In Search of the Acute Oxygen Sensor

Functional proteomics and acute regulation of large-conductance, calcium-activated potassium channels by hemeoxygenase-2

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1. INTRODUCTION

Detecting and reacting to acute perturbation in the partial pressure of atmospheric oxygen (pO_2), particularly hypoxia, is a fundamental adaptive mechanism which is conserved throughout the animal kingdom. In mammals, a number of cellular systems respond, often co-operatively as oxygen availability becomes compromised, with the express aim of maximising oxygen uptake by the lungs and of optimising its delivery to the metabolically most active tissues. Thus, during hypoxia, ventilation rate and depth are increased to maximize air flow across the gaseous exchange surface, local lung perfusion rates become rapidly matched to local alveolar ventilation and systemic arteriolar dilatation ensures that tissue and cerebral blood flow become swiftly optimized. Central to many of the oxygen-sensitive responses is hypoxic inhibition of large conductance, Ca^{2+}-activated potassium (BK, maxiK or *slo*) channels. Thus, BK channels are strongly implicated as critical components of the acute O_2 signalling cascade in; a) carotid body chemoreceptors (Peers, 1990; Riesco-Fagundo et al., 2001), where low arterial pO_2 is detected by BK channels and the resulting depolarizing signal is ultimately transduced into increased ventilation; b) fetal and postnatal pulmonary arteriolar myocytes, where BK channels may contribute to both persistent prenatal (Cornfield et al., 1996) and acute postnatal hypoxic pulmonary vasoconstriction (Peng et al., 1999; Cornfield et al., 1996) in order to match ventilation to perfusion; c) neonatal adrenomedullary chromaffin cells (Thompson & Nurse, 1998), where hypoxic inhibition of BK channels induces the huge surge in catecholamine secretion crucial for preparing the newborn's lung for air-breathing by activating alveolar fluid reabsorption and surfactant secretion and; d) central neurones (Liu et al., 1999; Jiang & Haddad, 1994b; Jiang & Haddad, 1994a), where hypoxic depression of BK channel activity may contribute to the excitotoxicity which results from increased neuronal excitability.

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Although a number of mechanisms of hypoxic regulation of K^+ channels in general, and BK channels in particular, have been proposed see (Lopez-Barneo et al., 2001) for recent review, the nature of the sensor is still unclear. Indeed, there are varying reports which have suggested involvement of either cytosolic factors e.g. (Wyatt & Peers, 1995), direct channel modulation e.g. (Liu et al., 1999; Riesco-Fagundo et al., 2001; Jiang & Haddad, 1994a) and membrane-delimited regulation via activation/inhibition of associated BK channel protein partners (Lewis et al., 2002). To investigate the later, we have employed HEK293 cells stably co-expressing identified α- and β-subunits of a human BK channel in order to examine the O_2-sensitivity of these recombinant K^+ channels at the single channel level. Furthermore, we have employed a sequential strategy (termed functional proteomics) to determine; 1) potential protein partners of BK α-subunit; 2) the role of particular protein partners in both BK regulation and hypoxic inhibition of BK activity and; 3) the effect of specific protein knock-down using post transcriptional gene suppression on BK regulation by hypoxia. Finally, we have used these data to inform experiments aimed at determining the role of hemeoxygenase-2 in hypoxic inhibition of BK channels natively expressed in rat carotid body glomus cells (Williams et al., 2004a).

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2. METHODS

2.1 Membrane Preparation and Immunoprecipitation

Cell pellets were homogenised in 1 ml ice-cold homogenization buffer and centrifuged at 1,000*g* for 5 minutes. The supernatants were transferred to fresh tubes and further spun at 16,000*g* for 30 minutes at 4°C. Pellets were solubilised by addition of Triton X-100. Suspensions were incubated with antibodies at 4°C for 1 hour before further incubation with Protein G beads for 4 hours. The beads were pelleted at 7,000*g*.

2.2 Electrophoresis and Protein Visualisation

For single dimension SDS-PAGE, pelleted immunoprecipitates were taken up in sample buffer, heated to 100°C for 3 minutes and loaded onto SDS-polyacrylamide gels comprising 4% stacking and 10% resolving gels For 2-D electrophoresis, the pelleted immunoprecipitates were resuspended in 250 µl rehydration buffer, sonicated for 20 minutes and loaded onto 11 cm, p*I* 4 - 7, IPG strips. After focussing, the IPG gel strips were equilibrated for 15 minutes in 15 ml reducing solution and 15 minutes in alkylating solution and then loaded onto 8 – 18% precast gels.

2.3 In-gel Trypsin Digestion and Peptide Mass Mapping

Bands of interest were excised from the gels, dehydrated in 0.5 ml acetonitrile for 10 minutes and dried in air. Proteins were reduced by incubation in 50 µl 10 mM DTT, 50 mM NH_4HCO_3 for 1 h at 56°C. Upon cooling, the DTT solution was replaced by 55 mM iodoacetamide, 50 mM NH_4HCO_3. The gel was then hydrated at 4°C for 45 minutes in trypsinisation buffer (20 ng/µl trypsin, 50

mM NH_4HCO_3). Digestion was carried out overnight at 37°C. 1.5 µl of the digest was mixed with 1.5 µl MALDI matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% v/v ethanol/50% v/v acetonitrile) and dried onto a MALDI target. The sample was analysed on a Micromass TofSpec MALDI/TOF-mass spectrometer. Monoisotopic peptide fingerprints were used to search databases.

2.4 siRNA Design, and Transfection into HEK293 Cells

Two short interfering (si) RNAs were designed to target nucleotides 212 - 232 (HO-2 siRNA1) and 481 - 501 (HO-2 siRNA2) of the HO-2 coding sequence and the following oligonucleotide templates were designed (underlined bases are complimentary to the HO-2 coding sequence):

$Antisense_1$; 5'- AGCACACGACCGGGCAGAAACCTGTCTC -3';
$Sense_1$; 5'- AATTTCTGCCCGGTCGTGTGCCCTGTCTC -3'.
$Antisense_2$; 5'- AGTACGTGGAGCGGATCCACCCTGTCTC -3';
$Sense_2$; 5'- AAGTGGATCCGCTCCACGTACCCTGTCTC -3'.

For transfections, 125 pmol duplex, Cy3-labelled siRNA was diluted into 490 µl Optimem and 10 µl Lipofectamine. The mixture was incubated at room temperature for 20 minutes before being added to the cells. Cells were cultured or a further 48 hours before electrophysiological analyses

2.5 Electrophysiology – Inside-out Patches

BK channels were recorded from inside-out patched of wild type HEK293, BKαβ HEK 293 and rat carotid body glomus cells (prepared as described previously (Hatton et al., 1997)). Pipette solution contained: 135 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5 mM HEPES, 10 mM glucose. Osmolarity of the solution was adjusted to 300 $mOsm.L^{-1}$with sucrose. pH was adjusted to 7.4 with 1M NaOH. Bath solution contained 10 mM NaCl, 117 mM KCl, 2 mM $MgCl_2$, 11 mM HEPES, 0.15 mM EGTA, 0.1 mM $CaCl_2$. The pH was adjusted to pH 7.2 using KOH. Free ionised Ca^{2+} $[Ca^{2+}]_i$ was 336 nM. Normoxic solutions (pO_2 ~ 150 mmHg) were bubbled with medical air and hypoxic (15-25 mmHg) solutions were bubbled with N_2. Patches were held at +20 mV (-Vp).

3. RESULTS AND DISCUSSION

Proteins which potentially associate with the α-subunit of recombinant human (BKα) were immunoprecipitated with a specific BKα antibody from lysates of HEK293 cells stably co-expressing both human $BK\alpha_1$ (KCNMA1) and $BK\beta_1$ (KCNMB1) and separated by 2-D (Figure 1A, right panel) and 1-D (Figure 1B, right lane) gel electrophoresis. Parallel immunoprecipitation experiments were performed on untransfected, wild type HEK293 cells for comparison purposes (Figure 1A, left panel; Figure 1B, left lane). Of the unique proteins that immunoprecipitated from the stable BKαβ cell line, peptide mass mapping using mass spectroscopy of trypsin digests consistently identified gamma glutamyl transpeptidase (GGT) and hemeoxygenase-2 (HO-2) as

potential protein partners. Although GGT associates directly with BKα, we have recently demonstrated that it is not involved in hypoxic inhibition of BK channels (Williams et al., 2004b).

Western blot of BKαβ cell lysates demonstrated the expected BKα subunit immunoreactivity only in transfected cells, whereas there was constitutive expression of HO-2 in both cell lines (Figure 1C). Biochemical interaction between BKα subunit and HO-2 was confirmed by co-immunoprecipitation of BKα with an HO-2 antibody, and *vice versa*, only in cells stably co-expressing either BKαβ (Figure 1D) or BKα alone (Figure 1E). Specificity of this interaction was demonstrated by the inability of BKα to immunoprecipitate either endothelial nitric oxide synthase (eNOS) or the α-subunit of the Na^+/K^+-ATPase (Figure 1D) despite both proteins being abundantly expressed (Figure 1C).

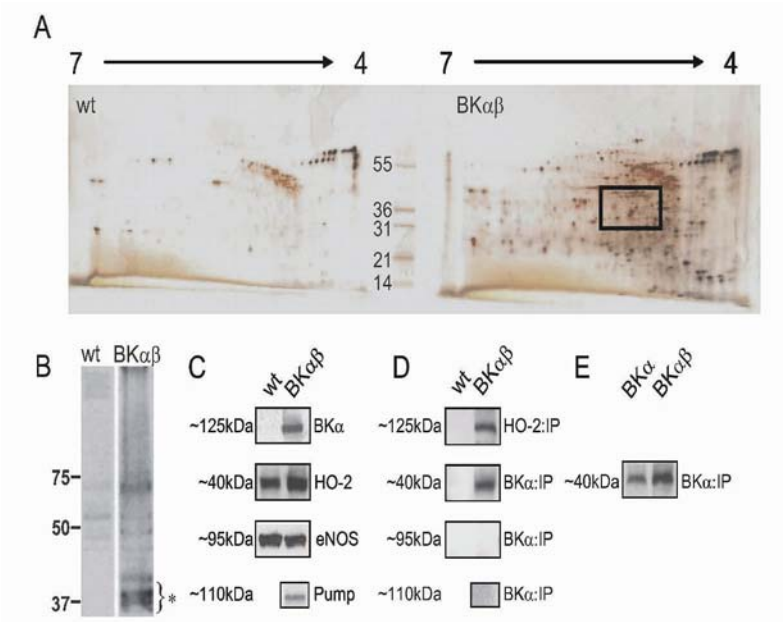

Figure 1. Hemeoxygenase-2 as a BKα protein partner. (A) 2-D gel electrophoresis of proteins immunoprecipitated with a BKα antibody from wild type (wt) and BKαβ HEK293 cells. Boxed area indicates location of protein spots selected for MALDI/TOF analysis. (B) SDS-PAGE of immunoprecipitates from wt and BKαβ cells. Bands removed for MALDI/TOF analysis are indicated by the asterisk. Linear pH gradients and/or molecular weight markers (in KDa) are shown. (C) Western blot analyses from lysates of wt and BKαβ cells show HO-2, endothelial nitric oxide (eNOS) and α-subunit of Na^+/K^+-ATPase (pump) are constitutively expressed. (D) Western blot identification of BKα and HO-2 following immunoprecipitation (IP) with the antibodies shown to the right (top two blots)**.** Neither eNOS nor the pump immunoprecipiated with BKα (lower two blots). (E) Western blot identification of HO-2 following IP with the BKα antibody using lysates from BKαβ cells and BKα cells (with no β subunit)**.** Adapted from Williams et al., 2004a.

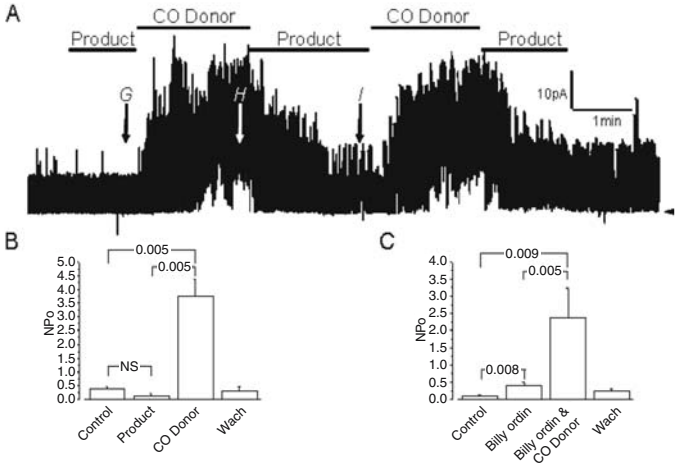

Figure 2. Hemeoxygenase metabolites activate BK channels. (A) Exemplar current recording from an inside-out patch excised from a BKαβ cell. Periods of application of CO-donor and its control (Product) are shown above the trace. (B) mean NPo plot showing effect of CO-donor. (C) Mean NPo plot showing additive effects of biliverdin and CO-donor. Patch potential (-Vp) = +20 mV, $[Ca^{2+}]_i$ = 335 nM, in this and all subsequent figures. P values are shown above bars and are from ANOVA/Bonferroni post hoc test. Adapted from Williams et al., 2004a.

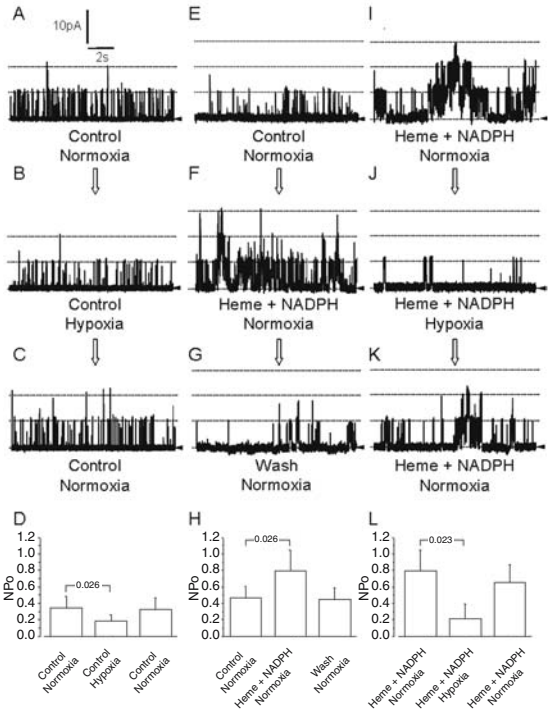

Figure 3. Hemeoxygenase substrates augment BKαβ channel activity and hypoxic inhibition. Exemplar traces and mean NPo plots indicating modest hypoxic channel inhibition in untreated patches (A-D), increased baseline channel activity by 1nM heme/1 μM NADPH (E-H) and augmentation of the hypoxic inhibition in the continued presence of heme/NADPH (I-L). All traces from inside-out patches excised from BKαβ cells. Student's t-test. Adapted from Williams et al., 2004a.

In the presence of O_2 and NADPH, hemeoxygenases catalyse the breakdown of heme to biliverdin, iron and CO (Prabhakar, 1999). BKαβ channel activity was robustly and reversibly activated by 30 μM of the chemical CO-donor, $[Ru(CO)_3Cl_2]_2$; 30 μM of the breakdown product of this compound, $RuCl_2(DMSO)_4$, which does not release CO and, therefore, acts as control, did not affect channel activity indicating that CO strongly activates BK channels in inside-out patches (Figure 2A). Normalised NPo was increased 15-fold by the CO-donor (Figure 2B; n = 13) 10 μM biliverdin evoked a more modest, but significant 4-fold activation of BKαβ channel activity (Figure 2C; n = 12). In patches treated sequentially with biliverdin and the CO-donor, the activation was additive with the CO-donor causing a further increase to 28-fold above control (Figure 2C; n=5). Wild type HEK293 cells did not display BK currents (data not shown but see (Lewis et al., 2002)) and no activation was observed upon addition of the CO-donor (data not shown).

Consistent with earlier reports (Lewis et al., 2002; Williams et al., 2004b), acute hypoxia resulted in a modest depression in NPo of inside-out patches excised from BKαβ cells (Figure 3A-2D; n = 14). In the presence of O_2, addition of the HO-2 co-substrates, heme (1nM) and NADPH (1μM), evoked a large increase in patch NPo (Figure 3E-2H; n = 15). 1nM heme alone has been previously shown not to modulate recombinant BKα channel activity (Tang et al., 2003), an observation which we have extended to BKαβ since NPo in the absence 0.252 ± 0.236 or presence 0.312 ±0.233of 1nM heme were not significantly different from each other ($P > 0.25$, n = 8, data not shown). Importantly, in the continued presence of the HO-2 co-substrates, hypoxia evoked a dramatic decrease in channel activity of over 70% suggesting that the enzymatic activity of HO-2 confers a significant enhancement to the O_2 sensing ability of the HO-2/BK protein complex (Figure 3I-L; n = 10). Thus, O_2 sensing by human BKαβ channels consists of two components of which the HO-2-dependent part is quantitatively more important.

Selective knock-down of HO-2 protein at 48 h was achieved by transfecting cells with siRNA species. Successful transfection of Cy3-labelled siRNA, designed against either a scrambled human GAPDH coding sequence or the human HO-2 coding sequence was followed using fluorescence microscopy. No knock-down of HO-2 immunoreactivity was observed using the scrambled siRNA. In complete contrast, almost total loss of HO-2 immunoreactivity was achieved with the specific HO-2 siRNA (data not shown). Identification of successfully transfected cells was achieved by observing Cy3 fluorescence prior to seal formation. The NADPH/heme-dependent hypoxic suppression seen in untreated cells was maintained following 48h incubation with the scrambled, control siRNA (Figure 4A-D; n = 10). Following post-transcriptional gene suppression of HO-2 for 48 h with HO-2 siRNA, mean patch NPo was dramatically depressed and NADPH/heme-dependent hypoxic suppression was completely absent (Fig 4E-H; n = 7). However, the CO-donor was able to rescue this loss-of-function in all patches tested (Fig. 4I-L; n = 3).

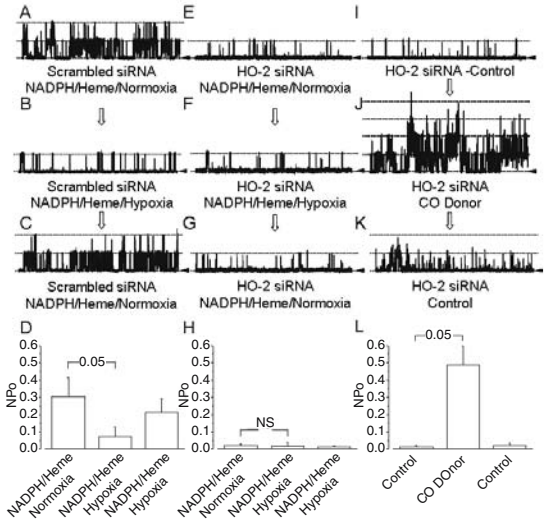

Figure 4. Modulation of heme/NADPH-dependent hypoxic inhibition in BKαβ cells following protein knock-down of HO-2 by siRNA. Exemplar traces and mean NPo plots NADPH/heme-dependent hypoxic channel inhibition in scrambled siRNA treated patches (A-D), almost complete loss of channel activity by in HO-2 treated patches (E-H) and rescue of channel activity by the CO-donor in HO-2 treated patches (I- L). All traces are from inside-out patches excised from BKαβ cells identified as siRNA-positive by Cy3 fluorescence prior to patch clamp. Statistical comparisons made by paired Student's t-test. Adapted from Williams et al., 2004a.

The physiological relevance of this novel enzyme-linked O_2 sensing by large conductance Ca^{2+}-dependent K^+ channels is illustrated in Figure 5 which shows the result of activating hemeoxygenase in inside-out patches excised from the membrane of rat carotid body glomus cells. Consistent with previous data obtained in native carotid body (Riesco-Fagundo et al., 2001), the large conductance K^+ channel was only modestly inhibited by hypoxia (Fig. 5A, B, C and G; n = 7). Similar to the recombinant system, supplying the channel complex with hemeoxygenase substrates (heme and NADPH - Fig 5G) or addition of the CO-donor increased patch NPo (35-fold, n = 7; data not shown). More importantly, NADPH/heme-dependent hypoxic inhibition was greatly augmented suggesting that the HO-2-dependent O_2 system is fully operable in native carotid body glomus cells (Figure 5 D - G; n = 7).

Amongst the numerous proteins which directly associate with the α-subunit of BK, HO-2 is notable in that it is concentrated in neuronal and chemosensing tissues, including carotid body glomus cells, where it is constitutively expressed (Prabhakar, 1999; Maines, 1997; Verma et al., 1993; Prabhakar et al., 1995). Such constitutive expression also holds true for the recombinant system in which we have chosen to study human BK channels, HEK 293 cells (Ahring et al., 1997). HO-1 immunoreactivity was not detected in HEK293 cells and has previously been discounted in rat carotid glomus cells (Prabhakar et al., 1995). Importantly, immunoprecipitation of proteins from BKαβ cells provides direct evidence that HO-2 is associated with the BK α-subunit. That the system is still

functionally intact in excised patches suggests strongly that the protein-protein interaction is membrane-delimited. Whether this interaction is direct or whether it occurs via intermediate proteins is uncertain; either way, it is clear that such a co-localization of BKα with HO-2 is necessary for both

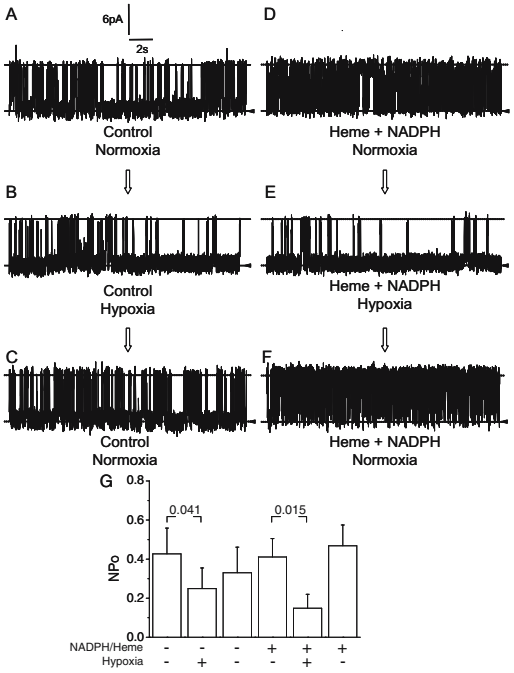

Figure 5. Augmentation of carotid body glomus cell BK channel activity by hemeoxygenase substrates. Exemplar traces indicating the modest hypoxic channel inhibition observed in untreated patches (A-C), increased baseline channel activity by 1nM heme/1 μM NADPH (C-D) and augmentation of the hypoxic inhibition in the continued presence of heme/NADPH (D-F). Corresponding mean NPo values are shown in (G). All traces are from inside-out patches excised from carotid body glomus cells. Patch potential (-Vp) = +20 mV, $[Ca^{2+}]_i$ = 335 nM. Statistical comparisons made by paired Student's t-test. Adapted from Williams et al., 2004a.

basal and O_2-dependent activity. That it is necessary for basal BK activity is demonstrated by the observation that HO-2 knock-out results in a dramatic loss of channel activity which is fully rescued by the HO-2 product, CO. Activation by CO gas has been reported in glomus cells, supporting our suggestion that HO-2 activity is crucial to native BK channel regulation (Riesco-Fagundo et al., 2001). Together with the data presented herein, the presence of HO-2 in the BK channel complex provides a molecular explanation for the observation that HO inhibition results in carotid body excitation (Prabhakar et al., 1995) and that HO-2 knock-out promotes blunted hypoxic ventilatory responses (Adachi et al., 2004). In the proposed model (Williams et al., 2004a), O_2-sensing is conferred upon the BK channel by co-localization with HO-2. In normoxia, tonic HO-2 activity generates CO and biliverdin, both of which maintain the open state probability of the channel at a relatively high level. Our data show that the

presence of CO and biliverdin together evoke BK channel activation which is more than additive, this may represent a means by which the normoxic signal is amplified. However, since biliverdin is rapidly broken down to bilirubin by the actions of biliverdin reductase, it seems likely that the physiological messenger is CO. At this juncture, one can only speculate on the mechanism of CO action. In the absence of other second messenger systems (such as gas-activation of guanylate cyclase) an appealing candidate, based on earlier data in native vascular tissue (Wang & Wu, 1997), is conformational regulation through direct interaction of CO with a histidine residue, potentially in the heme-binding domain of BKα (Wood & Vogeli, 1997; Tang et al., 2003). Whatever the molecular nature of the CO effect, cellular CO levels are reduced during hypoxic challenge as HO-2 substrate (O_2) becomes scarce, and rapidly fall below the critical threshold for the maintenance of BK channel activity at the tonically high level. In other words, HO-2 functions as a sensor of O_2 by regulating BK channel activity primarily through the production of CO.

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