Current Understanding of the O_2-Signalling Mechanism of Adrenal Chromaffin Cells.

- The Peter Baker's Lecture-

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Activation of the splanchnic nerve during stress stimulates the chromaffin cells of the adult adrenal gland to release catecholamines into the circulation. This vital component of the fight-or-flight response is characterized by several physiological changes that allow the animal to combat or flee the stressor. In some animals however, splanchnic innervation is immature at birth, yet adrenal catecholamine secretion has been shown to occur during physiological stresses, such as hypoxia. In this paper, I will review the current understanding of the mechanism of this non-neurogenic, hypoxia-induced secretion of catecholamines from neonatal chromaffin cells. Hypoxia induced catecholamine secretion is mediated through an O_2-signalling pathway that appears to be preferentially expressed in neonatal chromaffin cells, insofar as direct responses of the chromaffin cells to hypoxia are lost along a time course similar to the maturation of the splanchnic innervation. The O_2-sensing mechanism appears to involve a mitochondrial based O_2-sensor and reactive oxygen species intermediates that regulate the activity of several K^+ channels. This in turn, is thought to depolarize the chromaffin cell and broaden action potentials, which increases Ca^{2+} in the cytoplasm and evokes catecholamine secretion.

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Cell Biology of the Chromaffin Cell
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In adult animals, physiological or metabolic stress increases activity of the sympathetic nervous system, leading to acetylcholine release from splanchnic nerve endings that innervate the adrenal chromaffin cells (AMC). Released acetylcholine activates nicotinic receptors in the plasma membrane of AMC, which ultimately causes catecholamine exocytosis into the blood. The principle role of circulating catecholamines is to ensure that adequate blood flow to vital organs is maintained so that the animal may combat the stress. Interestingly, in species that are relatively immature at birth, such as rat and man, sympathetic innervation to target organs, including the adrenal is not functional[1]. However, despite this lack of neurogenic control of catecholamine exocytosis in neonatal animals, hypoxia is a potent physiological stress that evokes catecholamine release from AMC[2, 3, 4].

The hypoxic stimulus for neonatal animals appears to arise from two sources. The first is due to the lowered O_2 and elevated CO_2 experienced during birth, resulting from intermittent occlusions of the umbilical cord[5]. The second source of hypoxia arises from intermittent apneas due to interruption in the regular pattern of respiration; a process that is a normal component of the maturation of breathing. Both of these stimuli are thought to contribute to the transition from fetal to air breathing life because catecholamines, released from the chromaffin cells, play pivotal roles in the clearing of lung fluid and in the secretion of surfactant.

Indeed, Seidler and Slotkin[1] demonstrated that blocking □-adrenergic receptors during exposure of newborn rats to hypoxia severely compromised the animal's survivability. Adrenalectomy dramatically reduced neonatal rat survival during hypoxia, but blocking catecholamine release from sympathetic nerve endings did not[1]. In contrast, removing the adrenals from adult rats, did not compromise survival of these mature animals during hypoxia[1] and low O_2 also failed to initiate catecholamine secretion from adrenals that had received a more mature sympathetic innervation[7]. A final indication that sympathetic innervation plays an important role in regulating the direct

sensitivity of neonatal AMC to hypoxia comes from experiments were unilateral denervation of adult adrenals resulted in return of the non-neurogenic, hypoxia-induced catecholamine secretion from the denervated, but not the innervated gland[7].

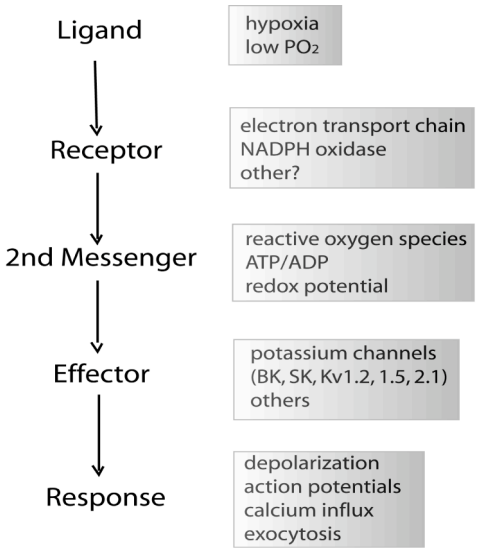

Figure 1. A general model of the O_2-signalling pathway. O_2-sensing occurs via a pathway that can be considered analogous to any ligand-receptor pathway. This model, and the proposed components (boxes), were developed from work on the carotid body glomus cell and pulmonary arteriole myocyte.

There also appears to be a role for adrenal-derived catecholamines in the activation of cardiac β-adrenergic receptors, which promotes survival of neonates during hypoxia. Administration of the β-blocker, phenoxybenzamine concomitantly with hypoxia resulted in a significant alteration of cardiac function. This was characterized by a decline in heart rate, slowing of sinus rhythm, and cardiac failure[6].

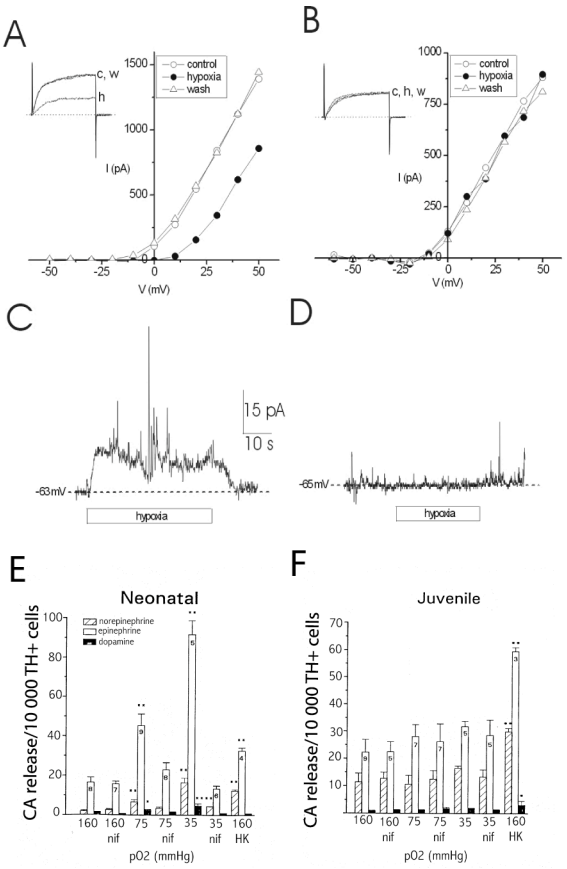

Figure 2. N**eonatal but not juvenile adrenal chromaffin cells are hypoxia-sensitive. A.** I/V plot showing the reversible suppression of outward currents by hypoxia, in neonatal AMC. Hypoxia (h) inhibits currents from the normoxic control (c) level, and the effects are reversible upon wash (w). **B.** hypoxia failed to suppress outward currents in juvenile AMC. The insets in A and B are current traces at a potential of +30 mV from the holding potential of –60 mV. **C**. hypoxia induces a receptor potential of ~ 15 mV in singly isolated neonatal, but not juvenile AMC (**D**). **E**.**&F.** hypoxia induces catecholamine secretion in cultures of neonatal but not juvenile AMC, which was inhibited by the L-type Ca^{2+} channel blocker, nifedipine (nif). Note that 30 mM K^{+} (HK) induced secretion from cells of both age groups, and indicates that juvenile AMC respond have a functional exocytosis mechanism. **C-F**

reproduced with permission from ref 3. These data demonstrate that O_2-sensing by AMC is developmentally regulated.

Taken together, these data suggest that AMC from neonatal animals express a mechanism for directly sensing blood PO_2, which can be considered an adaptation of the fight-or-flight response that is designed to promote survival at, or shortly following birth.

A general model of the O_2-signalling pathway. The work described above lead us to hypothesize that AMC express a developmentally regulated O_2-signalling pathway that mediates catecholamine secretion during hypoxia. O_2-sensing has been well characterized for a number of cell types. These include the prototypical O_2 chemoreceptor, the glomus cell of the carotid body[8], and the pulmonary arteriole smooth muscle cell, which is involved in hypoxic pulmonary vasoconstriction[9]. These specialized, or professional O_2-sensitive cells express an O_2-signalling pathway that can be considered analogous to any ligand-receptor pathway (Figure 1).

In the O_2-signalling pathway, the molecular identification of the O_2-receptor (colloquially known as the O_2-sensor) is a highly contentious issue. As indicated in Figure 1, the O_2-sensor has been proposed to constitute part of the mitochondrial electron transport chain[11-13], as a protein complex similar to the neutrophil NADPH oxidase[14,15], or as an unknown plasma membrane protein directly associated with ion channels[16]. Regardless of the identity of the O_2-sensor, which appears to be dependent upon the cell type being investigated, hypoxia (the ligand) alters that activity of the O_2-sensor, leading to alterations in a 2nd messenger. Several potential 2nd messengers have been proposed. These include reactive oxygen species (ROS) [11,12], cellular redox status[9], and a change in ATP concentration[13, 17].

The activation, or inhibition, of the 2nd messenger component of the O_2-signalling pathway during hypoxia leads to modulation of the effectors, which in turn evoke a physiological response. The effector molecules that ultimately lead to activation of the response have been

well characterized in numerous professional O_2-sensors, and are thought to be voltage-dependent K^+ channels. The types of K^+ channels known to play roles as the effectors in the O_2-signalling pathway are the large, and small conductance Ca^{2+}-dependent K^+ channels (BK and SK, respectively)[9,10,19] and delayed rectifier K^+ channels (K_v 1.2/1.5 and 2.1)[20]. Although other types of ion channels have been implicated[18], this paper will focus on K^+ channels. For AMC, the response is catecholamine secretion (see above). Whereas in glomus cells, it is the release of neurotransmitters to activate the respiratory reflex[10,18].

The O_2-signalling pathway of neonatal AMC. We initially tested the hypothesis that neonatal (postnatal day 1-2; non-innervated), but not juvenile (postnatal day 14-21; innervated) AMC express the O_2-signalling pathway. This was tested by preparing cultures of AMC from each age group and assaying them for hypoxia sensitivity using whole-cell patch clamp recording and HPLC to detect catecholamine secretion[3,13,21]. Figure 2 summarizes the effects of hypoxia on outward currents (Figure 2A and B), membrane potential (C and D) and catecholamine release (E and F) from neonatal and juvenile AMC, respectively.

Cultured AMC were initially exposed to normoxic conditions (PO_2 ~ 150 mmHg), followed by acute hypoxia (~5 mmHg for 2 min in patch clamp experiments and 1 hr for catecholamine release measurements), and then returned to normoxia. It can be seen in Figure 1A, C and E that neonatal AMC responded to hypoxia with a reversible inhibition of outward currents, membrane depolarization that was often associated with action potentials, broadening of action potential duration in spontaneously active AMC (data not shown; see reference 3), and catecholamine secretion[3, 21]. Note that in Figure 1 E, hypoxia-induced catecholamine release from neonatal AMC was blocked by 10 μM nifidepine, suggesting that Ca^{2+} influx through L-type Ca^{2+} channels may be an important step the O_2-signalling pathway. Juvenile AMC exposed to the same protocol failed to respond to hypoxia in any

of the parameters tested (Figures 1B, D and F), supporting the hypothesis that the O_2-sensitivity of AMC is regulated in some way by sympathetic innervation.

What types of K^+ channels are involved in mediating the acute O_2-sensitivity of AMC? Are the same channels involved in the inhibition of outward currents and initiation of the membrane depolarization? We used perforated patch whole-cell recording to address these questions[22]. Exposure of neonatal AMC to inhibitors of various classes of K^+ channels was used to pharmacologically identify the types of K^+ channels involved. We observed that ~ 60% of the hypoxia-sensitive outward current, IKO_2, was blocked by iberiotoxin[22], a potent and specific inhibitor of BK channels. The remaining ~ 40% of IKO_2 was blocked by TEA, indicating that it is likely composed of delayed rectifier channels. Interestingly, in the adrenal-derived MAH cell line, this delayed rectifier component appears to be comprised of 4-AP sensitive K_v 1.2/1.5 heteromultimeric K^+ channels[24]. It is still not known if K_v 1.2/1.5 channels are involved in the O_2 sensitivity of neonatal AMC. A third O_2-sensitive component of IKo_2 was found to be activated by hypoxia. This current was sensitive to glibenclamide and activated by pinacidil[22], or chromanklin[23], which tentatively identifies it as an ATP-dependent K^+ current (K_{ATP}). The molecular identity of these K_{ATP} channels is still unknown.

It was very interesting to us that none of the three components of IKO_2 appeared to contribute to the membrane depolarization (receptor potential) observed upon exposure of neonatal AMC to hypoxia[22]. Evidence for this came from current-clamp experiments in the presence of inhibitors of the various components of IKO_2. Neither iberiotoxin nor TEA blocked the receptor potential. Glibenclamide however, augmented the magnitude of the receptor potential, suggesting that K_{ATP} channels may function to limit the size of the depolarization during hypoxia, perhaps to reduce ATP consumption during this physiological stress[22, 25]. Evidence from two other labs suggests that hypoxia inhibits apamin-sensitive SK channels to initiate the receptor potential[19, 26], and

we observed that SK channels might be involved in generation of the receptor potential in MAH cells[24].

What is the O_2-sensor and 2nd messenger pathway that leads to K^+ channel modulation in neonatal AMC? To answer this, we again employed whole-cell recording, and monitored reactive oxygen species (ROS) levels in neonatal AMC with fluorometric and chemiluminescent indicators. Several lines of evidence suggest that the O_2-sensor is a component of the mitochondrial electron transport chain and the 2nd messenger is ROS. Firstly, the hypoxic inhibition of outward currents was reversed by exogenous application of H_2O_2, and blocked / mimicked by the ROS scavenger, N-acetyl-l-cysteine, which suggests that ROS are decreased during hypoxia. Second, by directly measuring ROS with 2, 7-dichlorofluorescine and luminol chemiluminescence we confirmed a role for ROS as the 2nd messenger (R.J. Thompson, J.A. Buttigieg and C.A Nurse, unpublished). Interestingly, hypoxia-induced changes in ROS were not observed in juvenile AMC. These observations, taken together with the well described modulation of K^+ channels by ROS suggests that H_2O_2 may be the important 2nd messenger in the O_2-signalling pathway of neonatal AMC (see Figure 3).

In parallel studies to the ones described in the preceding paragraph, we found that inhibitors of the mitochondrial electron transport chain that block electron flow at sites upstream (i.e. proximal) of the classical O_2 binding site in cytochrome C oxidase (complex IV) both mimic and attenuate responses of neonatal AMC to hypoxia. The proximal ETC inhibitors, rotenone, antimycin A and myxothiaxol were all found to suppress outward currents in neonatal AMC. Application of these ETC inhibitors concomitantly with hypoxia resulted in no further suppression of outward currents. Furthermore, the ETC inhibitors decreased ROS levels in neonatal AMC in a manner that was not additive with hypoxia, suggesting convergence of the two stimuli (R.J. Thompson, J.A. Buttigieg and C.A Nurse, unpublished). In contrast,

application of 5 mM cyanide, a competitive inhibitor for the O_2 binding site in complex IV, failed to mimic or block the effects of hypoxia.

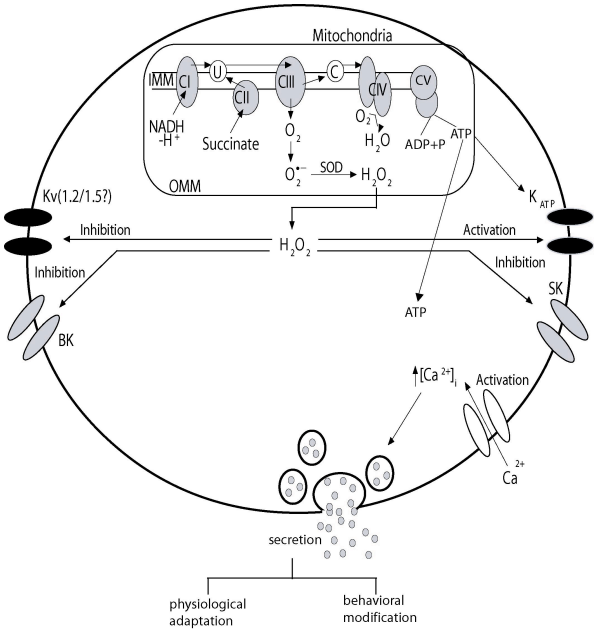

Figure 3. A working model of the O_2-sensing mechanism of neonatal adrenal chromaffin cells. Hypoxia is detected by the electron transport chain, a component of the inner mitochondrial membrane (IMM), reducing the production of superoxide radical (O_2^-). Although the image depicts ROS generation from complex III (CIII) a role of complexes I (CI) or II (CII) cannot be ruled out. O_2^- is rapidly dismutated to H_2O_2 by the mitochondrial enzyme, superoxide dismutase (SOD) and crosses the outer mitochondrial membrane (OMM). The overall decrease in cytoplamic H_2O_2 modulates plasma membrane ion channels. It is proposed that SK channels are inhibited and K_{ATP} channels activated, resulting in a receptor potential. Additionally, in spontaneously active AMC, H_2O_2 is hypothesized to inhibit large conductance Ca^{2+}-dependent (BK) and delayed rectifier (K_v 1.2/1.5) channels, which broadens action potentials. The combined receptor potential and modulation of the action potential waveform opens L-type Ca^{2+} channels, causing Ca^{2+} influx and catecholamine exocytosis into the blood.

A model of the O_2-signalling pathway of neonatal AMC, which was developed from our work and that of several other labs, is presented

in Figure 3. It can be seen that the hypoxic stimulus, occurring at birth or during apnea, is 'sensed' by the proximal mitochondrial electron transport chain. This evokes a decrease in mitochondrial ROS production and corresponding drop in ROS in the cytoplasm. It is then thought that decreased ROS differentially modulates at least four K^+ channels, resulting in a membrane depolarization, action potential generation / broadening, Ca^{2+} influx though L-type channels, and catecholamine exocytosis.

FUTURE DIRECTIONS

Some components of the O_2-signalling pathway require experimental confirmation, and several interesting questions remain unanswered. Despite an extensive understanding of the pathway at the whole-cell level, it is clear that a more molecular approach is needed to confirm the identity of the players. Future approaches will need to determine the molecular identity of the K^+ channels involved, the mechanism of how they are regulated by ROS, and if their expression is changed during the maturation of innervation.

Current evidence suggests that the loss of O_2-sensitivity during development is due to the inability of juvenile AMC to either detect hypoxia, or alter ROS levels during hypoxia. However, the identity of the O_2-sensor is not known and has only been tentatively located to a region of the electron transport chain that contains more than 50 proteins. These candidate O_2-sensors may be narrowed down to those that contain active groups such as Fe-S clusters or copper-containing proteins. This however, still leaves ~ 10 candidate proteins, and does not take into account the possibility that the loss of O_2-sensing in juvenile AMC is due to a multifaceted change in the relative abundance of several proteins and their isoforms.

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