REVIEW / SYNTHÈSE

Endothelial calcium-activated potassium channels as therapeutic targets to enhance availability of nitric oxide

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Abstract: The vascular endothelium plays a critical role in vascular health by controlling arterial diameter, regulating local cell growth, and protecting blood vessels from the deleterious consequences of platelet aggregation and activation of inflammatory responses. Circulating chemical mediators and physical forces act directly on the endothelium to release diffusible relaxing factors, such as nitric oxide (NO), and to elicit hyperpolarization of the endothelial cell membrane potential, which can spread to the surrounding smooth muscle cells via gap junctions. Endothelial hyperpolarization, mediated by activation of calcium-activated potassium (K_{Ca}) channels, has generally been regarded as a distinct pathway for smooth muscle relaxation. However, recent evidence supports a role for endothelial K_{Ca} channels in production of endothelium-derived NO, and indicates that pharmacological activation of these channels can enhance NO-mediated responses. In this review we summarize the current data on the functional role of endothelial K_{Ca} channels in regulating NO-mediated changes in arterial diameter and NO production, and explore the tempting possibility that these channels may represent a novel avenue for therapeutic intervention in conditions associated with reduced NO availability such as hypertension, hypercholesterolemia, smoking, and diabetes mellitus.

Key words: endothelium, calcium-activated potassium channels, nitric oxide, endothelial dysfunction, oxidative stress.

Résumé : L'endothélium vasculaire joue un rôle crucial dans la santé vasculaire en contrôlant le diamètre artériel, en régulant la croissance cellulaire locale et en protégeant les vaisseaux sanguins des conséquences néfastes de l'agrégation des plaquettes et de l'activation des réponses inflammatoires. Les médiateurs chimiques circulants et les forces physiques agissent directement sur l'endothélium pour libérer des facteurs de relaxation diffusibles comme l'oxyde nitrique (NO), et amorcer l'hyperpolarisation du potentiel membranaire des cellules endothéliales qui peut s'étendre aux cellules de muscle lisse environnant par l'intermédiaire des jonctions communicantes. L'hyperpolarisation endothéliale, qui dépend de l'activation des canaux potassiques activés par le calcium (canaux K_{Ca}), est généralement considérée comme une voie distincte de relaxation du muscle lisse. Cependant, des données récentes tendent à démontrer que les canaux K_{Ca} endothéliaux jouent un rôle dans la production de NO dérivé de l'endothélium, et indiquent que l'activation pharmacologique de ces canaux peut augmenter les réponses qui impliquent le NO. Dans cet article de revue, nous résumons les données actuelles concernant le rôle fonctionnel des canaux K_{Ca} endothéliaux dans la régulation des changements de diamètre artériel faisant intervenir le NO et dans la production de NO, et nous explorons la possibilité intéressante que ces canaux puissent représenter une nouvelle avenue d'intervention thérapeutique sur les conditions associées à une disponibilité réduite de NO comme l'hypertension, l'hypercholestérolémie, le tabagisme et le diabète sucré.

Mots-clés : endothélium, canaux potassiques activés par le calcium, oxyde nitrique, dysfonction endothéliale, stress oxydant.

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Introduction

The endothelium, the single layer of cells lining all blood vessels, plays a critical role in controlling arterial diameter via the release of diffusible relaxing factors such as nitric oxide (NO) and prostacyclin. In addition to regulating the contractile state of smooth muscle cells, NO and prostacyclin contribute to vascular homeostasis by modulating the function of many other cell types. Both NO and prostacyclin inhibit platelet aggregation (Riddell and Owen 1997; Gryglewski 2008), and NO also prevents platelet adhesion, limits oxidation of low density lipoprotein cholesterol, inhibits proliferation of vascular smooth muscle cells, and decreases the expression of proinflammatory genes that contribute to atherogenesis (for review see Förstermann and Münzel 2006; Gao 2010). Reduced availability of NO disrupts all of these processes and increases the risk of cardiovascular complications such as high blood pressure, accelerated atherosclerosis, stroke, and loss of limbs.

Production of endothelium-derived NO and prostacyclin is elicited by chemical mediators acting on receptors and by physical forces such as changes in flow and shear stress across the endothelial cell surface. These same stimuli also evoke hyperpolarization of the endothelial cell membrane potential mediated by activation of calcium-activated potassium (K_{Ca}) channels. Spread of this hyperpolarization to the surrounding smooth muscle cells via gap junctions (endothelium-dependent smooth muscle hyperpolarization; EDH) reduces the open probability of voltage-operated Ca^{2+} channels and so causes vasorelaxation (de Wit and Griffith 2010). The endothelium can also influence smooth muscle cell membrane potential via the production of hyperpolarizing factors such as C-type natriuretic peptide and epoxyeicosatrienoic acids. These factors act on receptors on the smooth muscle cells with subsequent activation of large conductance calcium-activated potassium (BK_{Ca}) channels and (or) adenosine-triphosphate (ATP)-dependent potassium channels (Campbell et al. 1996; Villar et al. 2007; Liang et al. 2010; Bukhari et al. 2011).

Activation of endothelial K_{Ca} channels and the subsequent EDH of smooth muscle has generally been regarded as a distinct pathway for vasorelaxation, separate from NO and other diffusible mediators, which assumes greater importance with decreasing vessel diameter (Tomioka et al. 1999; Hilgers et al. 2006). The role of endothelial K_{Ca} channels in physiological regulation of arterial diameter, blood flow, and blood pressure has been highlighted by studies of transgenic and knockout animals either over-expressing or lacking these channels (Taylor et al. 2003; Si et al. 2006; Brähler et al. 2009). Furthermore, a number of recent reviews have reflected the growing interest in targeting these channels to restore EDH-mediated dilation in conditions associated with endothelial dysfunction (Grgic et al. 2009; Dalsgaard et al. 2010; Köhler et al. 2010).

However, recent evidence supporting the notion that activation of these channels can also modulate the availability of endothelium-derived NO and, that pharmacological activation of these channels may enhance NO-mediated responses, has received far less attention. If this contention is correct, then it opens up the possibility that activation of endothelial K_{Ca} channels may be beneficial in conditions of endothelial dysfunction by affording a therapy that may not only improve regulation of blood flow and pressure, but also ameliorate the multiple consequences of loss of NO for vascular health. In this review we summarize the current data on the functional role of endothelial K_{Ca} channels in NO-mediated vasodilation, and discuss the recent evidence that supports the hypothesis that pharmacological activation of these channels may enhance NO availability.

Endothelial K_{Ca} channels

Endothelial cell hyperpolarization in response to chemical and physical stimuli is mediated by the opening of small (SK_{Ca}) and intermediate conductance (IK_{Ca}) calcium-activated potassium channels. K_{Ca} channels are formed by 4 pore-forming α-subunits, each consisting of 6 or 7 membrane spanning domains with a pore-forming loop bearing the K^+ selectivity filter located between the 5th and 6th segments. To date, 8 pore-forming α-subunits have been identified, which can be divided into 2 groups. The first group is composed of the 3 subunits, which give rise to SK_{Ca} channels with conductances around 5–10 pS (KCa2.1–2.3), and also the IK_{Ca} channel subunit, KCa3.1, homotetramers of which yield channels with a conductance of 20–40 pS. The second group is made up of the KCa1.1 subunit, homotetramers of which make up the BK_{Ca} (200–300 pS) found in vascular smooth muscle cells, and also the related subunits KCa4.1, KCa4.2, and KCa5.1 (Ledoux et al. 2006; Berkefeld et al. 2010).

A combination of mRNA and immunohistochemical techniques has revealed that KCa2.3 and KCa3.1 are expressed in endothelial cells of numerous blood vessels across a range of species, and thus are the most likely molecular correlates for endothelial SK_{Ca} and IK_{Ca} channels, respectively (Burnham et al. 2002; Bychkov et al. 2002; Köhler and Hoyer 2007). SK_{Ca} channels and IK_{Ca} channels are not expressed in native smooth muscle cells, but stimulation of cultured smooth muscle cells by mitogens is accompanied by de novo KCa3.1 mRNA and protein expression (Tharp and Bowles 2009).

Both SK_{Ca} and IK_{Ca} channels, as their names indicate, are activated by calcium. Unlike the BK_{Ca} channels of smooth muscle cells, which directly bind calcium in a region of the C-terminus (Schreiber and Salkoff 1997), SK_{Ca} and IK_{Ca} channels are regulated by calmodulin, which is constitutively associated with the C-terminus to confer submicromolar sensitivity to calcium (Fanger et al. 1999; Lee et al. 2003). Also, again unlike BK_{Ca} channels, SK_{Ca} and IK_{Ca} channels are voltage-independent and thus they can operate at negative membrane potentials close to the K^+ equilibrium potential (Berkefeld et al. 2010). This information has been derived from expression of cloned channels, little data being available on the electrophysiological characteristics of SK_{Ca} and IK_{Ca} channels in native endothelial cells. In porcine coronary artery and rat and rabbit aorta, endothelial cell SK_{Ca} channels with unitary conductances of 2.8–9 pS have been described (Sakai 1990; Marchenko and Sage 1996; Burnham et al. 2002). IK_{Ca} channels with unitary conductances of 17.1 pS and 31 pS have been recorded in endothelial cells from porcine coronary and human mesenteric arteries (Köhler et al. 2000; Bychkov et al. 2002). These values are close to the

unitary conductances of channels composed of KCa2.3 and KCa3.1 subunits when expressed in oocytes or mammalian cells. The density of SK_{Ca} and IK_{Ca} channels in mouse aortic endothelial cells was estimated to be ~307 and ~99 channels/cell, respectively, with IK_{Ca} current amplitude ~1.75-fold larger than the SK_{Ca} current, reflecting the higher single channel conductance of IK_{Ca} channels (Ledoux et al. 2008). The associated whole cell currents were voltage-insensitive, activated by calcium and blocked by apamin, a bee venom selective for SK_{Ca} channels, and either charybdotoxin or 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34), both inhibitors of IK_{Ca} channels. Charybdotoxin depolarized the membrane potential by ~8 mV, and blocking SK_{Ca} channels with apamin caused an additional ~3 mV depolarization. Isolation procedures necessarily remove the electrical contacts between endothelial cells and with surrounding smooth muscle cells. Such contacts can have a significant influence on control of membrane potential and their removal may exaggerate responses to channel modulators. However, in intact carotid and basilar arteries from rat, application of apamin and TRAM-34 depolarized endothelial cells by ~20 mV and ~12 mV, indicating that SK_{Ca} and IK_{Ca} channels likely play a significant role in regulating the resting membrane potential of endothelial cells (Eichler et al. 2003; Plane and Cole 2006; Ledoux et al. 2008).

Endothelial K_{Ca} channels and modulation of arterial diameter

The technical challenges associated with recording endothelial and (or) smooth muscle cell membrane potential in intact arteries means that the role of endothelial SK_{Ca} and IK_{Ca} channels in mediating changes in arterial diameter has, in the majority of cases, been inferred from the effects of apamin and either TRAM-34 or charybdotoxin on arterial tone. Historically it has been assumed that these channels only contribute to EDH-mediated responses, providing a parallel or compensatory pathway to NO-mediated dilation. Thus, in the vast majority of published studies these K_{Ca} channel inhibitors have only been applied to arteries treated with inhibitors of NO synthase (NOS) and cyclooxygenase or to vessels isolated from transgenic animals lacking NOS (Zygmunt and Högestätt 1996; Doughty et al. 1999; Miura et al. 1999; Eichler et al. 2003; Hilgers et al. 2006; Fitzgerald et al. 2007; Brøndum et al. 2010). Whilst these findings demonstrate the importance of endothelial K_{Ca} channels for endothelium-dependent vasorelaxation when NO and prostacyclin production are compromised, the potential contribution of these channels to NO-mediated vasodilation has been overlooked.

Generation of transgenic mice lacking either SK_{Ca} or IK_{Ca} channels provided another tool with which to investigate their role as regulators of vascular function in vivo. Suppression of SK_{Ca} channel expression in mice enhanced myogenic and phenylephrine-induced constriction of isolated mesenteric resistance arteries and increased systemic blood pressure, an effect that was reversed by over-expression of the channel (Taylor et al. 2003). Deletion of IK_{Ca} channels caused a similar rise in blood pressure to knockout of SK_{Ca} channels, but deletion of both channels caused only a small further increase, indicating that loss of one channel cannot be compensated for by the other, or by endothelium-derived factors such as NO or prostacyclin (Brähler et al. 2009). This approach, whilst supporting an important physiological role for endothelial K_{Ca} channels in regulating arterial diameter, generally does not allow discrimination between the role of SK_{Ca} and IK_{Ca} channels in EDH versus NO-mediated responses. However, the recent development of pharmacological agents that can selectively activate endothelial K_{Ca} channels has provided data to support the role of endothelial K_{Ca} channels in NO-mediated vasodilation. We will now review these data and discuss their potential implications for the development of pharmacological interventions to overcome loss of NO in endothelial dysfunction.

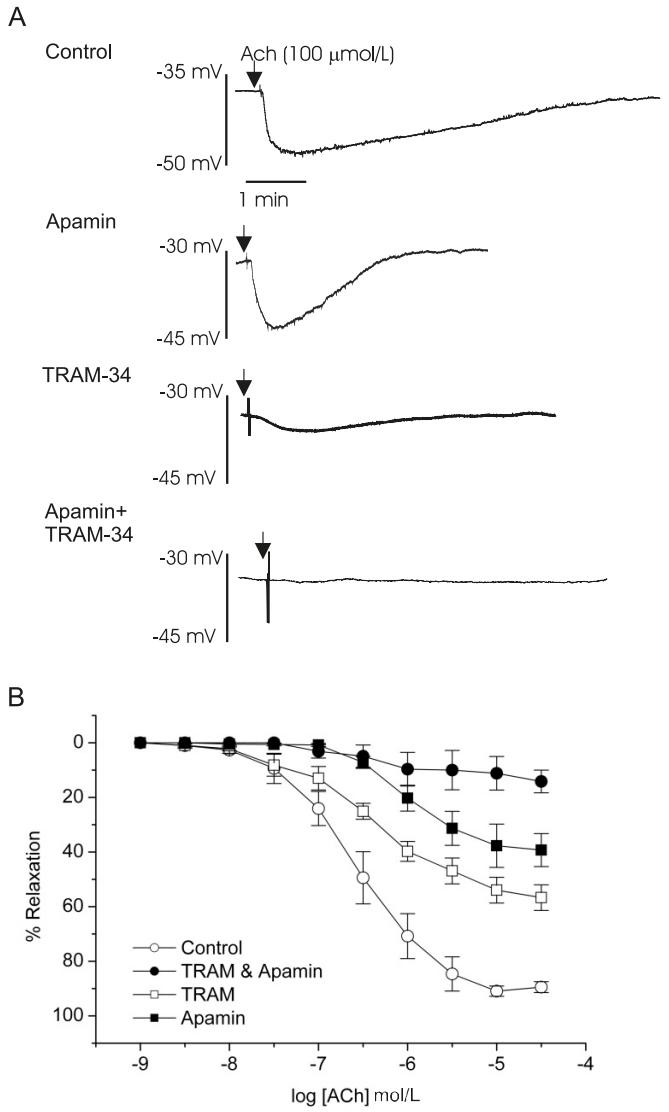
Evidence for the role of K_{Ca} channels in NO-mediated vasodilation

Early studies showed that acetylcholine stimulation of cultured endothelial cells was associated with a biphasic increase in intracellular Ca^{2+} levels; an initial transient rise due to inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from intracellular stores followed by a sustained elevation due to Ca^{2+} influx via store- and (or) receptor-operated non-selective cation channels (NSCCs; Adams et al. 1989; Schilling 1989). Simultaneous recordings of endothelial membrane potential and Ca^{2+} levels then led to the proposal that K_{Ca} channel activation is necessary to increase the driving force for the agonist-induced Ca^{2+} entry through NSCCs (Adams et al. 1989; Schilling 1989; Lückhoff and Busse 1990). At the time that most of these studies were carried out, NO had only just been identified as an endothelium-derived relaxing factor (Palmer et al. 1987) and methods to measure NO production had yet to be developed. This fact, combined with the explosion of interest in the EDH pathway elicited by the observation that in EDH-mediated responses could be blocked by application of SK_{Ca} and IK_{Ca} channel inhibitors to the endothelium (Doughty et al. 1999), led to the assumption that blockers of these channels are "selective inhibitors" of EDH and the potential link between K_{Ca} channels and NO being largely disregarded.

However, this assumption was challenged by reports that the NO scavenger oxyhemoglobin could inhibit responses attributed to EDH (Simonsen et al. 1999; Chauhan et al. 2003) and that blockers of endothelial SK_{Ca} and IK_{Ca} channels attenuated NO-mediated changes in arterial tone (Allen et al. 2002). We showed that in rat basilar artery, NO-mediated relaxations to acetylcholine are inhibited by endothelial depolarization (Allen et al. 2002). Subsequently we found that in the same artery, acetylcholine-evoked endothelial hyperpolarization and relaxation are both blocked by the combination of apamin and TRAM-34 (Plane and Cole 2006; Fig. 1) indicating that K_{Ca} channel-mediated hyperpolarization plays a significant role in regulating NO in this vessel. Similar findings have been reported in rat aorta (Qiu and Quilley 2001) and cremaster muscle arteries (Bakker and Sipkema 1997), rabbit carotid artery (Plane et al. 1998), and in piglet femoral artery (Støen et al. 2003).

In these studies, the role of NO in relaxation was inferred from the actions of NOS inhibitors. More recently, NO-sensitive electrodes or fluorescent dyes have allowed investigators to directly examine the effects of K_{Ca} channel modulators on

Fig. 1. Acetylcholine-evoked endothelial hyperpolarization and relaxation of rat basilar artery are inhibited by blockers of endothelial SK_{Ca} and IK_{Ca} channels. (*a*) Representative recordings of acetylcholine-evoked changes in endothelial membrane potential in the absence and presence of apamin (50 nmol/L) and (or) 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34; 1 μmol/L). (*b*) Mean concentration–response curves for acetylcholine-evoked relaxations of basilar artery segments precontracted with 5-hydroxytryptamine (1–3 μmol/L) in the absence and presence of apamin (50 nmol/L) and (or) TRAM-34 (1 μmol/L; n = 4–6 rat artery segments per test).



NO production. In human umbilical vein cells (HUVECs), blockade of SK_{Ca} and IK_{Ca} channels by apamin and either charybdotoxin or TRAM-34 abolished both NO production and membrane hyperpolarization elicited by agonists (Sheng and Braun 2007). Similarly, in rat superior mesenteric arteries, blockade of SK_{Ca} and IK_{Ca} channels abolished acetylcholine-evoked endothelial hyperpolarization and reduced NO production by 85% (Stankevičius et al. 2006).

The development of small molecule activators of SK_{Ca} and (or) IK_{Ca} channels (Wulff et al. 2007) has provided a major advance in allowing investigation of the functional consequences of opening K_{Ca} channels in cultured cells, intact arteries, and in vivo. The majority of studies have focused on EDH (Grgic et al. 2009; Sankaranarayanan et al. 2009; Köhler et al. 2010). But, the use of these agents has also provided further evidence that endothelial hyperpolarization due to activation of SK_{Ca} and IK_{Ca} channels contributes to NO-dependent changes in arterial tone.

The SK_{Ca}/IK_{Ca} channel activators 6,7-dichloro-1*H*-indole-2,3-dione 3-oxime (NS309; Strøbaek et al. 2004) and 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (DCEBIO; Singh et al. 2001) enhanced both ATP-evoked membrane hyperpolarization and NO synthesis by HUVECs and caused NO-dependent augmentation of acetylcholine-mediated vasodilation of skeletal muscle arterioles (Sheng et al. 2009). In the same study, DCEBIO and NS309 did not directly increase NO production by HUVECs, but in recent experiments, NS309 was shown to stimulate production of NO from HUVECs; an action that was blocked by the combination of SK_{Ca} and IK_{Ca} channel inhibitors (Stankevičius et al. 2011). The reason for this discrepancy is unclear, but stimulation of NO production by K_{Ca} channel activators is also supported by observations from intact arteries. 1-Ethylbenzimidazolin-2-one (1-EBIO), a less potent analogue of DCEBIO, caused NO-dependent dilation of the rat perfused mesenteric bed (Adeagbo 1999), and in rat isolated superior mesenteric artery NS309 evoked both NO production and NO-dependent relaxation, both of which were abolished by apamin and TRAM-34 (Stankevičius et al. 2011).

In the studies described above, the consequences of inhibiting K_{Ca} channels for NO-mediated responses were assessed by application of a combination of channel inhibitors. However, in a few recent studies, application of a single inhibitor has yielded interesting findings on the potential differential role of SK_{Ca} and IK_{Ca} channels in modulating NO production. In anesthetized dogs, NS309 caused NO-dependent increases in coronary blood flow that were abolished by TRAM-34, indicating that in this vascular bed, NS309 may preferentially activate endothelial IK_{Ca} channels to evoke NO production (Kurian et al. 2011). In contrast, in porcine retinal arterioles, NS309 caused relaxations that were mediated by both NO and prostacyclin, and inhibited by the SK_{Ca} channel inhibitor apamin (Dalsgaard et al. 2009). In the same vessels, NS309 and the selective SK_{Ca} channel opener cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine (CYPPA; Hougaard et al. 2007) enhanced NO-mediated relaxations to bradykinin via SK_{Ca} channel activation (Dalsgaard et al. 2010). Whether these differences are due to variations in channel expression between blood vessels is unclear.

It has been suggested that SK_{Ca} and IK_{Ca} channels may fulfil different functional roles within endothelial cells. Immunohistochemical techniques have shown SK_{Ca} and IK_{Ca} channels to be spatially separated within endothelial cells; KCa2.1 proteins are preferentially localized in caveolae and at endothelial cell junctions, whereas KCa3.1 proteins are concentrated within the endothelial projections that extend through the internal elastic lamina to contact underlying smooth muscle cells at myoendothelial gap junctions (Sandow et al. 2006, 2009). This arrangement is supported by biochemical evidence showing that in homogenates of mesenteric artery, caveolin-1 and KCa2.3 protein but not KCa3.1 protein migrated to the caveolin-rich fraction (Absi

et al. 2007). Also, caveolin-1 immunoprecipitates prepared from native porcine coronary artery endothelial cells contained KCa2.3 but not KCa3.1 protein (Absi et al. 2007). Localization of SK_{Ca} channels to caveolae, sites rich in NOS, G-protein coupled receptors, and Ca^{2+} permeable cation channels (Remillard and Yuan 2006; Grgic et al. 2009; Rath et al. 2009), could provide for microdomain-specific activation of NOS, and may indicate that localized changes in Ca^{2+} are more important than global increases in driving NO production. Being situated away from caveolae does not preclude the involvement of IK_{Ca} channels in NO production, but may indicate a more pronounced role in the gap junction communication pathway which underlies EDH.

How does activation of endothelial K_{Ca} channels enhance NO availability?

The possibility of enhancing the availability of endothelium-derived NO using small molecules to activate K_{Ca} channels is an exciting prospect, especially given that loss of NO contributes to many facets of cardiovascular disease. However, the question of how activation of K_{Ca} channels leads to NO availability remains unclear.

From the early studies of cultured cells, it was proposed that membrane hyperpolarization was necessary to increase the driving force for agonist-induced Ca^{2+} entry required for production of NO (Adams et al. 1989; Schilling 1989; Lückhoff and Busse 1990). Observed increases in intracellular Ca^{2+} in response to membrane hyperpolarization in cultured endothelial cells are consistent with this proposal (Li et al. 1999; Sheng and Braun 2007; Stankevičius et al. 2011). In intact arteries, however, changes in membrane potential in response to inhibitors or activators of K_{Ca} channels do not affect endothelial cell Ca^{2+} levels (Ghisdal and Morel 2001; Marrelli et al. 2003; Cohen and Jackson 2005; McSherry et al. 2005; Stankevičius et al. 2006; Dalsgaard et al. 2010).

In isolated endothelial cells, agonist-induced increases in intracellular Ca^{2+} were much more marked than those caused by NS309, despite comparable increases in NO production (Stankevičius et al. 2011). Thus, it is questionable whether the increase in Ca^{2+} can fully account for the increase in NO seen with NS309. Ca^{2+}-independent events may also be coupled to opening of K_{Ca} and to changes in NO availability. A caveat to this proposal is that the effects of K_{Ca} channel activators on changes in endothelial Ca^{2+} in intact arteries have been assessed using techniques for the measurement of bulk Ca^{2+}. As described above, it is becoming apparent that specialized microdomain complexes are crucial to cell signaling within the endothelium, and localization of K_{Ca} channels to specific cellular domains support the notion that local, spatially restricted regional increases in Ca^{2+} could be the important regulators of NO synthesis, rather than bulk Ca^{2+}. In terms of drug action, stimulation of small, spatially restricted increases in Ca^{2+} may be predicted to be beneficial, as it would confer a level of selectivity limiting activation of other pathways. Indeed, there are currently no reports of K_{Ca} channel openers increasing release of other mediators such as prostacyclin or endothelin.

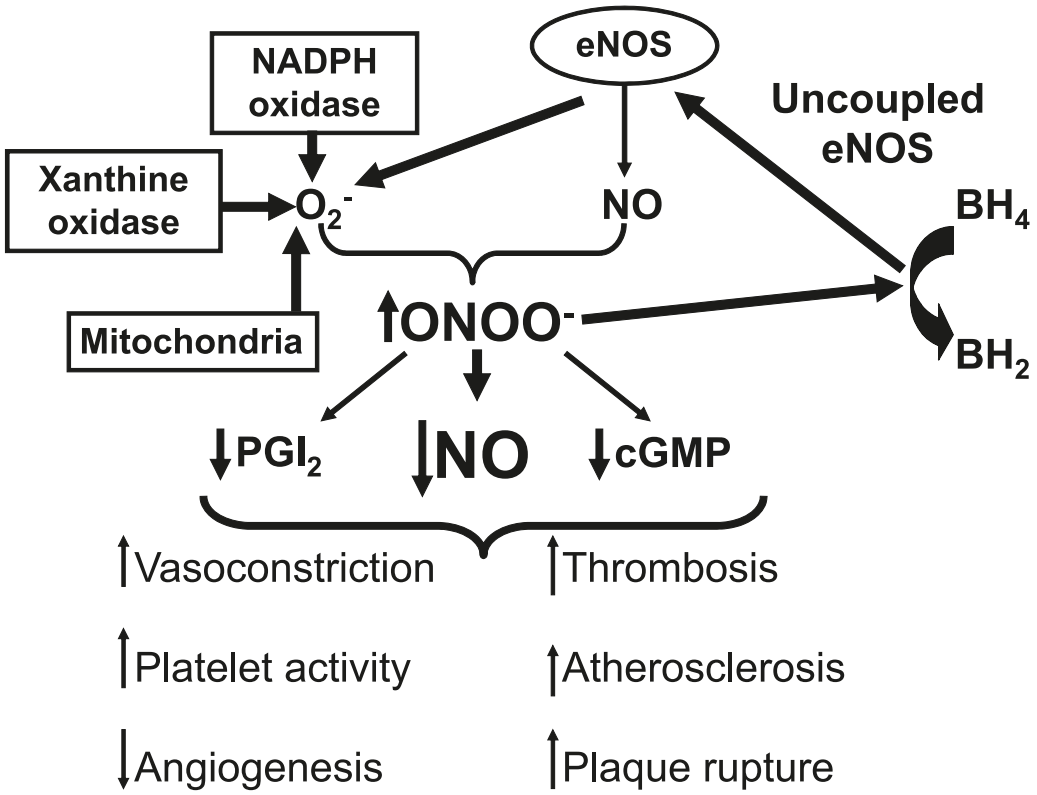
Other potential mechanisms linking opening of endothelial K_{Ca} channels and NO production include membrane potential dependent regulation of L-arginine uptake and (or) superoxide ($O_2^{\bullet-}$) production (Dalsgaard et al. 2010). L-Arginine is the substrate for NOS. Although intracellular concentration of L-arginine exceed the K_m of the enzyme, intracellular compartmentalisation, degradation by arginase, and the presence of endogenous inhibitors of NOS mean that L-arginine transport may be more important than intracellular L-arginine levels in supplying substrate to the membrane-bound enzyme (Chin-Dusting et al. 2007; Michell et al. 2011). L-Arginine transport can be regulated by membrane potential; in cultured endothelial cells, L-arginine uptake is increased by agents that elicit membrane hyperpolarization and inhibited depolarising stimuli (Bogle et al. 1991; Zharikov et al. 1997). There is no published data on the influence of K_{Ca} channel activators on L-arginine transport in intact arteries and, as shown by the data on membrane potential and endothelial Ca^{2+} levels described above, the situation in intact arteries may be very different. Indeed, animal and clinical studies examining the effect of L-arginine supplementation on endothelial function have been unconvincing (for review see Chrissobolis et al. 2011).

NO rapidly reacts with $O_2^{\bullet-}$ to form the highly reactive but relatively stable peroxynitrite ($ONOO^-$), a reaction that proceeds approximately 10 times faster than the dismutation of $O_2^{\bullet-}$ by superoxide dismutase (Huie and Padmaja 1993; Beckman and Koppenol 1996). Increased formation of $ONOO^-$ is a pivotal event in development of vascular disease, as it causes structural damage to vascular cells, inhibits prostacyclin synthesis, and disrupts NO signaling through protein kinase G (Bachschmid et al. 2003; El-Remessy et al. 2003; Maneen and Cipolla 2007; Szabó et al. 2007; Korkmaz et al. 2009; Aggarwal et al. 2011). Furthermore, $ONOO^-$ exacerbates $O_2^{\bullet-}$ production by oxidizing the NO synthase cofactor tetrahydrobiopterin (BH_4); in the absence of this cofactor, NO synthase reduces molecular oxygen to generate $O_2^{\bullet-}$ (Xia et al. 1998; Landmesser et al. 2003; Fig. 2).

$O_2^{\bullet-}$ is a product of normal metabolism generated from numerous sources within endothelial cells including xanthine oxidase, the mitochondrial electron transport chain, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Xanthine oxidase catalyses the sequential hydroxylation of hypoxanthine to xanthine and uric acid using molecular oxygen as an electron acceptor, and thereby producing $O_2^{\bullet-}$ (Harrison 2002). Similarly, while the normal function of the mitochondrial electron transport chain is to generate ATP, at several steps in the pathway, transfer of a single electron to oxygen results in formation of $O_2^{\bullet-}$ (Turrens 2003). Inhibition of xanthine oxidase has some beneficial effects for endothelium-dependent vasodilation in diabetes (Dogan et al. 2011), and increased mitochondrial $O_2^{\bullet-}$ formation and (or) decreased mitochondrial antioxidant systems have been proposed to contribute to endothelial dysfunction a number of settings (for review see Di Lisa et al. 2009).

The primary endothelial source of $O_2^{\bullet-}$, however, and the one which has received most attention in terms of contributing to formation of $ONOO^-$ in disease states, is NADPH oxidase, localized with NOS in endothelial caveolae (Mohazzab et al. 1994; Yang and Rizzo 2007). NADPH oxidase generates $O_2^{\bullet-}$ by transferring electrons from cytosolic NADPH to extracellular oxygen. NADPH oxidases are multisubunit enzymes composed of a flavin-containing catalytic "Nox" subunit with accessory proteins p22phox, p67phox, p47, and

Fig. 2. Central role of peroxynitrite ($ONOO^-$) in endothelial dysfunction. Increased formation of $O_2^{\bullet-}$ leads to increased formation of highly reactive $ONOO^-$ and decreased availability of nitric oxide (NO); NADPH, nicotinamide adenine dinucleotide phosphate; eNOS, endothelial nitric oxide synthase; BH, hydrobiopterin; PGI_2, prostcyclin; cGMP, cyclic guanosine monophosphate



Rac-1 or 2 (for reviews see Bedard and Krause 2007; Drummond et al. 2011; Montezano and Touyz 2012). Of the 7 mammalian Nox isoforms, Nox1, Nox2, Nox4, and Nox5 have been identified in endothelial cells. Under normal conditions, Nox 2 and 4 are the major endothelial sources of NADPH-derived $O_2^{\bullet-}$ (Görlach et al. 2000; Ago et al. 2004), but upregulation of Nox 1 and 2 is a major contributor to increased $O_2^{\bullet-}$ production and endothelial dysfunction associated with atherosclerosis and hypertension (Barry-Lane et al. 2001; Paravicini et al. 2002; Landmesser et al. 2002; Douglas et al. 2012). In contrast, a potential vasculoprotective role has been suggested for Nox4, which appears to predominatly generate H_2O_2 rather than $O_2^{\bullet-}$ (Dikalov et al. 2008; Ray et al. 2011).

The activity of NADPH oxidase at the plasma membrane is voltage-dependent, and in phagocytic cells that express high levels of the enzyme, oxidase activity at the plasma membrane can be measured as electron current (Petheö and Demaurex 2005). Such measurements have not been made for endothelial NADPH oxidase, but in both cultured endothelial cells and in intact tissues, membrane depolarization activates NADPH oxidase to increase production of $O_2^{\bullet-}$. In isolated aortic and pulmonary endothelial cells, cessation of shear stress results in production of $O_2^{\bullet-}$ by NADPH oxidase (Al-Mehdi et al. 1998; Song et al. 2001; Matsuzaki et al. 2005). This response is initiated by closure of ATP-sensitive potassium channels leading to membrane depolarization, and activation of phosphoinositide 3-kinase/Akt (Chatterjee et al. 2012). Potassium channel openers abrogate the depolarization, Akt phosphorylation and $O_2^{\bullet-}$ generation.

Recent data also indicate that in rat mesenteric arteries, SK_{Ca} and IK_{Ca} channels can control NO availability through modulation of NADPH oxidase. K_{Ca} channel blockers increased NADPH oxidase-dependent $O_2^{\bullet-}$ formation, which reduced NO availability and led to inactivation of NOS by phosphorylation at threonine 495 (Gaete et al. 2011). A limitation of the study is the use of apocynin, a nonselective inhibitor of NADPH oxidase that can also act as an antioxidant (Heumüller et al. 2008). Further mechanistic approaches are needed to clarify whether endothelial NADPH oxidase is modulated by membrane potential; indeed, whether K_{Ca} channels can affect other aspects of endothelial function. As described above, the NADPH subunit Nox4 may be vasculoprotective, raising the possibility that broad inhibition of NADPH oxidases by activation of K_{Ca} channels may have detrimental consequences. However, unlike other Nox isoforms, Nox4 has been localized to endoplasmic-reticulum-associated vesicular structures within the endothelium rather than at the plasma membrane (Chen et al. 2008), suggesting the intriguing possibility that activation of K_{Ca} channels may inhibit formation of $O_2^{\bullet-}$ by Nox 1 and 2 but leave H_2O_2 generation by Nox4 untouched.

Endothelial K_{Ca} channels in disease

For K_{Ca} channels to be considered as potential targets for drugs to overcome reduced availability of NO, then they must be functional in disease states displaying endothelial dysfunction. As described above, increased $ONOO^-$ production plays a central role in endothelial dysfunction and inhib-

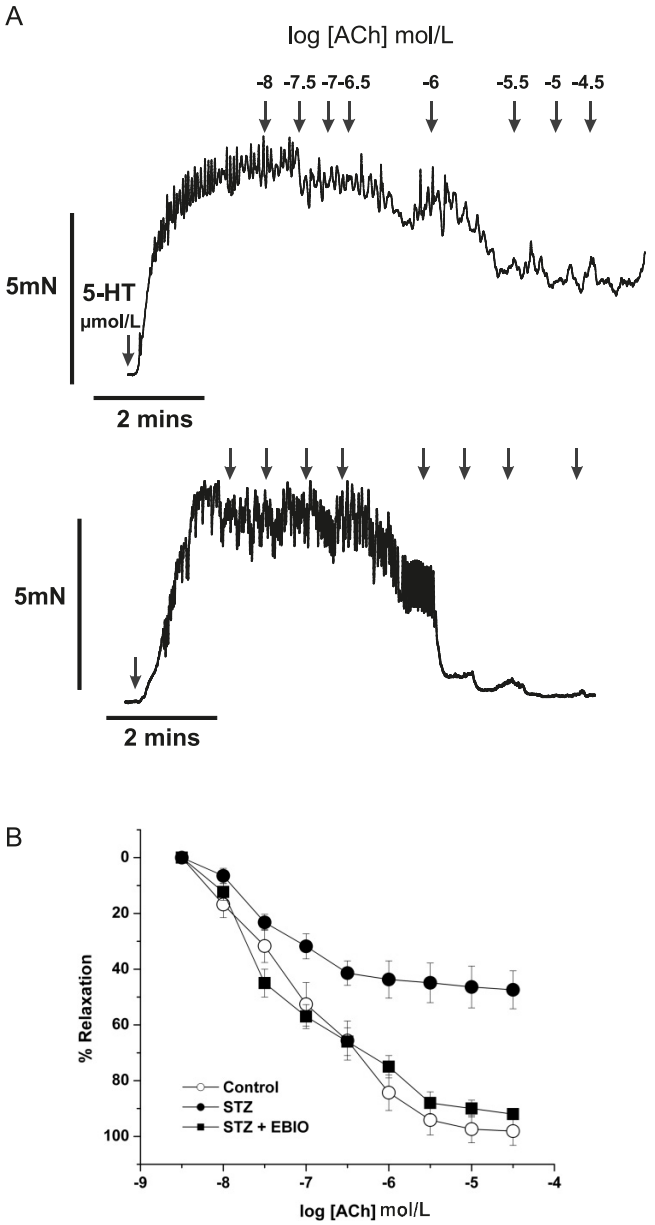
its the activity smooth muscle BK_{Ca} channels (Liu et al. 2002). The effects of $ONOO^-$ on endothelial SK_{Ca} and IK_{Ca} channels has yet to be examined, but maintenance of EDH-mediated responses in disease models such as *db*/*db* mice, rats treated with streptozotocin (STZ), and rats with congestive heart failure, implies the presence of functional endothelial K_{Ca} channels (Pannirselvam et al. 2002; Ueda et al. 2005; Shi et al. 2006; Park et al. 2008). Direct investigation of SK_{Ca} and IK_{Ca} channel expression and function has been limited and provided mixed results. In rats with monocrotaline-induced pulmonary hypertension, Zucker diabetic fatty (ZDF) rats, and *db*/*db* mice, expression of mRNA for K_{Ca} channels was unaltered or increased (Burnham et al. 2006; Pannirselvam et al. 2006; Morio et al. 2007; Brøndum et al. 2010), whereas in rats subjected to 5/6 nephrectomy, angiotensin II infusion, or common bile-duct ligation, channel mRNA expression was reduced (Köhler et al. 2005; Hilgers and Webb 2007; Dal-Ros et al. 2010). In mice lacking endothelial NOS, compensatory increases in EDH-mediated responses were not associated with concomitant changes in the expression of K_{Ca} channel mRNA (Ceroni et al. 2007), and in ZDF rats, the apamin-sensitive component of EDH to acetylcholine was reduced even though although KCa2.3 mRNA expression was increased in the same arteries (Burnham et al. 2006). Thus, comparison of mRNA levels does not necessarily provide a good indication of changes in channel function, and future work should focus on K_{Ca} channel protein localization and function.

Therapeutic potential of endothelial K_{Ca} channel activators in cardiovascular disease

Currently, there is little data available on whether K_{Ca} channel activators can improve endothelial function in disease models. Exposure to NS309 (1 μmol/L) restored relaxations to acetylcholine in isolated mesenteric arteries from ZDF rats without changing endothelial intracellular Ca^{2+} concentration (Brøndum et al. 2010). The ZDF is a model of type 2 diabetes in which endothelial dysfunction is associated with oxidative stress (Oltman et al. 2005). Endothelial hyperpolarization to acetylcholine is attenuated in this model, but the effect of NS309 on membrane potential or production of NO or $O_2^{\bullet-}$ was not investigated (Brøndum et al. 2010). We have found that 1-EBIO can restore NO-mediated relaxations to acetylcholine in isolated basilar arteries from STZ-treated diabetic rats (Fig. 3). This effect was blocked by apamin and TRAM-34, indicating that it was due to activation of K_{Ca} channels and not a nonselective action of 1-EBIO. STZ-induced diabetes is also associated with increased oxidative stress, but we have yet to determine whether this acute enhancement of NO-mediated relaxations is due to reductions in $O_2^{\bullet-}$ production.

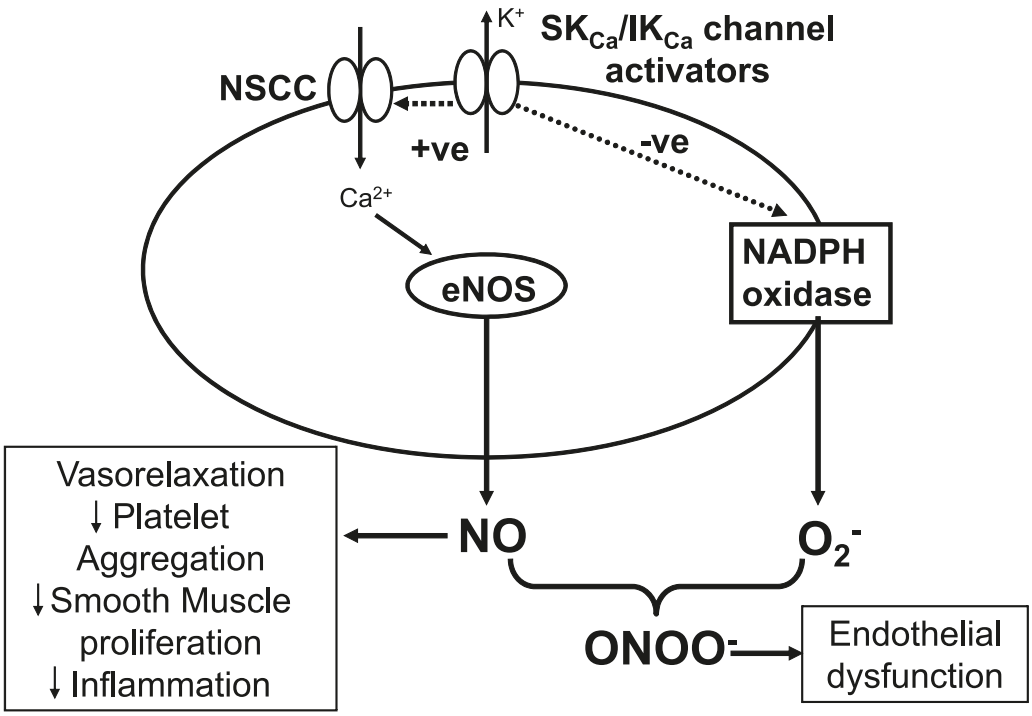
Although these data are supportive, evidence that in-vivo exposure to activators of endothelial K_{Ca} channels can improve endothelial function in disease models is still awaited. Certainly preliminary results show that K_{Ca} channel activators can have effects on vascular function in vivo. In dogs, intravenous injection of naphtho[1,2-d]thiazol-2-ylamine (SKA-31; 2 mg/kg body mass) caused a rapid, transient decrease in blood pressure followed by a fast and pronounced increase in heart rate reflecting baroreceptor activation (Damkjaer et al. 2012). Also, intraperitoneal injection of a SKA-31

Fig. 3. Restoration of acetylcholine (ACh)-evoked relaxation in arteries from streptozotocin (STZ)-treated rats by 1-ethylbenzimidazolin-2-one (1-EBIO). (*a*) Representative trace showing ACh-evoked relaxations in basilar artery from STZ-treated rat. (*b*) Representative trace showing enhancement of by 1-EBIO (1 μmol/L) of ACh-evoked relaxations in basilar artery from STZ-treated rat. (*c*) Mean concentration–response curves for ACh-evoked relaxation of basilar arteries from control and STZ-treated rats, and arteries from STZ rats pretreated with 1-EBIO (n = 4–5 rat artery segments per test); 5-HT, 5-hydroxytryptamine.



(10–30 mg/kg body mass) depot lowered blood pressure in both normotensive and angiotensin-II-infused hypertensive mice over 24 h (Sankaranarayanan et al. 2009). Hypertension is associated with endothelial dysfunction but, in the latter study, this was not assessed. Although the contribution of NO to the observed effects of SKA-31, in vivo, was not examined, the results are encouraging and suggest that SKA-31 could provide a lead compound for more extensive long term studies in disease models.

Fig. 4. Schematic representation of proposed model of K_{Ca}-channel-mediated modulation of nitric oxide (NO) availability. Hyperpolarization of the endothelial cell membrane potential enhances the driving force for entry of calcium through nonselective cation channels (NSCCs) to enhance production of NO by endothelial notric oxide synthase (eNOS), and inhibits formation of $O_2^{\bullet-}$ by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to reduce formation of peroxynitrite ($ONOO^-$).



Perspective

Drug-induced activation of endothelial SK_{Ca} and IK_{Ca} channels represents an attractive possibility as a new strategy to enhance NO-mediated responses in disease states associated with endothelial dysfunction. This proposal is supported by in-vitro studies showing enhancement of NO production and NO-mediated relaxations in isolated endothelial cells and arteries treated with K_{Ca} openers. The current dogma is that activation of endothelial K_{Ca} channels causes hyperpolarization, which modulates intracellular Ca^{2+} levels. This model is based on studies of isolated cells, but reports of K_{Ca}-channel mediated dilation in the absence of changes in endothelial Ca^{2+} suggest that hyperpolarization may modulate other cellular processes, such as L-arginine uptake and $O_2^{\bullet-}$ generation (Fig. 4).

The wide distribution of SK_{Ca} and IK_{Ca} channels raises the issue of potential side effects to drugs targeted to these channels. SK_{Ca} channels modulate the excitability of central dopaminergic neurons (Ji et al. 2009; Herrik et al. 2012) and play an important role in controlling sensory input into the spinal cord (Bahia et al. 2005). Cardiac SK_{Ca} channels have been proposed as new drug targets for treatment of atrial fibrillation (Diness et al. 2010; 2011), and these channels have also been identified as important regulators of the fate of neural stem cells, activation of these channels driving pluripotent cells toward mesoderm commitment and cardiomyocyte specification (Kleger et al. 2010). IK_{Ca} channels provide the necessary driving force for chloride secretion by colonic epithelial cells (Warth et al. 1999) and for calcium entry into T cells and mast cells (Duffy et al. 2001; Fanger et al. 2001). Production of free radicals by neutrophils relies on membrane depolarization, and so activation of K_{Ca} channels may be predicted to inhibit this process. However, contradictory data has been presented with increased free radical production reported in response to both K_{Ca} channel activators and inhibitors (Fay et al. 2006; Patel et al. 2009). Such varied roles for SK_{Ca} and IK_{Ca} channels underlines the need for development of vascular-specific channel modulators, but does not preclude targeting of these channels for therapeutic purposes, as illustrated by the success of inhibitors of L-type voltage-operated calcium channels in the treatment of cardiovascular disease.

The precise relationship between endothelial cell membrane potential and NO production needs to be clarified, but the lack of effect of K_{Ca} channel openers on endothelial bulk Ca^{2+} may actually be beneficial. Oxidative stress is accepted as a major contributor to endothelial dysfunction; increased production and (or) decreased scavenging of reactive oxygen species leads to reduced bioavailability of NO. In addition to the direct interaction between NO and $O_2^{\bullet-}$, oxidative stress can lead to uncoupling of oxygen reduction to NO synthesis by NOS turning it into a $O_2^{\bullet-}$-generating enzyme (Xia et al. 1998; Landmesser et al. 2003; reviewed by Förstermann 2010). Generation of NADPH-oxidase-derived reactive oxygen species can trigger the uncoupling of NOS, and the uncoupled enzyme significantly contributes to oxidative stress. In this setting, enhancing endothelial Ca^{2+} levels may exacerbate the situation by further enhancing $O_2^{\bullet-}$ production. Indeed, the potentially deleterious consequences of an increase in bulk Ca^{2+} for endothelial function was revealed in a study using an opener of the Ca^{2+} permeable endothelial TRPV4 channel (a candidate NSCC involved in Ca^{2+} entry; Ma et al. 2011). Activation of this channel increased both NO- and

EDH-mediated vasodilation in mice, but caused massive endothelial damage, ascribed to increased production of reactive oxygen species, with fatal consequences (Willette et al. 2008).

Preliminary findings in in-vivo studies with K_{Ca} channel activators indicate that these drugs are not toxic to endothelium, supporting the notion that these drugs do not cause changes in endothelial Ca^{2+} levels but modulate endothelial function by other mechanisms (Sankaranarayanan et al. 2009; Damkjaer et al. 2012). If it is confirmed that K_{Ca} channel openers can limit NADPH-mediated $O_2^{\bullet-}$ production, these agents may provide a means of not only increasing the bioavailability of NO but also preventing uncoupling of NOS. Thus, it is essential that further work is carried out to establish whether endothelial K_{Ca} channel openers can actually enhance NO in vivo in models displaying loss of endothelial dysfunction and to define their mechanism of action. Despite the pivotal role of $O_2^{\bullet-}$ in endothelial dysfunction, clinical trials with antioxidants such as vitamin C, vitamin E, or folic acid have been disappointing, owing to poor bioavailability at the site of disease and paradoxical pro-oxidant effects (Heart Protection Study Collaborative Group 2002). The therapeutic potential of drugs targeted against vascular NADPH oxidase, to decrease $O_2^{\bullet-}$ generation and so increase NO availability and decrease formation of $ONOO^-$, has recently been highlighted, but as yet no compounds have reached clinical trials (for reviews see Paravicini and Touyz 2008; Drummond et al. 2011). In this context, the possibility that that small molecule K_{Ca} openers, in addition to being able to stimulate vasodilation via the EDH pathway, may provide a endothelium-targeted antioxidant strategy to enhance NO availability, is an exciting prospect.

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