I: Hemocytometer

A. Hemocytometer with surface up prepared for sample introduction.

9 Cell proliferation assay as determined using MTT method

Materials required
● Micro-plate Reader with a 450 - 550 nm filter
● 96 well micro-plate, 24-well plate sterilized clear plate for cell assay
● Multi-channel pipette (8 or 12 channel: 10-100 μl)
● Pipette tips for 10-100 μl
● CO2 incubator
● Hemocytometer or cell counter
● Centrifuge and rotary for a 15 ml centrifuge tube

Reagents
● Isopropanol
● Concentrated hydrochloric acid
● Cell culture media
● Substances/or drug to be tested
● Phosphate buffered saline (PBS)
● MTT solution (3-(4, 5-Dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide powder) was prepared 5 mg/ml in 1×PBS, wrapped with aluminum foil and stored at 4°C until use.

Procedure
Endothelial cells were cultured in 100 mm petri dish until 80-90% confluence was reached. Cells were trypsinized and counted using Neubauer hemocytometer as described; (Papadimitriou et al; 2001; Giannopoulos et al; 2001; Pyrihou et al; 2006).

1. The cells were then seeded in 96-well plate at a density of 8000 cells/well in 100 μl of medium using multi-channel pipette. Cells were incubated for 24 h at 37°C to attach.
2. The cells were then treated with molecules or compounds for 48 h.
3. At the end of incubation time (48 h), the MTT solution was added to each well (To the volume equal 10% of the medium in the well) under the hood.
4. The plate was incubated for 4 h at 37°C.
5. At the end of 4 h the content of the plate was sucked using a sterile Pasteur pipette. A multi-channel pipette was used to add 100 μl of acidified isopropanol (0.33 ml of concentrated HCL was added in 100 ml of isopropanol) to each well to dissolve the purple coloration of formed formazan crystals.
6. The sample’s supernatant optical density was counted within 30 minutes using micro-plate reader at 550nm.

10. Migration assay
Cell migration assays were done as described; (Boyden, 1962; Hatziapostolou et al, 2005; Papapetropoulos et al, 2009).

**Materials**
- 24-well micro-chemotaxis chambers (Costar) with uncoated polycarbonate membranes with 8 μm pores.
- Cotton swap
- Glass slides and cover slips
- Optech microscope

**Reagents**
- 0.2 % bovine serum albumin (BSA)
- 0.1 % toluidine blue
- Saline-buffered formalin (carson's fixative)

**The procedure**
The experimental set up was shown in (figure 8I) below

1. Cells were prepared according to standard cell culture procedure
2. Cells were starved overnight in serum-free medium contained 0.2 % BSA
3. Cells were pre-treated with the appropriate compounds, washed with 1×PBS and harvested using 0.1% trypsin.
4. Cells were re-suspended in serum-free medium containing 0.2 % BSA to an appropriate final cell concentration.
5. Cells were placed in a 24 well plate, and in the thin cell culture inserts in the multi wells cell culture plate to form two compartments.
6. 600 μl of culture medium with or without chemo attractant was added to the lower chamber of each well of the cell culture plate.
7. Cells were counted and 100,000 cells added to the upper chamber though the center of each transwell insert in 100 μl volume, air bubbles were avoided.
8. The cell culture plate was placed in a cell culture incubator at 37°C and 5 % CO₂, for cells to migrate, for 4 h.
9. At the end of incubation time the medium was removed, and cells were fixed using phosphate buffered formalin (carson’s fixative).

10. Culture insert was removed and non-migrated cells were cleaned using cotton swap

11. Cells were stained with 0.1 % toluidine blue. The migrated cells were counted and an average was taken from 8 random fields.

![Figure 8I: Experimental setup for in vitro cell migration assay](image)

A thin cells culture inserts were placed in the well of a multi wells cell culture plate which form a lower and upper chamber with a porous pore size 8.0 µm membranes in-between, which allows the cell to actively migrate from the upper to the lower compartment.

11. **In vitro matrigel tube formation assay**

The in vitro matrigel assays were done as previously described;
(Ribeiro et al; 1995; Maniotis et al ;1999; Shao et al; 2004; Pyriohou et al; 2006).

**Materials**

- 96-well plate and endothelial cells
- Reduced growth factor matrigel
- Cell culture medium and humidified tissue culture incubator, 37°C, 5 % CO₂ atmosphere.
- Automated imager (Camera) and stereoscope.

**The Procedure**

The procedure was carried out under aseptic condition as described below:

The matrigel is stored at -20°C.
1. The matrigel was removed from -20°C and allowed to thaw at 4°C overnight on flat surface on ice, before it is opened.

2. Under laminar flow hood, the matrigel was opened, aliquotted and labeled properly.

3. 45 µl of the reduced growth factor matrigel was added to each well of a 96-well plate.

4. The plate containing the matrigel was left to polymerize for 30 minutes at 37°C and 5 % CO₂ environment.

5. Cells were counted and seeded on the reduced growth factor matrigel at a density of 15,000 cells/well and the tested agents were applied immediately.

6. Cells were incubated for 4-8 hours and the plate was examined for tube like network formations.

7. The formed tubular structures were carefully stained with 0.1 % toluidine blue and pictures were taken with Canon camera mounted on the stereoscope.

12. Cell Transfection with siRNAs

Procedure

The re-suspension of lyophilized siRNA duplex was done in 330 µl of the RNAase free water making a 10 µM stock solution which was aliqouted in 60 µl per vial and stored at –20°C until used. 40 nM from the stock of siRNA specific for kir6.1 or 6.2 were used for the transfections. Briefly, HUVECs were transfected with the appropriate siRNA for 24 hours and 4 hours after the transfection the medium was replaced by fresh full M199 (containing 10% FBS). At the end of incubation time cells were washed twice with 1xPBS, trypsinized and used for the in vitro assays or the cell lysates were collected and used for western blots.

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

mRNA expression was evaluated using Real-Time Quantitative PCR (qRT-PCR). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) and quantified by a NanoDrop 2000 spectrophotometre (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 (K_6.1): FW 5’-CTGGCTGCTCTCGCTATC-3’ and REV 5’-AGAATCAAAACCGTGATGGC-3’; and RPS18: FW 5’-TCGGAAGTGGCCATGA-3’ and REV 5’-GAACCTCCGACTTTCGTC-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 ΔΔCT method (Livak and Schmittgen, 2001).

14. Western blotting

Western blotting identified proteins of interest using specific antibodies. Proteins were separated from one another according to their size by SDS-PAGE. The proteins were transferred in a nitrocellulose poly vinylidene fluoride (PVDF) membrane. The gel was placed next to the membrane and application of an electrical current induced the proteins in the gel to move to the membrane where they adhere. The membrane was a replica of the gel’s protein pattern and was subsequently incubated with an antibody. The process includes sample preparation, gel electrophoresis, transfer from gel to membrane and immunostain of the blots. The procedure was carried out as previously described (Pyriohou et al; 2006; Papapetropoulos et al; 2009).

Sample preparations: Consist of the following five (5) stages:

● Lysis buffers
● Protease and phosphatase inhibitors
● Preparation of lysates from cell culture
● Determination of protein concentration
● Preparation of samples for loading into gels

Preparation of lysis buffer

● 0.605 g of Tris in 40 ml of de-ionized H_2O
● 0.290 g of NaCl.

The solutions were stirred and pH was adjusted to 7.5

Then, the following solutions were added:

● 10 ml of 10% NP-40
● 5 ml (10% Sodium deoxycholate). Stirred, until solution become clear.
• 1 ml of 100 mM EDTA
• 1 ml of 10% SDS
• 1 ml of 100 mM EGTA

• Note: EGTA does not easily dissolve. NaOH solution or pellets required to dissolved it. The lysis buffer was wrapped in an aluminum foil and stored at 4°C.

**Protease and phosphatase inhibitors**

Protease and phosphatase inhibitors were prepared and stored at -20°C, consisted of the following:

• Aprotinin 2 µg/ml
• Leupeptin 5-10 µg/ml
• Pepstatin10µg/ml
• EDTA 5 mM
• EGTA 1 mM
• PMSF 1 mM
• Na Fluoride 5-10 mM
• Na orthovanidate 1 mM

**Preparation of lysates from cell culture**

Cells for western blots were cultured in 60 mm cell culture plate until 80-90% confluence was reached. Cell lysates were prepared and collected as follow:

1. Cells in 60 mm petri dish were placed in the hood; the medium was removed and washed with 1×PBS.

   The PBS was drained and then ice-cold lysis buffer was added (0.5ml per 5x10^6 Cells / 60 mm dish) and allowed to remain on the ice block for 20-30 minutes

2. The adherent cells were scraped off from the dish using cold plastic cell scraper and gently cells suspension was transferred in pre-cooled 1 ml eppendorf tube.

3. The eppendorfs containing cell lysates were placed on a rotor at 4°C overnight.

4. The eppendorfs contain the cell lysates were then stored at -80°C for later use or kept on ice cold for immediate homogenization or immersed in liquid nitrogen for long storage.
Determination of protein concentration

The amount of protein in the sample for western blots was determined using Bradford assay as described; (Bradford; 1976). Bovine serum albumin was serially diluted and used as a standard. The procedure is as follows;

Materials
- Protein samples
- Micro-plate for protein assay
- Eppendorfs tubes
- 10-1000 µl pipettes and tips

Reagents
- Reagent A for protein assay (BioRad)
- Reagent B for protein assay (BioRad)
- Bovine serum albumin (BSA) diluted (10 mg/ml – 0.325 mg/ml).

For Bradford assay 1000 µl of reagent A was added to 20 µl of reagent S to form Å. Then, 25 µl of Å was added to 96-well micro plate contained 5 µl of the respective samples or standard, finally 200 ul of substrate reagent B was added to each well. The plate was covered with aluminum foil and incubated for 15 minutes at room temperature (RT).

The plate contained the samples were immediately taken to micro-plate reader and the absorbance was measured at 550 nm. The standard curve for the protein assay was then calculated.

Sample preparation
Gels were loaded with 30 µg to 40 µg of protein depending on the concentration of the protein in the sample. The procedure is as follows:

When the amount of protein in the sample was determined by Bradford assay, the amount to be loaded per gel was prepared with the standard loading buffer in eppendorf tubes and the protein samples were boiled for 5 – 7 minutes at 95to 100°C, to denature the proteins.

Note: (pro-long boiling avoided, as proteins get destroyed).

Loading buffer: Loading buffers were prepared as 2×, 4× or even 6× depend on how much protein to be loaded. The standard 2× loading buffer preparation is as follows:
- 4 % SDS
- 10 % 2-mercaptoehanol
- 20 % glycerol
- 0.004 % bromophenol blue
- 0.125 M Tris HCl
The pH was adjusted to 6.8.

**Gel electrophoresis - SDS-PAGE (Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis).**

The procedure used to separate proteins according to their molecular weight (Hames and Rickwood; 1998).

**Gel preparation**

Gel was prepared depending on the molecular weight of the protein. The higher the molecular weight of the protein the lower the percentage of the gel. 10 % Separating gel and 5 % stacking gel was generally used and the recipes are shown in the table 3 below.

**Table 3: Gel recipes**

<table>
<thead>
<tr>
<th>Gel</th>
<th>15 ml</th>
<th>Stacking gel</th>
<th>6 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 % Gel</td>
<td>15 ml</td>
<td>5 %</td>
<td>6 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>5 ml</td>
<td>30% Acrylamide</td>
<td>0.95 ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>3.75 ml</td>
<td>0.5M Tris (pH 6.8)</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>De-ionized water</td>
<td>6.5 ml</td>
<td>De-ionized water</td>
<td>2.9 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150 µl</td>
<td>10% SDS</td>
<td>50 µl</td>
</tr>
<tr>
<td>10% AMPS</td>
<td>55 µl</td>
<td>10% AMPS</td>
<td>31.5 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>TEMED</td>
<td>7.5 µl</td>
</tr>
</tbody>
</table>

The gel was run at 100 volts for 20-30 minutes, as the blue dye gets down (into the separating gel), then it was changed at 150 volts and 400 mA for 1 h.

**The recipe for the running buffer**

10x Running Buffer

- 30.3 g (0.25 M) Tris Base
- 144 g (1.92 M) Glycine
- 10 g (1%) SDS or appropriate for concentrated stock

Volume adjusted to 1000 ml with de-ionized H₂O

The pH was adjusted to 8.3

**The recipe for the transfer buffer**

- **10x Transfer buffer**
- 30.3 g Tris Base
- 144 g Glycine
- 20 % Methanol

**Volume adjusted to** 1000 ml with de-ionized H₂O

- **1x Transfer buffer**
- 100 ml 10x stock
- 200 ml (20%) Methanol
- Make it 1 L with 700 ml of de-ionized H₂O

**Optional:** 1 ml (10% SDS) can be added.

The materials for the transfer were pre-wet in transfer buffer and the PVDF was first soaked in methanol for 1 minute then rinsed in 1×transfer buffer. They stacked in the following order: Case clear side > Sponge> Whatman Paper > Membrane > Gel > Sponge > Case (Black side) as shown in (figure 9I) below.

**Figure 9I: Arrangement (Stacking gel) for transfer**

The transfer was run at 200 volts and 400 mA for 2 hours.

After the transfer the set up was detached and the membrane was immediately washed three times (3×) for 5-10 minutes each, in 1×TBST to get rid of methanol.

**Recipe for TBS**

For 10×TBS in 1 litre
- Trizma® base 200 mM (24.22g)
- NaCl 137 mM (80.06g)
- The pH was adjusted to 7.6
- 1×TBST (Tris buffered saline 0,05% T-ween-20)

for 1 liter
- 10×TBS = 100 ml
- De-ionized water = 900 ml
- Tween-20 = 500 µl

**Coomassie Stain Protocol**

Sometimes the membrane was normally stained with coomassie blue, to check the equal loading or the presence of the protein of interest.

**Procedure**

- The gel or membrane was stained with 0.5% (or less) coomassie blue in 10% acetic acid, 30% methanol and 60% de-ionized water on a shaker for a minimum of 20 minutes, enough to visualized the bands.
- The gel or membrane can be de-stained with at least two changes of the solvent until is cleared from coomassie stain. **Coomassie blue recipes (RG-250)**
  - Coomassie brilliant blue 2.5 g (0.5%)
  - Methanol 150 ml (30%)
  - Acetic acid 50 ml (10%)
  - De-ionized water 300 ml
- **De-Staining recipes**
  - Methanol 150 ml (30%)
  - Acetic acid 50 ml (10%)
  - De-ionized water 300 ml

**Blocking of the membrane**

The membranes were normally blocked to prevent non-specific background binding of the primary or secondary antibodies to the membrane (that has high capacity to bind proteins). Two blocking solutions were used, non-fat milk or BSA (Cohn fraction V). Milk is not recommended for studies of phospho-proteins.

**Procedure**

5 g of milk or BSA was weighed per 100 ml of Tris Buffer Saline Tween-20 (TBST) buffer. Mixed well and filtered. Failure to filter sometimes can lead to “spotting” where tiny dark grains could contaminate the blot during development. The membrane was incubated for 1 hour at room
temperature under agitation. The membrane was then washed three times (3X) for 5 - 10 minutes each with 1×TBST

**Primary antibodies and Secondary antibodies used**

The membranes were incubated in 1×TBST at 4°C overnight with primary antibodies. The antibodies used and their dilutions were given in the table (4) below:

### Table 4: List of Primary and secondary antibodies used

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Primary Antibodies</th>
<th>Secondary Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erk1/2</td>
<td>Rabbit polyclonal (1:1000)</td>
<td>Anti-rabbit (1:2500)</td>
</tr>
<tr>
<td>p38</td>
<td>Rabbit polyclonal (1:1000)</td>
<td>Anti-rabbit (1:2500)</td>
</tr>
<tr>
<td>Akt</td>
<td>Rabbit polyclonal (1:1000)</td>
<td>Anti-rabbit (1:2500)</td>
</tr>
<tr>
<td>Kir6.1</td>
<td>Goat polyclonal (1:200)</td>
<td>Donkey anti-goat (1:10000)</td>
</tr>
<tr>
<td>Kir6.2</td>
<td>Goat polyclonal (1:200)</td>
<td>Donkey anti-goat (1:10000)</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Mouse Monoclonal (1:1000)</td>
<td>Anti-mouse (1:2500)</td>
</tr>
</tbody>
</table>

The membrane was washed three times (3×) in 1×TBST while agitating for 5 minutes or more per wash and incubated with specific secondary antibodies at room temperature (RT) for 1 h. The dilution depends on the antibodies used.

**Method of Development**

The bands were detected using commercially available detection kits from Thermo Scientific. Enhanced chemiluminescent (ECL) for detection of horseradish peroxidase (HRP) enzyme activity-conjugated antibodies was used. It consists of two (2) kits; they were mixed in 1:1 dilution at a proper amount enough to cover the entire membrane and incubated for 5 minutes at RT before they were developed in dark room. Ordinary X-ray films (Agfa) were used as the films and the exposure time varies; depend on the protein of interest.

**Note:** An over-exposed film is not suitable for analysis as determination of the relative amount of protein is not possible. Overexposed films show totally black bands with no contrast or numerous non-specific bands.

**Developer:**

For ½ liter: 80 ml of the developer in 400 ml of de-ionized water was used.

**Fixer:**
For ½ liter: 100 ml of the fixer in 400 ml of de-ionized water was used.

15. Statistical Analysis

All experiments were performed at least three independent times. Data are expressed as the mean ± SEM of the given number of observations. Results were compared between groups using Student’s t-test using SPSS 10.0 software (IBM, Armonk, NY) under Windows XP. A value of P<0.05 was considered to be significant.
1. In vivo effects of $K_{ATP}$ modulation in CAM

1.1 Can $K_{ATP}$ activators promote angiogenesis in vivo in CAM?

To determine whether direct $K_{ATP}$ activation is able to elicit angiogenic responses, we used one of the best-established in vivo models, the chick chorioallantoic membrane (CAM) assay, which provides in vivo information regarding the angiogenic potential of test compounds. This model allows for application of small molecules or polypeptide factors in a restricted area of the CAM and quantification of the resulting effect, by using software which determines the length of the vascular system and the number of branches within the rubber ring-delineated area.

We first tested a direct channel opener, the Nicorandil derivative SG-209. We chose SG-209 over Nicorandil, because it lacks Nicorandil’s NO-donor properties, which are distinct from $K_{ATP}$ activation (Ishibashi et al., 1991) and which may have confounded the interpretation of the results. As shown in fig. 1, SG-209 was able to stimulate angiogenesis dose-dependently, by upregulating both total vascular length and branching points in the CAM (fig. 1). At 1 nmole/cm², SG-209 increased the length of the vascular network to 129.0±13.6% and the number of branching points to 131.9±14.6% of basal (100%), while at 10 nmole/cm, the respective numbers were 145.1±14.6% and 144.3±16.6% (fig. 1).
Figure 1: Effect of SG-209 on CAM angiogenesis. CAMs were treated with the indicated concentrations of the \( K_{\text{ATP}} \) channel opener SG-209. Vessel length and number of branching points were determined via NIH image analysis software 48 h 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 vs Vehicle; #: p<0.01 vs Vehicle.

To test whether activation of \( K_{\text{ATP}} \) channels indirectly, via receptor-mediated mechanisms, has a similar effect, we decided to use C-type natriuretic peptide (CNP), because CNP is a well-established activator of \( K_{\text{ATP}} \) channels and for this reason has been proposed to account for part or whole of the experimentally-observed activity of Endothelial Derived Hyperpolarizing Factor (EDHF). CNP produced a robust increase in the length of vascular network and the number of branching points, which were equivalent at both 300 and 3000 nmole/cm\(^2\), while it did not elicit a response at 30 nmole/cm\(^2\) (results not shown).

The pro-angiogenic effect of CNP in this system is comparable to that produced by the “archetypal” pro-angiogenic factor VEGF at 500 nmole/cm\(^2\), which increased the length of vascular network and the number of branching points to 135.3±11.4% and 135.5±13.2% of basal, respectively (fig. 2).

![Graph showing CAM angiogenesis](image)

Figure 2: Effect of CNP and VEGF in vivo on CAM angiogenesis. CAMs were treated with the indicated concentrations of either CNP or VEGF. Vessel length and branching point numbers 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, using Student’s t-test.
1.2 Effects of K\textsubscript{ATP} channel inhibition on *basal* CAM angiogenesis

The next step was to test whether inhibition of the K\textsubscript{ATP} results in the opposite effects in the same test model. For this, we initially selected to use one first generation (Tolbutamide) and one second generation (Glibenclamide) sulfonylurea compound, which are both well-characterized K\textsubscript{ATP} inhibitors at the concentrations used. Because these molecules can non-selectively inhibit both membrane and mitochondrial K\textsubscript{ATP} channels, we decided to test in addition a mitochondrial channel-selective inhibitor, 5-HD (5-hydroxy decanoate). The first step was to test these compounds on *basal* CAM angiogenesis, i.e. in the absence of an exogenous angiogenic compound like VEGF or CNP.

Tolbutamide, a member of first generation sulfonylureas, at 10 and 100 nmole/cm\textsuperscript{2} decreased the length vascular network to 76.7±8.6% and 69.0±7.2% and the number of branching points to 67.2±8.3% and 57.5±8.0%, respectively. No significant decrease was observed at 1 nmole/cm\textsuperscript{2} (fig. 3), denoting a concentration-dependent effect.

Figure 3: Effect of the K\textsubscript{ATP} channel inhibitor Tolbutamide (1-100 nmole/cm\textsuperscript{2}) on CAM Angiogenesis. Tolbutamide (1-100 nmole/cm\textsuperscript{2}) was applied on CAMs in vehicle and vessel length and branching point numbers 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, #: non-significantly different from Vehicle, using Student’s t-test.
Glibenclamide, a second-generation sulfonylurea, also dose-dependently (1-100 nmole/cm²) decreased basal vessel formation and branching points in the CAM (fig. 4). Glibenclamide, tested at three different concentrations (1, 10 and 100 nmole/cm²), significantly reduced the length vascular network to 78.8±11.6%, 65.4±11.4% and 60.8±8.7%, respectively and the number of branching points was decreased to 75.9±5.8%, 60.9±10.0% and 53.2±7.8%, respectively.

Figure 4: Effect of the K<sub>ATP</sub> channel inhibitor, Glibenclamide (1-100 nmole/cm²) on CAM angiogenesis. Vessel length and branching point numbers 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 vs Vehicle, **: p<0.01 vs Vehicle

Next, we proceeded to test the effect of the mitochondrial-selective molecule 5-Hydroxydecanoate (5-HD) on the CAM angiogenesis. 5-HD, at 100 and 1000 nmole/cm², reduced the length of vascular network to 76.9±8.4% and 70.5±7.0% of Vehicle Control and the number of branching points to 75.4±12.3% and 60.1±10.2% of Vehicle Control respectively, while no significant effects were observed at 10 nmole/cm² (figure 5).
Figure 5: Effect of the mitochondrial-selective inhibitor 5-HD (10-1000 nmole/cm²) on CAM angiogenesis. 5-HD (10-1000 nmole/cm²) was applied on CAMs in vehicle the vessel length and branching points 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.

1.3 Effects K_{ATP} channel inhibition on polypeptide-induced angiogenesis on CAM

To test whether the angiogenic effects of CNP in the CAM model depend on its reported K_{ATP} activation, we treated CAMs with Glibenclamide prior to the application of CNP. In this series of CAM experiments, at the lowest concentration used (1 nmole/cm², fig 6), Glibenclamide did not significantly affect basal angiogenesis, but was nonetheless able to suppress the angiogenic effects of CNP, indicating that the effects of CNP are sensitive to K_{ATP} blockade (fig 6).
Figure 6: Effect of the K$_{ATP}$ channel inhibitor Glibenclamide on CNP-induced angiogenesis in CAM. CAMs were pretreated with Glibenclamide (1 nmole/cm$^2$) 30 min before application of CNP (300 nmole/cm$^2$) and vessel length and branching point numbers 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 vs Vehicle; #: non-significant vs. Vehicle.

2. *In vitro* effects of pharmacological K$_{ATP}$ modulation

2.1. Does direct K$_{ATP}$ channel modulation regulate for bEnd.3 cell proliferation and migration *in vitro*?

To further explore the link between K$_{ATP}$ activation and triggering of angiogenic responses at the cellular level, we next employed a number of endothelial cell-based *in vitro* assays, since endothelial activation is of paramount importance in angiogenic responses. We used two different channel activators i.e nicotimidoethyl acetate (SG-209) and Nicorandil.

SG-209 (1µM) enhanced cellular growth, determined as cell numbers in the individual wells, by 83.9±11.3%. While at the concentrations used neither channel inhibitor we employed had a significant effect on basal endothelial proliferation, both Glibenclamide and 5-HD effectively abrogated the proliferative response to SG-209 (fig 7).
Figure 7: SG-209 enhances bEnd.3 cell growth in vitro. bEnd.3 cells were maintained in DMEM + 2.5% FBS in 24-well plates and pretreated with either Glibenclamide (10 μM) or with 5-HD (100 μM) for 20 min before addition of SG-209 (1 μM). 48h later, cells were trypsinized and counted using a hemocytometer. Data are expressed as means ± S.E.M, n=5. **: p<0.01 versus vehicle control; #: p<0.05 versus SG-209 alone, using Student’s t-test.

SG-209 also increased another important parameter that controls angiogenesis: cell motility. This was determined by quantitating the transmigration of endothelial cells through a Transwell compartment. SG-209 stimulated migration to 2.5-fold of vehicle control (fig 8). Pretreatment of bEnd.3 cells with either Glibenclamide (10 μM) or with 5-HD (100 μM), while it failed to significantly affect basal responses (fig. 8), abolished the motility effects of SG-209 in the Transwell migration model.

Figure 8: Modulation of bEnd.3 cell migration by K_{ATP} channel openers and inhibitors in vitro. bEnd.3 cells were resuspended in serum-free medium and pre-treated with either Glibenclamide (10 μM) or 5-HD (100 μM) for 20min, before being placed in transwells and allowed to migrate for 4h in response to SG-209 (1µM). Data are expressed as means ± S.E.M, n=5. *: p<0.05 versus Vehicle; #: p<0.05 versus SG-209 alone, using Student’s t-test.

Nicorandil (10 μM) also increased b.End.3 cell growth by 112.2±7.37% (fig 9) and motility by almost 3-fold (fig 10). Nicorandil, in addition to the direct effect on K_{ATP} activation has a nitrate group and thus is a NO donor. Despite this “combined” potential activity, Nicorandil’s effects
were abolished (similar to those of SG-209) by either Glibenclamide (10 µM) or 5-hydroxydecanoate (5-HD 100 µM, fig 9 and fig 10). The increase in bEnd.3 growth was more robust in cells treated with 10 µM Nicorandil than with 1 µM SG-209. This may have been the result of our possible use of a sub-maximal concentration of SG-209 or of the contribution of the NO-releasing effects of Nicorandil in motility. However, such a difference between Nicorandil and SG-209 was not observed in terms of increases in motility.

Figure 9: Nicorandil induces bEnd.3 cell growth in vitro. bEnd.3 cells were maintained in DMEM + 2.5% FBS in 24-well plates and pretreated with either Glibenclamide (10µM) or with 5-HD (100µM) for 20 min before addition of Nicorandil (10 µM). 48h later, cells were trypsinized and counted using a hemocytometer. Data are expressed as means ± S.E.M, n=5. **: p<0.01 versus Vehicle control; #: p<0.05 versus Nicorandil alone, using Student’s t-test.
2.2. Can indirect, receptor-mediated $K_{\text{ATP}}$ activation influence proliferation, migration and capillary-like network formation in vitro?

Subsequently, we also tested whether C-type natriuretic peptide (CNP), a peptide that can induce hyperpolarization, likely as a result of inducing receptor-mediated $K_{\text{ATP}}$ activation, would produce similar effects to those shown by direct $K_{\text{ATP}}$ activators.

CNP at 100 and 1000 pM increased b.End.3 cell proliferation by 20.5± 6.6% and 34.1±6.7%, respectively (fig 12). At 1000 pM, the effect produced by CNP is comparable to that elicited by the “archetypal" angiogenic factor, VEGF, at 500 pM (fig 11).

Figure 11: The indirect $K_{\text{ATP}}$ activator CNP induces bEnd.3 cell proliferation in vitro. bEnd.3 cells in 2.5% FBS were treated with the indicated concentrations of CNP (1-100 pM) or VEGF (500 pM) and allowed to proliferate for 48 hours. Cell proliferation was determined by cell counting with a hemocytometer. n=6. Data are expressed as means ± S.E.M *: p<0.05 versus Vehicle, using Student’s t-test.
CNP (100pM) also increased cell motility resulting in up-regulation of Transwell migration by approximately 2-fold, compared to the vehicle control. This response was abolished by 10 µM Glibenclamide (fig. 12).

Figure 12: The indirect K_{ATP} activator CNP induces bEnd.3 cell migration. bEnd.3 cells in serum-free medium were pretreated with Glibenclamide (10 µM) for 20min, placed in well inserts and then exposed to either CNP (100 pM). Migrated cells were stained and counted 4h later. n=5 for each condition. Data are expressed as means ± S.E.M *: p<0.05 versus Vehicle, #: p<0.05 versus CNP alone, using Student’s t-test.

2.3. Can inhibition of K_{ATP} channels influence the responses of bEnd.3 endothelial cells to the pro-angiogenic growth factor VEGF?

As expected, VEGF (500 pM) also induced increases in bEnd.3 cell growth as well as migration/motility through Transwells. Both of these responses were abolished by either Glibenclamide 10 µM or 5-HD 100 µM (Fig. 13 and 14), indicating a dependence of critical endothelial angiogenesis processes to VEGF on K_{ATP} activation. Of note, the pro-migration effects of CNP using this type of cells are comparable with those of VEGF (compare fig. 12 and 14), indicating, again, that under the experimental conditions we have used thus far, CNP is indeed a bona-fide angiogenic factor.
Figure 13: VEGF-induced bEnd.3 cell growth depends on K\textsubscript{ATP} channels. bEnd.3 cells were pretreated with Glibenclamide (10 µM) or 5-HD (100 µM) before being exposed to VEGF (20 ng/ml). 48h later cells were trysinized and counted using a hemocytometer (n=6). Data are expressed as means ± S.E.M *: P<0.05 vs. vehicle; #: P<0.05 vs. VEGF alone, using Student’s t-test.

Figure 14: VEGF-induced bEnd.3 cell motility/migration depends on K\textsubscript{ATP} channels. bEnd.3 cells were pretreated with Glibenclamide (10 µM, 20min) or 5-HD (100 µM, 20min) and then placed in transwells and allowed to migrate in response to VEGF (20 ng/ml) for 4h, then cells were stained and counted (n=5). Data are expressed as means ± S.E.M *: P<0.05 vs. vehicle; #: P<0.05 vs. VEGF alone, using Student’s t-test.
2.4. Effects of K$_{\text{ATP}}$ channel modulators on endothelial cell network formation \textit{in vitro}

To further assess this dependence of angiogenic responses on K$_{\text{ATP}}$ channel activation, we used a well-characterized \textit{in vitro} angiogenesis assay, namely the ability of endothelial cells to form capillary tube-like structures when placed in a Matrigel preparation. For this, we employed reduced-growth Matrigel, which has been treated to eliminate a proportion of the growth factors it contains, as these would obscure any angiogenic effects of purified test substances. In addition, in order to address any (legitimate) concerns regarding cell type-selectivity of the observed responses, we also decided to incorporate HUVECs in this assay for the sake of comparison and because HUVECs are a “gold standard” type of endothelial cell used in experimentation.

As can be seen in fig. 15 below, the direct K$_{\text{ATP}}$ channel opener SG-209 (1 µM) elicited capillary-like formation in bEnd.3 cells (increase by 72±4.4%). This was effectively blocked by either Glibenclamide (10 µM) or by 5-HD (100 µM). These data demonstrate that direct activation of K$_{\text{ATP}}$ channels can stimulate the requisite endothelial processes that result in the complex formation of a vascular network. Furthermore, they show that the data obtained in the CAM assay were not an indirect result, via, e.g vasodilatation via activity on vascular smooth muscle cells in that \textit{in vivo} model, but indeed can be directly attributed to endothelial actions.

![Figure 15: SG-209 promotes endothelial formation of capillary-like structure in reduced growth Matrigel. bEnd.3 cells were placed in 96-well plates in reduced-growth matrigel and were treated with SG-209 (1 µM) in the presence or absence of Glibenclamide (10 µM) or 5-HD (100 µM) for 24h. The tubular network length was determined from microphotographs using the Scion image software (n=5). Representative photomicrographs of control and SG-209 treated cells (40× magnification). Data are expressed as means ± S.E.M *: P<0.05 vs. vehicle; #: P<0.05 vs. SG-209 alone.](image-url)
When HUVECs were used in this assay, CNP (100 pM) elicited a stimulatory effect (an increase of 131.7±10.9% above basal). This effect was also abrogated by the two K<sub>ATP</sub> blockers (fig. 16), while there was no significant effect of Glibenclamide or 5-HD on basal capillary-like tube formation (fig. 15 and 16). These results demonstrate that CNP can elicit complex cell behavior on primary human endothelial cells (HUVECs) or in a mouse brain-derived endothelial cell line (b.End.3), in a manner that can be pharmacologically modulated by K<sub>ATP</sub> blockers.

Figure 16: CNP promotes endothelial formation of capillary-like structure in reduced growth Matrigel. HUVECs were placed in 96-well plates in reduced-growth Matrigel and were treated with CNP in the presence or absence of Glibenclamide (10 μM) or 5-HD (100 μM) for 24h. The tubular network length was determined from microphotographs using the Scion image software (n=5). Representative photomicrographs of control and CNP treated cells (40× magnification). Data are expressed as means ± S.E.M * : P<0.05 vs. vehicle, # : P<0.05 vs. CNP alone.

3. The role of the K<sub>ATP</sub> channel subunit K<sub>i</sub>6.1

3.1 Knock-down of the K<sub>i</sub>6.1 K<sub>ATP</sub> subunit expression using specific siRNA.

Since the contribution of specific channel subunits in angiogenic responses is virtually unexplored, we decided to further investigate the contribution of the pore-forming K<sub>ATP</sub> K<sub>i</sub>6.1 subunit. To do this, we introduced by transfection a siRNA specific for the human sequence of K<sub>i</sub>6.1 or siRNA vehicle (as a Control), in HUVECs. 24 hours later, analysis of cell lysates by western blotting or by qRT-PCR indicated that the siRNA targeting K<sub>i</sub>6.1 effectively reduced the
mRNA abundance for this subunit by more than 70 % (fig. 17) and down-regulated Kᵦ6.1 protein content by 53% (fig. 18).

Figure 17: Down regulation of the Kᵦ6.1 subunit expression by a Kir 6.1-specific siRNA. HUVECs were transfected with 40nM of siRNA specific for Kir6.1 for 24h. At the end of 24h, mRNAs were collected for qRT-PCR analysis. n=3 independent experiments. Data are expressed as means ± S.E.M **: p<0.01 versus vehicle Control, using Student’s t-test.

Figure 18: Down regulation of the Kᵦ6.1 subunit expression by siRNAs at the protein level. HUVECs were transfected with 40nM of small interfering (si) RNA specific for Kᵦ6.1. After 24h, cell lysates were collected for protein analysis. Cell lysates were analyzed from three independent transfection experiments. Insert is a representative western blot photomicrograph probed with Kᵦ6.1 and β-actin antibodies. n=3 independent experiments. Data are expressed as means ± S.E.M **: p<0.01 versus vehicle Control, using Student’s t-test.
3.2 Is the K\textsubscript{i,6.1} subunit required for HUVEC responses to SG-209 and CNP?

In parallel experiments, we transfected HUVECs with either Control (siRNA vehicle) or siRNA specific for K\textsubscript{i,6.1} for 24h and determined their responses in transwell migration and Matrigel assays. CNP (100pM) or SG-209 (1µM) alone produced 2-fold increases in cell motility (fig. 19). These effects were suppressed in cells transfected with the K\textsubscript{i,6.1} siRNA (fig. 19), indicating a dependence on this subunit.

Figure 19: Requirement of the K\textsubscript{i,6.1} subunit in HUVEC migration in response to SG-209 and CNP. HUVECs were transfected with 40nM siRNA specific for K\textsubscript{i,6.1}. 24h later, cells were trypsinized, placed in transwells and allowed to migrate in the presence or absence of SG-209 (1 µM) or CNP (100 pM) for 4h, after which migrated cells were stained and counted. n = 5 for all group. Data are expressed as means ± S.E.M *: p<0.05 versus Vehicle control, #: p<0.05 versus CNP or SG-209 alone, using Student’s t-test.

Similarly, in the Matrigel assay, tube-like length formation induced by SG-209 (1µM, 60.5±12.5% increase) or CNP (100pM, 58.3±8.2% increase) was reduced to 29.2 % and 28.4 % above Control respectively, in cells transfected with the K\textsubscript{i,6.1}-specific siRNA (Fig. 20). These results strongly suggest that full expression of the K\textsubscript{i,6.1} subunit is required in the production of maximal angiogenic effects by both angiogenic molecules.
Because we wanted to investigate more strictly the involvement and requirement of the Kir6.1 subunit in these responses, we decided to use side-by-side 2 different Kir6.1-specific siRNAs (one from Santa Cruz and one from Ambion), as well as 2 different Control siRNAs (again one from Santa Cruz and one from Ambion). We repeated the experimentation regarding knock-down of Kir6.1 expression at the mRNA level and evaluated their effects on the same functional responses, i.e. migration and Matrigel tube formation.

As can be seen below, neither of the Control siRNAs affected the expression of the Kir6.1 subunit in three different transfection experiments, while both of the Kir6.1 specific siRNAs knocked-down its expression by approximately 60% (fig 21).

In parallel experiments we also probed for potential function impairment of HUVECs transfected with the siRNAs. Globally, the additional experiments confirm that knock-down of Kir6.1 results in impairment of endothelial responses to either the direct K$_{ATP}$ channel activator
SG-209 (figs 22-26) or the indirect activator, CNP, demonstrating beyond doubt the dependence of these angiogenic responses on the adequate expression of this subunit.

Figure 21: Down regulation of the K_{ir}6.1 subunit expression by siRNAs. HUVECs were transfected with 40nM of either CTL siRNA or siRNA specific for Kir6.1 for 24h and 4h after the transfection medium was replaced with full M199. At the end of 24h, cells were washed twice with 1×PBS and RNAs were collected for qPCR analysis. n=3 independent experiments. CTLsi #1 and siKir6.1 #1 denote siRNA reagent pair from Santa Cruz, while CTLsi#2 and siKir6.1#2 denote siRNA reagent pair from Ambion. Each siRNA pair was tested in three independent transfections. Data are expressed as means ± S.E.M, n=3. **: p<0.01 versus the respective vehicle Control (CTL#1 or CTL#2), using Student’s t-test.

Figure 22: Requirement of the K_{ir}6.1 subunit in HUVEC migratory responses to CNP. HUVECs were transfected with either CTL siRNA or siRNA specific for Kir6.1 from Ambion. 24h later, cells were trypsinized, placed in transwells and allowed to migrate in the presence or absence CNP (100 pM) for 4h, after which migrated cells were stained and counted. CTLsi#1 and siKir6.1#1 denote siRNA reagent pair from Ambion. n = 5 for all groups. Data are expressed as means ± S.E.M *: p<0.05 versus. Vehicle control, #: p<0.05 versus CNP alone, using Student’s t-test.
Figure 23: HUVECs require the K\textsubscript{ir}6.1 subunit to form capillary-like structures in responses to CNP in Matrigel. HUVECs were transfected with a siRNA specific for K\textsubscript{ir}6.1. 24h later, they were placed in 96-well plates in reduced-growth Matrigel and treated with CNP (100 pM) for 8 h. Tubular network formation was determined and quantified from microphotographs using the Scion image software. n=5 for all groups. CTLsi #1 and siKir6.1 #1 denote siRNA reagent pair from Ambion. Insert is representative photomicrographs of cells treated with the Ambion siRNAs. (40× magnification). Data are expressed as means ± S.E.M. *: p<0.05 versus. Vehicle control, #: p<0.05 versus CNP alone, using Student’s t-test.

Figure 24: HUVECs require the K\textsubscript{ir}6.1 subunit to form capillary-like structures in responses to SG-209 in Matrigel. HUVECs were transfected with a siRNA specific for K\textsubscript{ir}6.1. 24h later, they were placed in 96-well plates in reduced-growth Matrigel and treated with either SG-209 (1 μM) for 8 h. Tubular network formation was determined and quantified from microphotographs using the Scion image software. n=5 for all groups. CTLsi#1 and siKir6.1#1 denote siRNA reagent pair from Ambion. Data are expressed as means ± S.E.M. *: p<0.05 versus. Vehicle control, #: p<0.05 versus SG-209 alone, using Student’s t-test.
Figure 25: HUVECs require the Kir6.1 subunit to form capillary-like structures in responses to CNP in Matrigel. HUVECs were transfected with a siRNA specific for Kir6.1. 24h later, they were placed in 96-well plates in reduced-growth Matrigel and treated with CNP (100 pM) for 8h. Tubular network formation was determined and quantified from microphotographs using the Scion image software. CTLsi#2 and siKir6.1#2 denote siRNA reagent pair from Santa Cruz. n=5 for all groups. Data are expressed as means ± S.E.M. *: p<0.05 versus. Vehicle control, #: p<0.05 versus CNP alone, using Student’s t-test.

Figure 26: HUVECs require the Kir6.1 subunit to form capillary-like structures in responses to SG-209 in Matrigel. HUVECs were transfected with a siRNA specific for Kir6.1. 24h later, they were placed in 96-well plates in reduced-growth Matrigel and treated with either SG-209 (1 μM) for 8 h. Tubular network formation was determined and quantified from microphotographs using the Scion image software. CTLsi#2 and siKir6.1#2 denote siRNA reagent pair from Santa Cruz. n=5 for all groups. Insert is representative photomicrographs of cells treated (40× magnification). Data are expressed as means ± S.E.M. *: p<0.05 versus. Vehicle control, #: p<0.05 versus SG-209 alone using Student’s t-test.
4 Dependence of the effects of SG-209 on Erk1/2, p38 and Akt kinases

While the various angiogenic molecules may use proximally diverse pathways to initiate angiogenesis, there are common cellular biochemical pathways that are employed by the majority of them and underpin endothelial angiogenic responses. In order to characterize the possible mobilization of such known downstream effectors by $K_{\text{ATP}}$ activation, we focused on the direct activator SG-209. 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), in concentrations that have been shown to inhibit the respective kinases in our laboratory.

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. 27 and fig. 28). This indicates that the angiogenic effects induced by activation of $K_{\text{ATP}}$ channels depend on the activity of this kinase, although whether ERK1/2 lies directly downstream or not is an open question.

Figure 27: In vitro endothelial proliferation in response to SG-209 depends on functional cell kinase Erk1/2. 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. n=6 for all groups. Data are expressed as means ± S.E.M. *: p<0.05 versus Vehicle, #: p<0.05 versus SG-209, using Student’s t-test.
Figure 28: *In vitro* endothelial migration in response to SG-209 depends on functional cell kinase Erk1/2. bEnd.3 cells were serum-starved and pretreated for 20 min with the Erk1/2 inhibitor U0126 (10 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. Data are expressed as means ± S.E.M *: p<0.05 versus Vehicle, #: p<0.05 versus SG-209, using Student’s t-test.

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 as shown in figs. 30 and 29, respectively. In this series of experiments, SG-209 increased motility by 1.5-fold, an effect which was abolished by both pharmacological kinase inhibitors. These results indicate that angiogenic-type responses to direct K$_{ATP}$ activation depend on the function of Akt, p38 and Erk1/2 kinases.

Figure 29: The effects of the K$_{ATP}$ opener SG-209 depend on p38 activation enhanced bEnd.3 cells migration, bEnd.3 cells were serum-starved and pretreated for 20 min with the p38 inhibitor SB230963 (10 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. Data are expressed as means ± S.E.M *: p<0.05 versus Vehicle, #: p<0.05 versus SG-209 alone, using Student’s t-test.
Figure 30: The effects of the K<sub>ATP</sub> opener SG-209 depend on Akt activation enhanced bEnd.3 cells migration. bEnd.3 cells were serum-starved and pretreated for 20 min with 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. Data are expressed as means ± S.E.M *: p<0.05 versus Vehicle, #: p<0.05 versus SG-209 alone, using Student’s t-test.
**DISCUSSION**

$K_{ATP}$ channels are members of a family of membrane-spanning proteins found to be intermediate conduction channels gated by ATP (Caroll et al., 1999). They play an important role in maintenance of cellular integrity and cell function, including smooth muscle contractility, epithelial electrolyte transport, hormonal secretion and neurotransmitter release, among others (Ko et al., 2008). Several hundred genes encoding for K$^+$ channels have been identified and these broadly fall into three families; voltage-gated (including Kv and calcium-activated), inward rectifiers (Kir) and twin pore K$^+$ channels (Ko et al., 2008; Bilman, 2008; Kohler et al., 2010). Whereas the role of Kir channels has been well-described in certain physiological processes, such as insulin secretion by pancreatic β-cells and in vascular smooth muscle hyperpolarization (Baron et al; 1999; Burke et al; 2008; Akrouh et al., 2009), their contribution to endothelial mobilization in angiogenesis has not been explored in any significant depth yet.

Indeed, identification of new, basic molecular mechanisms in angiogenesis, especially if they are amenable to pharmacological targeting, may have significant implications regarding the therapeutic use of molecules that modify these mechanisms. This is desirable in situations where up-regulation of angiogenesis will exhibit a beneficial effect, e.g. in alleviating the sequellae of ischemic heart disease and managing the progress of peripheral arterial disease. In contrast, angiogenesis inhibitors are useful when reduction of ectopic or excessive angiogenic growth is desirable, such as in stemming the growth of solid tumors and in checking diabetic retinopathy (Carmeliet and Jain, 2011; Coultas et al., 2005; Ferrara and Kerbel, 2005).

We were prompted to start the present research work by the intriguing observation that two molecules that possess angiogenic properties, CNP (Yamahara et al., 2003) and H$_2$S (Papapetropoulos et al., 2009) can both elicit $K_{ATP}$ activation, albeit via not well-understood mechanisms. CNP is the third member of the natriuretic peptide family (Baliga et al., 2012; Margulies and Burnett, 2006; Lumsden et al., 2010) that also comprises ANP and BNP. ANP, BNP and CNP are structurally-related peptides in which the bioactive domains reside in the C-terminal region of the propeptides. CNP is widely expressed in many organs, tissues and their constituent cells. CNP has a well-described role (Ramachandran et al., 2011) as an important anabolic regulator of cartilage growth, stimulating chondrocytes to increase collagen and proteoglycan synthesis and proliferate. In the vasculature, CNP is especially highly expressed by the endothelial cells (Baliga et al., 2012; Moyes et al., 2014). Izumiya et al. (2012) have reported that CNP can act as an autocrine/paracrine factor to regulate endothelial cell activities.
and is thought to exert an overall vascular protective role. CNP can fine-tune vascular cell growth, upregulating endothelial proliferation while inhibiting smooth muscle cell growth (Khambata et al., 2011). It can also establish tone and flow in resistance arteries (Lumsden et al., 2010; Villar et al., 2007), can positively influence remodeling of the vessel wall following chronic or acute injury (Itoh et al., 2004; Moyes et al., 2014) and accelerate re-endothelialization in damaged vessels (Komeda and Nakao, 2002; Ohno et al., 2002). An additional important beneficial effect of CNP may be reduction of inflammation (Lumsden et al., 2010; Itoh et al., 2004; Moyes et al., 2014). CNP-triggered vasodilatation has been well-described and is thought to be directly linked to its stimulation of $K_{ATP}$ activity (Burley et al., 2014). Initially, the identification of CNP’s production by endothelial cells, and the characterization of its activity via a guanylyl cyclase-coupled receptor (Sellitti et al., 2011), indicated that endothelial CNP performs some of the same functions as NO in the vasculature. Like NO, endothelial CNP leads to increased cGMP production, through the NPR-B receptor (Sellitti et al., 2011) expressed in the underlying smooth muscle, and consequently to activation of vasoprotective protein kinase G-dependent pathways. This may be an important protective action of CNP in the cardiovascular system, since inhibition of this pathway leads to defective cardiac and vascular remodeling and fibrosis after injury (Sellitti et al., 2011). This concept about CNP’s role has been widely supported by both in vivo and in vitro studies and involves the ability of CNP to inhibit myocyte hypertrophy (heart) or proliferation (smooth muscle) and to limit the synthesis of fibrotic matrix proteins in either cell type after tissue damage. CNP has in addition been found to function as an NPR-C-dependent endothelium-derived hyperpolarizing factor: this distinct activity contributes to CNP’s regulation of local blood flow and systemic blood pressure by hyperpolarizing smooth muscle cells (Rose and Giles; 2008).

Whether, in addition to these vascular effects, CNP can control or modulate angiogenic responses is not known, nor is it understood what is the contribution of $K_{ATP}$ channel activation in CNP’s angiogenic effects, assuming that they can be demonstrated. For all these reasons, we included this peptide in our studies.

There are a number of compounds that interact and modulate the activity of the $K_{ATP}$ channels, which are important pharmacological tools in both the experimental research and in the therapeutic arena. For example, 2-Nicotinamidoethyl acetate (SG-209) is a pyridine derivative in which the nitrate moiety of Nicorandil (also known as SG-75) has been replaced with an acetate residue (Ishibashi et al; 1991) and which retains Nicorandil’s ability to act as a $K_{ATP}$ channel opener. The existence of a N-ethyl-nicotinamide moiety determines not only the
vasodilator potency but also the mechanism of action of this compound: both nicorandil and its congeners, i.e. N-ethylnicotinamide derivatives which have either nitroxyl or acetoxyl groups at C2 of the parent structure of Nicorandil, play a pivotal role in making these compounds act as \( \text{K}_{\text{ATP}} \) channel openers (Satoh et al., 1991; Sakai and Saito; 1998). Consequently, \( \text{K}_{\text{ATP}} \) channel opening by such compounds might play a key role in regulating the electrical status of the endothelial cell membrane and thus significantly affect endothelial functions, although the role of these channels in endothelial cells is incompletely understood. It is well-known that Nicorandil is an orally efficacious anti-anginal drug (Sakai and Saito; 1998), possessing a dual mechanism of action, consisting of both \( \text{K}_{\text{ATP}} \) channel activation and stimulation of guanylate cyclase. The current thinking is that Nicorandil’s therapeutic benefit is linked to activity exerted on smooth muscle cells (Davie and Standen; 1998; Jahangir and Terzic; 2005).

Our results overall have shown that opening of \( \text{K}_{\text{ATP}} \) channels, whether this is operated directly or indirectly, can upregulate basal, ongoing angiogenesis \textit{in vivo} in the CAM model. In contrast, basal angiogenesis or angiogenesis triggered by CNP was reduced by \( \text{K}_{\text{ATP}} \) inhibitors. Together, these results indicate that modulation of the \( \text{K}_{\text{ATP}} \) channel can influence angiogenesis \textit{in vivo}. The lower potency of the mitochondrial \( \text{K}_{\text{ATP}} \) channel-selective molecule, 5-HD, in our hands, compared to the non-selective inhibitors, may be attributable to a number of possible factors: inadequate effects from mitochondrial-selective \( \text{K}_{\text{ATP}} \) inhibition alone, lower affinity in inhibiting \text{mitoK}_{\text{ATP}} or suboptimal tissue and cell penetration of 5-HD. Our current results do not point to any particular possibility.

In interpreting the CAM results, we considered whether vasodilatation, an action shared by SG-209 and CNP (Andrade et al., 2014; O’Rourke, 1996; Ishibashi et al., 1991), could indirectly promote angiogenesis in this model. However, a) increase in CAM angiogenesis is not a general property of vasodilators and b) the wealth of our \textit{in vitro} results unequivocally indicate that SG-209 and CNP act directly on endothelial cells. For these reasons, the CAM effects of both reagents can be attributed to their direct activity on endothelial cells.

CAM responses to CNP were blocked by Glibenclamide, revealing for the first time a critical involvement of \( \text{K}_{\text{ATP}} \) channels in CNP’s endothelial effects, in addition to their well-described involvement in CNP’s smooth muscle 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 cell proliferation (Khambata et al., 2011; Kun et al., 2008; Villar et al., 2007). In the future, specific inhibition of the NPR-C receptor by synthetic molecules (Khambata et al., 2011),
or knockdown via specific siRNAs may elucidate the role of this particular receptor in the angiogenic, endothelial-mediated effects of CNP.

In bEnd.3 cells, growth and migratory responses were both upregulated by direct K\textsubscript{ATP} openers. In these experimental settings, nicotinamido-ethylacetate (SG-209) seemed less effective in promoting proliferation than the parent molecule, Nicorandil, although both compounds influenced cell motility/migration equally. We think that this discrepancy is unlikely due to the additional ability of Nicorandil to “donate” NO, based on our findings that K\textsubscript{ATP} inhibitors reduced equally the effects of both agents. Perhaps this may be related to stability differences that become apparent in the longer (48h) proliferation assay, in contrast to the 4-hour migration assay. In addition to pro-migratory effects, SG-209 could also stimulate the complex processes that result in capillary morphogenesis, and was able to drive network-like formation by endothelial cells in Matrigel \textit{in vitro} (fig. 24). In conclusion, SG-209 mobilizes endothelial cells to acquire a fully angiogenic phenotype.

Endothelial cell proliferation and motility were comparably increased by CNP and VEGF (fig. 20) and were abrogated by K\textsubscript{ATP} inhibition by Glibenclamide and 5-HD (fig. 21). These results establish CNP as a \textit{bona fide} angiogenic polypeptide, with comparable efficiency to VEGF (Papapetropoulos et al., 2009). These effects of both polypeptides are controlled by K\textsubscript{ATP} channel function. Papapetropoulos et al. (2009) attributed part of VEGF’s dependence on K\textsubscript{ATP} channels to synergy with endogenously-produced H\textsubscript{2}S, which, as we know, can trigger K\textsubscript{ATP} activation. It is unknown if such a mechanism is also used by CNP, but future experiments can address this issue, by including inhibitors of enzymatic H\textsubscript{2}S production in the assays, such as amino-oxyacetic acid (AOAA, Asimakopoulou et al., 2013).

The molecular pathway that drives from CNP-receptor stimulation to opening of K\textsubscript{ATP} channels is only partly elucidated. However, data in smooth muscle, cardiac and endothelial cells support a critical role played by 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\textsuperscript{+} channels (Simon et al., 2009). The extent to which cGMP is indeed involved (possibly \textit{via} the NRP-B receptor), the requirement of the NPR-C and the possible contribution of endogenous H\textsubscript{2}S in shaping the angiogenic responses of CNP are all the subject of ongoing investigations in our laboratory that should extend our understanding of the molecular mechanisms via which CNP elicits its angiogenic effects.

Our present observations, which establish unequivocally CNP as an angiogenic molecule, are in agreement with fragmentary evidence offered by Khambata et al. 2011 and Yamahara et al.
2003. However, these and our data are in disagreement with the report by Del Ry et al. (2013), which showed that CNP can inhibit HUVEC responses in Matrigel. The discrepancy is very likely due to the high (10-1000 nM) CNP concentrations used by Del Ry et al. in contrast to those used by us and by Khambata et al. (pM range). The latter concentrations are closer to the reported circulating levels in human and mice (Karla et al., 2003; Moyes et al., 2014) and therefore have arguably more physiological significance.

Taken together, our in vitro and in vivo data support a regulatory role for \( K_{\text{ATP}} \) channels in angiogenesis, shared among widely different angiogenic molecules. This invites the question of their molecular composition of these channels and their cellular localization.

\( K_{\text{ATP}} \) channel composition can be variable, depending on the cell- and tissue-expression of the specific pore-forming (inwardly rectifier, \( K_r \)) and regulatory (sulfonylurea-binding, SUR) subunits which participate in the formation of the channel complex (Aschcroft, 1988; Flagg et al., 2010). This variability results in different sensitivities to nucleotides as well as to pharmacological agents. For example, there is tissue-specific heterogeneity regarding expression of the SUR subunit (Hiraoka and Furukawa; 1998), which exists in several isoforms generated from different genes and/or via incompletely characterized tissue-specific alternative splicing, named SUR1, SUR2A and SUR2B. These forms exhibit structural differences resulting in unique pharmacological and functional characteristics of \( K_{\text{ATP}} \) channels in each tissue (Brady and Terzic; 1998). The SUR1 isoform, when heterologously coexpressed and coassembled with Kir6.2, reconstitutes the pancreatic \( K_{\text{ATP}} \) channel “phenotype”, while in cardiomyocytes \( K_{\text{ATP}} \) are composed of SUR2A and Kir6.2, in smooth muscle cells they are formed by SUR2B and Kir6.1 (Seino and Miki, 2003) and in the coronary endothelium SUR2B forms a heteromultimeric complex with both Kir6.1 and Kir6.2 subunits (Yoshida et al., 2004). The composition in other endothelial beds is not well described. In agreement with Yoshida et al., we have been able to detect both Kir6.1 (this study) and Kir6.2 (B. Umaru, unpublished observations) subunits in HUVECs by qPCR or/and western blotting analysis.

This overall cell- and tissue-variability can theoretically allow for development of sulfonylurea compounds that interact exclusively with the pancreatic \( K_{\text{ATP}} \) channels, leaving the endothelial channels unaffected. “Selective” targeting of pancreatic \( K_{\text{ATP}} \) channels by “beta-cell–selective sulfonylureas” is already a strategy in the pharmacologic management of diabetic patients with ischemic heart disease, since it seems that both sulfonylurea and non-sulfonylurea molecules achieve selectivity at least based on their ability, when recombined in cells, to preferentially inhibit the pancreatic versus the cardiac recombinant channels (Stephan et al., 2006).
The unequivocal determination of the type of subunits expressed in endothelial cells and a comparative study of SUR-targeting molecules in sparing endothelial $K_{\text{ATP}}$ channels are still matters that need future elucidation.

The formation of capillary-like structures elicited by exposure of HUVECs to either SG-209 or to CNP (fig. 24, fig. 25) required unperturbed expression of the $K_{i\text{r}}6.1$ subunit (fig. 26, fig. 27). These results suggest a) a contribution of $K_{i\text{r}}6.1$ activation in these responses, and b) the possible existence of an “expression-dosage”-sensitive effect for $K_{i\text{r}}6.1$, since knockdown of this subunit by half impaired the responses to both SG-209 and to CNP. Furthermore, reduction in the expression of $K_{i\text{r}}6.1$ cannot be compensated by the $K_{i\text{r}}6.2$ subunit, which is also expressed in HUVECs (as seen by RT-PCR, B. Umaru, unpublished results). As a consequence of this, any molecule that can inhibit the activity of $K_{i\text{r}}6.1$-containing $K_{\text{ATP}}$ channels should theoretically impact endothelial function, or at least angiogenesis, quite effectively.

Whether the endothelial $K_{\text{ATP}}$ channels that mediate angiogenesis are at the plasma membrane or associated with mitochondria is another open question, which we tried to address by employing the mitochondrial-selective $K_{\text{ATP}}$ channel inhibitor, 5-HT. The function and role in pathophysiology of the mitochondrial $K_{\text{ATP}}$ (mito$K_{\text{ATP}}$) channels has been extensively characterized, especially in the heart (O’Rourke et al., 2004). Although progress has been made in elucidating the mechanisms involved in mito$K_{\text{ATP}}$-mediated cardioprotection, the molecular composition of the mito$K_{\text{ATP}}$ channel is not universally agreed upon and has delayed structure-function studies of this intracellular channel. Nevertheless, the recent development of novel, more specific, mitochondrial $K_{\text{ATP}}$ channel openers and inhibitors is in agreement with the notion that increased mitochondrial $K^+$ influx is an important protective mechanism from cellular ischemic injury (O’Rourke et al., 2004). The mitochondria constitute critical “targets” of oxidative stress, because survival of endothelial cells can be compromised by opening of the mitochondrial permeability transition pore (Davidson and Duchen, 2007). In addition, evidence suggests that endothelial mitochondria may play a “reconnaissance” role. For example, even though the exact mechanism remains obscure, it has been proposed that endothelial mitochondria may sense levels of oxygen in the blood and relay this information to cardiac myocytes as well as modulate the vasodilatory response mediated by endothelial-derived nitric oxide (Davidson and Duchen, 2007).

The presence of $K_{i\text{r}}6.1$ in mito$K_{\text{ATP}}$ is therefore still a matter of debate. While its existence has been detected in mitochondrial membranes by antibodies and its inclusion is also supported by pharmacological approaches (O’Rourke, 2004; Suzuki et al., 1997), genetic deletion of $K_{i\text{r}}6.1$ in
mice fails to disrupt mitoK$_{ATP}$ opening (Miki et al., 2002; Flagg et al., 2010), thus putting the functional role of Kir6.1 in mitochondria in doubt.

Our own evidence suggests that mitoK$_{ATP}$, comprising Kir6.1 subunits, are key mediators of angiogenesis and may underpin the endothelial-protective effects of both H$_2$S (Suzuki et al., 2011) and of CNP (Lumsden et al., 2010), based on two findings: a) the ability of 5-HD, a mitoK$_{ATP}$ inhibitor (O’Rourke et al., 2004; Sato et al., 1998), to antagonize the responses due to K$_{ATP}$ activation by either direct or indirect activators, as effectively as the non-selective inhibitor Glibenclamide (fig. 25 to 28) and b) the requirement for undiminished expression Kir6.1 subunits to obtain these responses (Fig. 26).

The angiogenic activities of the direct K$_{ATP}$ activator, SG-209, fit well in the current framework of molecular and biochemical pathways that contribute to angiogenesis. The increased endothelial proliferation elicited by SG-209 was almost abolished by Erk1/2 pathway inhibition (fig. 30), while the motility responses were abrogated by inhibition of the Erk1/2, the Akt or the p38 kinases (fig. 31 to 33), at concentrations that have been previously shown to block kinase phosphorylation/activation (Papapetropoulos et al., 2009). These findings agree with reports (Khambata et al., 2011; Papapetropoulos et al., 2009) that H$_2$S or CNP induce their activation of endothelial responses through mobilization of some of these kinases. In contrast, the inability of Akt inhibition to block H$_2$S-induced migration (Papapetropoulos et al., 2009) suggests an only partial overlap between H$_2$S-triggered and K$_{ATP}$-triggered mechanisms. Further work is required to elucidate how exactly K$_{ATP}$ channel activation impacts these well-characterized molecular mechanisms of angiogenesis.

Nicorandil, an anti-angina agent, combines ATP-dependent potassium (K$_{ATP}$) channel opening and nitrate-like activity, and reportedly improves prognosis in patients with angina pectoris via pre-conditioning effects. Nicorandil has been shown to be cardioprotective through inhibition of cardiomyocyte apoptosis that can be induced by oxidative stress and hypoxia (Abdel-raheem et al., 2013), in addition to its other well-characterized effects: the unequivocal vasodilatation it causes, the endothelial-protective and anti-apoptotic effects that it displays both in clinical settings and in animal studies (Serizawa et al; 2011), attributable in some cases to the opening of mitochondrial K$_{ATP}$ channels in endothelial cells (Minamino and Hori, 2007). These experimental studies suggest that endothelial K$_{ATP}$ channels play an important role in protecting endothelial function as well as myocardial health. This line of thought is shared by the IONA group, (Impact of Nicorandil in Angina) who have demonstrated that Nicorandil therapy in patients with stable angina (Minamino and Hori; 2007) displayed its clinical benefits via the
activation of $K_{ATP}$ channels rather than the nitrate-dependent effects. Our present results are in agreement with the above and also raise the additional possibility that part of the benefit associated with the use of Nicorandil 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 to longer-term, angiogenic effects. These last may be attributable to activation of $K_{ATP}$ channels, thus allowing for better myocardial recovery after the insult.

Importantly, our results also suggest some intriguing implications regarding the use of $K_{ATP}$ inhibitors (sulfonylureas) in diabetes. Diabetes has long been considered a risk factor for vascular disease with hyperglycemia itself being a well-characterized risk factor. In diabetes, there is elevated risk of arterial disease (especially peripheral artery disease, PAD) and the increased incidence of occlusion of major vessels (such as the coronary arteries) is attributable to an atherosclerotic (Rodger, 1999) as well as a thrombotic process. Most of the major cardiac risk factors (glucose intolerance, dyslipidemia, truncal obesity and hypertension) are clustered in individuals said to present a syndrome associated with insulin resistance (Laakso and Kuusisto, 2014). These changes are initiated by or simply coexist with endothelial impairment. In PAD, physiological collateral angiogenesis is characteristically deficient and therefore its preservation is important therapeutically. The current work shows that sulfonylureas can inhibit endothelial cell survival and function (e.g. proliferation in response to angiogenic agents). For this reason, it is not surprising that heart ischemic events in diabetic patients are associated with worse outcome if they have been previously under sulfonylureas treatment (Riddle, 2003). In addition, diabetic patients that have been treated with sulfonylureas show a loss of ischemic preconditioning during vascular surgery (Kottenberg et al., 2014). Intriguingly, sulfonylureas have a varying profile regarding inhibition of endothelial-mediated ischemic post-conditioning in humans (Okorie et al., 2011). From the above, it is apparent that sulfonylurea drugs have important actions within the cardiovascular system that go beyond their ability to induce release of insulin, even though at the clinical level, sufficient data on the effect of sulfonylureas on clinical outcomes, on which to base comprehensive recommendations for the use of these drugs in diabetic patients, are still lacking.

The above considerations, in the light of our own data, can be interpreted as meaning that in choosing the sulfonylurea-type drug to treat diabetes, one should consider not only the known negative cardiovascular effects (Proks et al., 2002) but also its potentially negative angiogenic effects, based on non-selective inhibition of endothelial $K_{ATP}$ channels. Furthermore, our results
suggest that new compounds should be designed and evaluated, with an endpoint of inhibiting more selectively the pancreatic and sparing the endothelial K$_{\text{ATP}}$. These characteristics would logically present a better vascular protective profile in diabetics.

In conclusion, our work has shown for the first time that pharmacological interference with the function of K$_{\text{ATP}}$ in vitro and in vivo, either directly, by K$_{\text{ATP}}$ openers or inhibitors, or indirectly, via CNP-cognate receptor interactions, results in the modulation of angiogenic responses. The endothelial K$_{\text{ATP}}$ blockade by sulfonylureas may partly explain their divergent undesirable effects on vascular pathophysiology in diabetic patients.

K$_{\text{ATP}}$ activation thus appears to be a novel common mechanism underpinning angiogenesis to various physiological stimuli including VEGF, H$_2$S and CNP. K$_{\text{ATP}}$ channels should therefore be considered as a valid therapeutic target in angiogenesis and molecules that modulate their activity, especially if they are already in clinical use, should be re-examined under this new light.
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