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How does the heart sense changes in oxygen tension – a role for ion channels?

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Abstract

**Significance:** Oxygen occupies a key role in cellular metabolism and function. Oxygen delivery to cells is crucial and a lack of oxygen such as occurs during myocardial infarction can be lethal. Cells must therefore be able to respond to changes in oxygen tension. **Recent advances:** Since the first studies examining the acute cellular effect of hypoxia on activation of transmitter release from glomus or type I chemoreceptor cells, it is now known that virtually all cells are able to respond to changes in oxygen tension.

**Critical Issues:** Despite advances made in characterising hypoxic responses, the identity of the “oxygen sensor” remains debated. Recently, more evidence has evolved as to how cardiac myocytes sense acute changes in oxygen. This review will examine the available evidence in support of acute oxygen sensing mechanisms providing a brief historical perspective and then more detailed insight into the heart and the role of cardiac ion channels in hypoxic responses. **Future Direction:** Further understanding these cellular processes should lead to interventions that assist in preventing the deleterious effects of acute changes in oxygen tension such as alterations in contractile function and cardiac arrhythmia.
I. Introduction

All aerobic organisms require O$_2$ for life. Cells must therefore possess a mechanism for sensing changes in O$_2$ tension and eliciting a response. It is assumed that acute changes in O$_2$ tension will result in adaptive responses that are designed to restore O$_2$ to tissue and maintain normal cellular function. This is true of cardiorespiratory reflexes in the brain that respond rapidly to discharge levels from afferent chemosensory fibres in the carotid body as a result of alterations in blood levels of O$_2$, CO$_2$ or pH. In addition, acute changes in hypoxia may also contribute to maintaining cell survival until normoxia is restored. Prolonged periods of hypoxia however lead to more distinct remodelling of cells including changes in transcription and translation of proteins that can contribute to pathophysiological states such as dementia (1-3), heart failure (4, 5), vascular disease (6-8) and cancers (9, 10).

Cardiovascular disease remains the leading cause of death in the western world and the majority of deaths occur as sudden cardiac deaths (11). An acute change in cellular oxygen tension represents a trigger for cardiac arrhythmia where an appropriate substrate such as myocardial infarction or a defect in a gene encoding an ion channel exists (12, 13). Although significant advancement has been made in understanding how changes in O$_2$ tension alter the function of glomus cells of the carotid body (14, 15), and the pulmonary (16) and renal vasculature (17, 18), the identity of the “oxygen sensor” remains controversial. This is particularly pertinent with respect to the heart since it is well recognised that this organ is critically dependent on O$_2$ for normal excitation and contraction. A great deal has been studied and written about how the heart responds to a hypoxic environment. Despite this, it is still
not clear how cardiac myocytes sense changes in $O_2$ tension. That is, the mechanism/s by which the cell detects acute changes in oxygen tension and translates this into a physiological response. Over the past 15-20 years a number of mechanisms have been proposed for oxygen sensing in the heart. This review will revisit the early hypotheses of oxygen sensing with reference to ion channels and argue the evidence for and against oxygen sensing sites in cardiac myocytes with recent data as support for the arguments. The review will examine the mechanisms for acute oxygen sensing only. That is, physiological responses that occur within seconds of exposure to hypoxia and are not prolonged for hours or days. Ion channels are capable of responding very rapidly to changes in the cell’s environment, and rapid changes in oxygen tension can represent acute triggers for sudden cardiac death. Since the mechanism for oxygen sensing is largely unresolved in cardiac tissue, some evidence for mechanisms of oxygen sensing will be drawn from experimental data from other systems. Therefore the review will initially provide some background to mechanisms that have been debated in other systems such as a role for a heme protein in hypoxic sensing or hydroxylation of a protein with reference to ion channels. Possible direct effects of oxygen and carbon monoxide on ion channels will be discussed followed by the evidence for indirect effects of hypoxia via alterations in cellular reactive oxygen species. These include a role for the reactive oxygen species generating mechanisms NAD(P)H-oxidase and mitochondria. Finally the implications for acute hypoxia on the cardiac action potential are discussed.

II. **Oxygen sensing mechanisms**
A. A heme protein or hydroxylation of a protein?

For some time it was thought that a reduction in binding of O\textsubscript{2} to a heme protein initiated the synthesis of erythropoietin in the kidney and an increase in red blood cell production (19). This was based on the observation that rats injected with cobalt chloride exhibited polycythemia and increased erythropoietin production. It was concluded that cobalt bound to heme and mimicked hypoxia by changing the protein to a deoxy configuration. In addition, it was thought that the posttranslational stabilization of the hypoxia-inducible factor-1 (HIF-1) \( \alpha \) subunit during hypoxia involved a heme protein. HIF-1 binds to DNA sequences within hypoxia response elements as a basic helix-loop-helix PAS heterodimer and appears to be involved in a wide range of cellular responses involving DNA binding and transcription of a number of genes during hypoxia. Some of these include glucose transporters, glycolytic enzymes, vascular endothelial growth factor, erythropoietin, inducible nitric oxide synthase (NOS II), and insulin-like growth factor 2 (IGF-2) (20).

However, the induction of erythropoeitin and the stabilization of HIF-1 do not involve a heme protein. Prolyl hydroxylation at a conserved core LXXLAP motif of the oxygen degradation domain of HIF-1 results in proteasomal degradation of the HIF-1 \( \alpha \) subunit (21). The prolyl hydroxylase is a 2-oxoglutarate-dependent dioxygenase that has an absolute requirement for dioxygen as co-substrate. In addition, the prolyl hydroxylation requires iron as a co-factor. This accounts for the effect of transition metals such as cobalt, manganese and nickel, and iron chelators such as desferrioxamine on HIF-1 induction. HIF-1 \( \alpha \) protein levels are induced in an organ-specific manner with expression in the mouse heart clearly detectable after 1 hour of exposure to
hypoxia (22). The effects of HIF-1 on the regulation of cellular transcription typically occur over many hours. However the hydroxylation reaction (and degradation of HIF-1 α occurs much more rapidly. Therefore prolyl hydroxylase serves as an excellent candidate for acute O₂-sensing.

The onset of hydroxylation of prolines within HIF-1 is a rapid reaction. An involvement of protein hydroxylation in the regulation of ion channel function during hypoxia is not well characterised. However hydroxylated residues may be important determinants of channel function since removal of hydroxyl groups in the pore forming region of the α₃ subunit of the human glycine receptor is crucial to receptor channel inactivation (23). In addition, hydroxylated amino acids form the narrow region of the pore of the acetylcholine receptor channel and a hydroxylated serine is a main determinant of channel conductance (24). It was recently identified that the transient receptor potential channel TRPA1 is hydroxylated by HIF during normoxia in Drosophila Melanogaster leading to reduced channel activity and internalization of the channel protein (25). During hypoxia the channel becomes activated and internalization is reversed. TRPA1 channels are not expressed in the heart. However, the channels can influence cardiovascular responses through effects on the vasculature (26). Despite the rapid nature of the prolyl hydroxylation reaction, it does not appear to play a significant role in regulation of cardiac function during acute hypoxia.

A heme protein has been implicated in hypoxia responses and in the regulation of maxiK channels in the carotid body (27, 28) and hypoxic pulmonary vasoconstriction (29). Inhibition of maxiK channels occurred in excised patches leading to the conclusion that the response (and location of
the heme \( O_2 \) sensor) was membrane-delimited. The protein responsible for sensing the change in oxygen was identified as a heme oxygenase-2 (30). The role of heme oxygenase-1 and heme oxygenase-2, both of which are expressed in the heart is still being fully elucidated (31). Heme oxygenase-1 over expressing mice are protected against ischemia reperfusion injury and develop less remodelling in the heart (32) while heterozygote knockout mice have larger infarcts after ischemia reperfusion (33). Heme oxygenase-2 may partner with biliverdin reductase to prevent \( \beta \)-adrenergic receptor induced apoptosis in the heart (34). However these responses occur over many hours to days and are adaptive responses to chronic hypoxia.

With respect to rapid responses, a direct binding of heme oxygenase to the \( Ca^{2+} \)/calmodulin-dependent protein kinase II-specific motif on the C terminal end of the L–type \( Ca^{2+} \) channel has been implicated in hypoxic regulation of the channel in the heart (35). The degradation of heme leads to the generation of ferrous iron, biliverdin and carbon monoxide (Fig. 1). Significant attention has been placed on understanding the roles of these three products in mediating physiological responses to hypoxia. Ferrous iron induces ferritin expression, that is necessary for iron sequestration. It is recognised that biliverdin has antioxidant and antiapoptotic properties and may also act through the regulation of microRNA’s (36). Carbon monoxide mediates cardioprotection and appears to act in a similar manner to nitric oxide. Although conflicting responses have been reported, carbon monoxide can regulate the function of a number of ion channels (37). Much of the data describing effects of carbon monoxide on ion channel function have been derived from expression systems and therefore the net effect on native cell
function or system physiology is still being elucidated. With reference to
cardiac ion channels, since the large conductance Ca\(^{2+}\) -activated K\(^{+}\) channel
is expressed in the heart it can be assumed that carbon monoxide activates
the channels, while decreasing K\(_{v}2.1\) channels (38) and L-type Ca\(^{2+}\) channels
(39). The net effect of carbon monoxide on the action potential is to induce
early afterdepolarizations as a result of increasing Na\(^{+}\) conductance through
the late Na\(^{+}\) current (40). Although the 2 pore domain potassium channel
TREK-1 is expressed in the heart it has not been demonstrated that carbon
monoxide regulates cardiac TREK1 channels.

The mechanism by which carbon monoxide confers changes in ion
channel function remains unclear. One mechanism is through activation of
signalling molecules such as nitric oxide synthase (that produces nitric oxide)
(37), MAP-kinase (41) and by altering mitochondrial production of reactive
oxygen species through inhibition of mitochondrial cytochrome oxidase C
(39). The alteration in channel function occurs as a result of reactions of
signalling molecules with cysteines, threonines or serines on the channel.
Since carbon monoxide is produced by a heme oxygenase-2 that can co-
localise with BK\(_{Ca}\) channels (30), it has been suggested that carbon monoxide
can directly alter channel function by binding to a metallo-thiol region within
the RCK2 domain of the channel (42, 43). However this region also confers
sensitivity to calcium (44) so could be regulated indirectly through an effect of
carbon monoxide on calcium regulatory proteins. It has been suggested that
carbon monoxide reacts directly with amino acids on the channel protein.
However a direct effect of carbon monoxide on channel function must be
channel specific in order to explain the variability in responses reported. We
tested whether carbon monoxide can directly alter the function of the L-type Ca\textsuperscript{2+} channel alpha subunit. We expressed the human long NT isoform of Ca\textsubscript{v}1.2 in HEK cells, purified the channel protein and then reconstituted the protein in liposomes for functional assessment using patch-clamp technique. Under these conditions we can assess changes in single channel activity in the absence of other regulatory proteins and assess whether carbon monoxide can directly bind and alter channel function. In each patch, we confirm that the current is derived from Ca\textsubscript{v}1.2 channels by measuring the magnitude of the current while voltage stepping the channel, calculating open probability (P\textsubscript{o}) and assessing the sensitivity of the channel to L-type Ca\textsuperscript{2+} channel antagonist nisoldipine (Fig. 2). The channel protein is also identified as a 240 kDa band on immunoblot when probed with the L-type Ca\textsuperscript{2+} channel antibody (Fig. 2D). We applied the carbon monoxide donor CORM-2 at a concentration of 30 µM directly to the channel incorporated in liposomes in the bath for at least 30 minutes and recorded changes in current. This is a concentration that is effective at inducing vasorelaxation in aortic rings (45) and positive inotropic effects in isolated perfused hearts (46). We found no alteration in P\textsubscript{o} or magnitude of current at voltage steps of -200mV to +200mV suggesting that carbon monoxide cannot directly alter channel function (Fig. 3). This is consistent with the finding that nifedipine failed to prevent the increase in systolic pressure and inotropic effects in isolated hearts exposed to carbon monoxide donors (46). It also suggests that the effects of carbon monoxide on L-type Ca\textsuperscript{2+} channel function occur via secondary or indirect modulation of signalling pathways in the heart (rather than direct effects of CO on the channel protein) and is consistent with effects mediated via alterations
in reactive oxygen species (39). The mechanisms for the effect of carbon monoxide on K⁺ channels and Na⁺ channels in the heart are still being elucidated.

B. The “O₂-sensitive ion channel”

Ion channels have been considered O₂ sensors because their modulation by hypoxia is rapid and occurs in excised membrane patches where cytosolic variables such as second messengers, ATP and Ca²⁺ are thought to be absent. A number of studies have reported modulations in ion channel activity during hypoxia. The first report of an effect of hypoxia on an ion channel was the characterization of O₂-sensitive K⁺ channels in glomus or type I cells of the rabbit carotid body (47). O₂-sensitive K⁺ channels have been found in a number of neurosecretory cells (48-51). The closure of K⁺ channels by hypoxia results in membrane depolarization and Ca²⁺ influx, transmitter release to the innervated organ and activation of afferent sensory fibres.

In neurosecretory cells such as chromaffin cells, hypoxia does not appear to influence Ca²⁺ channels directly. Ca²⁺ influx occurs as a result of membrane depolarization. A similar response is observed in hypoxic pulmonary vasoconstriction as a mechanism to reduce poor ventilation and prevent systemic hypoxemia where atelectasis is present (8, 52). This is not the case however in some pulmonary resistance vessel myocytes where hypoxia increases Ca²⁺ channel conductance (53-55). In fact, ion channel responses to hypoxia appear to vary considerably depending on the cell type (56). Ion channels with PAS domains such as the human ether-a-go-go related (HERG) K⁺ channel do not appear to be modulated by hypoxia (57,
In arterial smooth muscle cells, hypoxia appears to inhibit L-type Ca\(^{2+}\) channels resulting in relaxation of vessels (59, 60). The varied responses by ion channels to changes in O\(_2\) tension has made it difficult to assign a universal O\(_2\)-sensing component to the channel. In addition, there is no experimental or molecular evidence to suggest that the channel protein is capable of directly sensing changes in oxygen tension.

Hypoxia can induce changes in protein function in the absence of cellular metabolic inhibition. Because oxidative phosphorylation and ATP production does not become compromised until oxygen tension falls below 2 mmHg (61, 62) many hypoxic responses occur in the absence of alterations in cellular ATP or adenosine concentrations. ATP-sensitive K\(^+\) channels contribute to repolarization of cardiac myocytes and were originally described as being activated upon depletion of cellular ATP (63). However, it has been suggested that the plasmalemmal channels may also be activated during hypoxia/ischemia in the absence of depletion of ATP and influence cardiac contractility (64). The role of the channels in hypoxic responses remains unclear because myocardial infarct size is unchanged in Kir6.2 null mice suggesting that the channels are not protective against necrotic injury (64). Activation of mitochondrial but not plasmalemmal ATP-sensitive K\(^+\) channels has been implicated in ischemic preconditioning and the mechanisms involve protein kinase C (PKC) (65-68).

Whether hypoxia directly alters the function of ATP-sensitive K\(^+\) channels is unclear. A considerable amount of evidence is now available to suggest that ion channel function can be modified by redox state. The ATP-sensitive K\(^+\) channel may undergo modification of thiol groups during hypoxia.
resulting in an alteration in channel function. In support of this, the sulfhydryl agents 5,5′-dithio bis-(2-nitro-benzoic acid) (DTNB) and N-ethylmaleimide have been shown to alter channel function (69). Other cardiac ion channels appear to be modified during hypoxia as a result of altered cellular redox state. Hypoxia has been shown to increase a persistently inactivating Na⁺ current in rat ventricular myocytes and central neurones that is postulated to involve redox modification of the channel protein or an associated nearby protein (70-72).

In cardiac myocytes, there is good evidence that hypoxia inhibits the L-type Ca²⁺ channel. This has been demonstrated in HEK 293 cells expressing the α₁C subunit of the human cardiac L-type Ca²⁺ channel (35, 73) and in native channels in guinea-pig cardiac myocytes (74, 75) suggesting that there is a requirement for the α₁C subunit of the channel in the O₂-sensing mechanism. In search for the identity of the O₂-sensing site, Fearon et al (76) studied 3 splice variants of the α₁C subunit of the human cardiac L-type Ca²⁺ channel. They were able to isolate a 39 amino acid segment of the C terminal domain of the hHt isoform that appeared to be required for inhibition of the channel during hypoxia.

We tested whether the alpha subunit was sufficient for hypoxic inhibition of the human cardiac L-type Ca²⁺ channel. We reconstituted purified human long N terminal (NT) isoform of the Ca₂⁺,1.2 in proteoliposomes and tested the effect of altering oxygen tension on single channel activity (Fig. 4). The long NT isoform lacks exon 45 that is proposed to confer oxygen sensitivity (76). Decreasing oxygen tension from room oxygen (150 mmHg) to 15 mmHg had no effect on P₀ of the long NT isoform. In addition hypoxia did
not alter the $P_o$ of the short NT hHt isoform that contains exon 45, despite inducing a reversible effect on the native channel in guinea pig myocytes (Fig. 5). These data indicate that the $\alpha_{1C}$ subunit of the channel cannot respond directly to changes in oxygen tension implicating an alternative mechanism or regulatory intermediate protein in hypoxic inhibition of the L-type Ca$^{2+}$ channel.

The L-type Ca$^{2+}$ channel does not possess an O$_2$ binding domain. However the $\alpha_{1C}$ subunit contains a number of critical thiol groups that can be modified in response to alterations in cellular redox state that occurs during hypoxia. In fact there are approximately 53 cysteine residues on the Cav1.2 although not all of these will be freely available to react because some are embedded in the lipid bilayer (Fig. 6). Therefore alterations in channel function may occur because of modifications in thiol groups critical to function. Consistent with this, we and others have shown that alterations in cellular production of O$_2^-$ and H$_2$O$_2$ can modulate ion channel function (77-80). Redox modification of channel function has been demonstrated in native channels where all channel subunits are present (74, 75, 79, 81) and in the purified Cav1.2 subunit of the channel reconstituted in proteoliposomes (82). Direct modification of thiol groups on the channel with application of DTNB or oxidised glutathione (GSSG) directly increases $P_o$ while dithiothreitol and reduced glutathione (GSH) decrease $P_o$ (Fig. 7). We have demonstrated that oxidative stress is associated with an increase in channel activity as a result of direct glutathionylation of the Cav1.2 subunit (82). In addition Cav1.2 is glutathionylated in human ischemic heart (82). Given that O$_2$-sensitivity is not a universal characteristic of ion channels, and ion channels respond
differentially to hypoxia, it appears that ion channels are not oxygen sensors
per se but effectors responding as a consequence of the signal from the
putative sensor. Reactive-oxygen species are capable of acting as signalling
molecules. In addition, changes in cellular oxygen tension are associated with
alterations in cellular redox state. Therefore, possible candidates for O$_2$-
sensing must also include those that are capable of generating reactive-
oxygen species as signals.

C. Reactive-oxygen species as signals

Reactive-oxygen species (ROS) can act as mediators of hypoxic responses.
The term ROS generally groups oxygen molecules in different redox states
and electronic excitation as well as compounds of oxygen with hydrogen and
nitrogen. The production of ROS begins with the reduction of O$_2$ to superoxide
anion (O$_2^-$). O$_2^-$ is not an oxidant at physiological pH. It is highly solvent in
water and does not cross lipid membranes (83). O$_2^-$ is dismutated to H$_2$O$_2$ by
superoxide dismutase (SOD). Cardiac myocytes express a cytosolic copper-
zinc form of SOD and a mitochondrial manganese form of SOD (MnSOD)(84).
The presence of these forms of SOD ensure that O$_2^-$ concentrations are
maintained at picomolar concentrations within the cell (85). H$_2$O$_2$ is however
an excellent candidate for a signalling molecule as it is small and stable and
freely crosses membranes to its target cells. It is itself an oxidant but can also
react with iron via a Fenton reaction to produce hydroxyl radical, a powerful
oxidant. Other candidates for ROS include nitric oxide and peroxynitrite anion
(ONOO$^-$) formed as a result of reaction between O$_2^-$ and nitric oxide. Cardiac
myocytes possess 3 isoforms of nitric oxide synthase (NOS) resulting in the
production of nitric oxide (NO). Types I and III are constitutive and type II is inducible by exposure to cytokines (86, 87). Both NO and ONOO\textsuperscript{−} represent oxidising agents as they can react with target proteins and modify function by oxidation of critical thiol groups.

ROS play an important role in hypoxic responses by modulating cell signaling pathways, and when produced at high levels inducing cell death. ROS interact with cell signaling pathways by way of modification of key thiol groups on proteins that possess regulatory functions. The target proteins may be second messengers such as tyrosine and MAP kinases, tyrosine or serine/threonine protein phosphatases and transcription factors such as NF-κB (88). ROS also interact with lipids to produce lipid radicals and phospholipases to modulate the activity of arachidonic metabolites that play an integral role in ischemic myocardium (88).

Considerable controversy exists as to whether cellular ROS production increases or decreases during hypoxia. The high reactivity, variable diffusion rate across cell membranes and the instability of ROS make them extremely difficult to detect in cellular systems. Whether cellular ROS increase or decrease during hypoxia it is clear that ROS act as signaling molecules and they are capable of altering cellular redox state and in turn altering protein function. The activity of intracellular second messengers is under the tonic regulation of cellular ROS. For example, epidermal growth factor and insulin-induced intracellular H\textsubscript{2}O\textsubscript{2} formation are dependent upon the inhibition of protein tyrosine phosphatase activity by H\textsubscript{2}O\textsubscript{2}. Tyrosine phosphatase activity is inhibited via reversible oxidation of its catalytic cysteine (89). Under conditions where cellular levels of H\textsubscript{2}O\textsubscript{2} are decreased, oxidation of the
catalytic cysteine is reversed and tyrosine phosphatase activity would then increase. Therefore the cellular redox state, being a balance between cellular ROS generation and antioxidant activity determines protein catalytic activity and ultimately functional responses. This is particularly pertinent in the heart where glutathione is highly abundant and is a potent antioxidant when ROS levels are increased such as during reperfusion after ischemia and in the failing heart.

Two major contributors to the production of ROS are the NAD(P)H-oxidase complex and the mitochondria. These two contributors of ROS production are also potential \( \text{O}_2^- \)-sensors.

\section*{D. NAD(P)H-oxidase as \( \text{O}_2^- \)-sensor}

NAD(P)H-oxidase has been proposed to be a putative \( \text{O}_2^- \)-sensor because the complex is a major source of production of \( \text{O}_2^- \) in vascular tissue as compared with ROS produced from xanthine oxidase, arachidonic acid and mitochondrial oxidases (90). The complex is a membrane-associated enzyme that reduces \( \text{O}_2 \) to \( \text{O}_2^- \) with the transfer of one electron from NADH or NAD(P)H. \( \text{O}_2^- \) is then converted to \( \text{H}_2\text{O}_2 \) by superoxide dismutase. Therefore \( \text{H}_2\text{O}_2 \) serves to link the \( \text{O}_2^- \)-sensor with the target protein such as an ion channel.

Although the orientation and subunit composition of the enzyme may vary in different tissues, cardiac myocytes appear to express a membrane-bound NAD(P)H-oxidase that is a source of cytosolic \( \text{O}_2^- \) production (91, 92). The complex is composed of the electron transfer components (gp91phox and
p22phox) and the regulatory p67phox and p47phox subunits. The low molecular weight G protein rac also regulates function of the enzyme.

The oxidase has been postulated to act as a sensor in the regulation of ion channels because the oxidase inhibitor diphenylene iodide (DPI) prevents the inhibition of K+ channels by hypoxia (49, 93). The problem with this hypothesis is that DPI also inhibits K+ channels (94), and a wide range of flavin-containing oxidases within the cell. In addition, DPI should serve to mimic the hypoxic response. Knockout mice that lack the gp91phox subunit demonstrated a decrease in the generation of ROS but retained the inhibition of K+ currents and hypoxic pulmonary vasoconstrictor response (95). Additional evidence against its role as a sensor comes from patients who suffer from chronic granulomatous disease where one or all of the subunits of the oxidase are absent have normal erythropoietin levels and retain hypoxic vascular responses. Cells derived from these patients are still able to express vascular endothelial growth factor and aldolase mRNA in the presence of 1% O2 (96). These studies suggest that other isoforms of the oxidase may be involved in O2-sensing in these patients.

A role for NAD(P)H-oxidase in acute O2-sensing in cardiac myocytes has not been established. In vascular smooth muscle angiotensin II is a potent activator of NAD(P)H-oxidase and there is good evidence that ROS produced as a result of activation of the oxidase mediates the development of vascular smooth muscle cell hypertrophy, proliferation and migration (97). In cardiac myocytes it has been shown that hypertrophy induced by chronic exposure to glucose (98), α1-adrenergic receptor stimulation (99), endothelin-1 or phenylephrine (100, 101) involves signalling by ROS produced from activation
of NAD(P)H-oxidase. Since the rate at which NADP(H)-oxidase produces O$_{2}^{$- is dependent upon the available substrate (oxygen), hypoxia is associated with less production of O$_{2}^{$- and a reduced cellular redox state. Reoxygenation results in an increase in production of O$_{2}^{$- and a shift to an oxidized cellular redox state. NAD(P)H-oxidase has been implicated as being responsible for bursts of ROS associated with reperfusion injury in cardiac myocytes however this remains contentious (102-104). The heart expresses Nox2 and Nox4 isoforms but knockout mice exhibit similar injury after ischemia reperfusion to wt mice (105). We tested whether acute activation of NAD(P)H-oxidase with angiotensin II or thrombin could alter cellular superoxide in ventricular myocytes in the presence or absence of hypoxia. Hypoxia (a decrease in oxygen from 150 to 15 mm Hg) decreased superoxide in myocytes assessed as changes in dihydroethidium fluorescence or 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (DCF; Fig. 8A-C)(74, 106). The myocytes possessed functional NAD(P)H-oxidase protein because DPI and apocynin decreased consumption of NADPH (Fig. 8A). However addition of angiotensin II up to 5 µM concentration could not acutely increase dihydroethidium signal in the myocytes (Fig. 8D and E) despite a robust increase recorded in rat neurons (Fig. 8F). This would suggest that although the protein is functional, NAD(P)H-oxidase does not participate in acute oxygen sensing in guinea pig myocytes. However there is evidence that the oxidase participates in prolonged (chronic) alterations in cellular redox state when the expression of the protein is increased in response to agonists such as angiotensin II and endothelin I during hypertrophic growth (107).

E. Mitochondria as O$_{2}^{-}$-sensors
It has been argued that the mitochondria are good candidates for $O_2^-$ sensing since they are capable of rapidly binding and utilising $O_2$. In addition, electron transport and oxidative phosphorylation cease in the absence of $O_2$. The original basis for the mitochondrial $O_2$-sensing hypothesis was that cyanide (which blocks cytochrome oxidase c) stimulated the carotid body (108, 109). It has been suggested that hypoxia induces a decrease in $V_{max}$ for cytochrome oxidase resulting in an increase in $O_2^- \cdot$ production due to electron transfer at ubisemiquinone site (110, 111). Under hypoxic conditions, it is proposed that electron transfer by cytochrome oxidase becomes inhibited and more ubisemiquinone is generated resulting in an increase in generation of superoxide due to electron transfer to oxygen by ubisemiquinone. The response is lost in cells depleted of mitochondrial DNA (rho(0) cells). However the shift in $V_{max}$ for cytochrome oxidase is dependent upon the available oxygen, the pool of ubisemiquinone, the reduced state of the electron transfer proteins, the mitochondrial membrane potential and limitations regarding the measurement of $O_2^- \cdot$ production (112-114). Increases in ROS production were not evident until 1-2 hours after induction of hypoxia in the cardiomyocytes implying that the duration of hypoxia is also an important consideration (115, 116). Acute exposure to hypoxia (less than hour) does not cause a decrease in $V_{max}$ of cytochrome oxidase. In addition the response of ion channels occurs more rapidly than the rate of inhibition of cytochrome oxidase and a number of ion channels are responsive to levels of hypoxia well above the level that compromises mitochondrial respiration (80 mm Hg vs 2-3 mm Hg). Thiol-specific reducing agents and reduced glutathione mimic the effect of hypoxia on ion channels (74, 75, 79, 81, 117, 118) and consistent with a
decrease in availability of molecular oxygen, a number of groups have also reported that hypoxia results in a decrease in production of ROS (119-123). At least for the renal and pulmonary arteries, it has been argued that the differential responses to hypoxia are due to differential expression of mitochondrial electron transport chain components between cell types (18). It is well recognised that the electron transport chain contributes to the production of superoxide anion by transferring an electron to molecular oxygen. It is on this basis that the electron transport chain is considered to be the oxygen sensor in mitochondria and capable of responding rapidly to acute changes in O_2 tension in cardiac myocytes. When the reduction of oxygen is limited at complex III with application of the complex III inhibitor myxothiazol, or when the electron transport chain is collapsed with application of the cyanide derivative FCCP, superoxide production is decreased (18, 74, 106). This mimics the effect of hypoxia in ventricular myocytes (74, 79, 106).

Superoxide anion is very rapidly dismutated to H_2O_2 and H_2O_2 is a stable signalling molecule capable of altering cysteines on target proteins. A decrease in H_2O_2 associated with hypoxia then alters ion channel function either by direct modification of critical thiol groups on the channel protein or by modification of an intermediate protein. This is postulated because dithiothreitol mimics the effect of hypoxia on ion channels (see also Fig. 7) (74, 75, 79, 81, 82, 117, 118) and decreasing intracellular H_2O_2 by perfusing myocytes intracellularly with catalase, (a naturally occurring antioxidant that converts H_2O_2 to water and oxygen) also mimics the effect of hypoxia (79). Therefore mitochondria can rapidly alter cellular redox state in response to
variations in availability of oxygen that then leads to alterations in ion channel function.

**III. Implications of hypoxia on the action potential**

Variability in delivery of oxygen can lead to electric instability in the myocardium and the generation of arrhythmias (13). A rapid decrease in oxygen supply to cardiac myocytes from 150 to 15 mm Hg is not energy limiting and does not deplete ATP (61) but can alter the function of a number of cardiac ion channels (57, 70, 71, 73-75, 79, 124-126). Under these conditions hypoxia increases late Na$^+$ current while decreasing fast Na$^+$ current in rat ventricular myocytes (70, 71, 125). It has been proposed that the increase in late Na$^+$ current may be arrhythmogenic (127). In addition, acute hypoxia decreases the basal current through L-type Ca$^{2+}$ channels (73-76, 79, 124, 128) and the slow component of the delayed rectifier K$^+$ channel without affecting the rapid component (57). Application of reduced glutathione or dithiothreitol mimic the effects of hypoxia on late Na$^+$ current (125), L-type Ca$^{2+}$ channel(74, 75) and delayed rectifier K$^+$ channel (57) confirming that the ion channels can respond to changes in cellular redox state in response to alterations in oxygen tension. However when the net effects of acute hypoxia (pO$_2$ of 15-20 mmHg) on Na$^+$, Ca$^{2+}$ and K$^+$ channels are taken into consideration, very little change in action potential duration occurs (Fig. 9A) (129). It is well recognised that ischemic heart disease and angina are associated with an increase in circulating catecholamines that further increases the risk of developing ventricular tachyarrhythmias (130). When the effects of β-adrenergic receptor stimulation on Na$^+$, Ca$^{2+}$ and K$^+$ channels are taken into account, hypoxia causes a prolongation of the action potential that
leads to induction of early afterdepolarizations and spontaneous tachycardia. This can be induced experimentally in ventricular myocytes (Fig. 9B-D) and in silico (129). The data are consistent with an increase in sensitivity of the L-type Ca\(^{2+}\) channel to isoproterenol when myocytes are exposed to dithiothreitol (74) or perfused intracellularly with catalase (79). This provides clinically relevant insight into the effects of acute hypoxia in the heart during increased adrenergic stimulation. β-Blockers are the only class of antiarrhythmics that can decrease mortality and the mechanism by which they appear to reduce arrhythmias is by decreasing adrenergic stimulation of the L-type Ca\(^{2+}\) channel during hypoxia (130). This is consistent with studies that demonstrate that decreasing calcium influx through L-type Ca\(^{2+}\) channels or inhibition of protein kinase A (130) or Ca\(^{2+}\)/calmodulin-dependent protein kinase II (131-133) can reduce the incidence of early afterdepolarizations.

IV. Concluding remarks

The energy demands of the heart are high. Mitochondria are abundant in the myocardium and are required to meet the energy demands of the heart by supplying ATP during oxidative phosphorylation. In the process they also reduce molecular oxygen to superoxide anion that is rapidly converted to H\(_2\)O\(_2\). The production of reactive species then influences the redox state of the cell and target proteins such as ion channels that posses reactive cysteines. Therefore mitochondria are ideal candidates for acute oxygen sensing in the heart. However, many proteins (including ion channels) can also be modified by nitric oxide and carbon monoxide that can vary during alterations in oxygen tension. It is recognised that there are many mechanisms by which cells respond to acute changes in oxygen tension.
Understanding the mechanisms associated with $O_2$-sensing and the interaction between sensors and effectors in cardiac myocytes will unravel the pathophysiological processes involved in the induction of arrhythmia. Chronic hypoxia can induce remodelling of the myocardium and development of cardiac hypertrophy and cardiac failure. Under these circumstances alternative oxygen sensing mechanisms may predominate. Understanding oxygen sensing that is specific to the disease process will enable strategies to promote adaptive responses to hypoxia in the myocardium and prevent the deleterious effects associated with $O_2$ deficits.
Acknowledgments

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Author Disclosure Statement

There are no disclosures.
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Figure Legends

Figure 1. Proposed mechanisms of action of heme oxygenase-1 and heme oxygenase-2 in ventricular myocytes. Heme oxygenase can directly bind to the Ca\(^{2+}\)/calmodulin-dependent protein kinase II-specific motif on the C terminal end of α subunit of the L–type Ca\(^{2+}\) channel (Ca\(_v\)1.2). Alternatively heme oxygenase is converted to ferrous iron (Fe\(^{2+}\)), carbon monoxide and biliverdin. Of the three products carbon monoxide regulates signalling through alterations in production of reactive oxygen species (ROS) from the mitochondria and nitric oxide (NO) production via soluble guanylate cyclase (sGC) or nitric oxide synthase (NOS). Carbon monoxide also influences MAPK and protein kinase A (PKA) activity. For further detail please see text.

Figure 2. Purified human long NT isoform of Ca\(_v\)1.2 channel reconstituted in liposomes can be assessed using single channel patch-clamp technique. (A) Representative single-channel currents recorded at +100 mV. The channel is open in an upward direction. ‘C’ denotes the channels’ closed state and ‘O’ the open state of the respective channel (n) contributing to the total current obtained in the recording. A current-amplitude frequency histogram constructed from 20 s of single-channel data recorded at +100 mV is shown below. (B) Representative single-channel currents recorded at varying pipette potentials as indicated. Nisoldipine (nisol) abolished channel activity in the patch. External and pipette solutions contained 100 mM BaCl\(_2\), 50 mM NaCl, 10 mM Hepes, 2 µM BayK8644, pH 7.4 at 22 °C. (C) I–V relationship recorded from the same patch in the absence and presence of nisoldipine. (D) Immunoblot of the long NT isoform
of the Ca\textsubscript{v}1.2 channel protein indicated at approximately 240 kDa when probed with anti-Ca\textsubscript{v}1.2 antibody (lane 1) and protein from HEK293 cells expressing pcDNA3.1 plasmid minus α1C,77L-His (lane 2). Reproduced with permission from Ref. 82.

**Figure 3.** The carbon monoxide donor CORM2 does not directly alter the open probability (P\textsubscript{o}) of the purified human long NT isoform of Ca\textsubscript{v}1.2 reconstituted in liposomes. (A) Representative single-channel currents recorded at +100 mV in control Ca\textsubscript{v}1.2 protein (upper trace) and in Ca\textsubscript{v}1.2 channel protein exposed to 30 µM CORM2 (middle trace). The L-type calcium channel antagonist nisoldipine (Nisol) attenuated the openings (lower trace). External and pipette solutions contained 100 mM BaCl\textsubscript{2}, 50 mM NaCl, 10 mM Hepes, 2 µM BayK8644, pH 7.4 at 22 °C. (B) Mean ±SE of alterations in P\textsubscript{o} relative to P\textsubscript{o} recorded in control Ca\textsubscript{v}1.2 (at left) and I–V relationship recorded from Ca\textsubscript{v}1.2 channels in the absence and presence of CORM2.

**Figure 4.** Hypoxia does not directly alter the open probability (P\textsubscript{o}) of the purified human long NT isoform of Ca\textsubscript{v}1.2 or the short NT isoform of Ca\textsubscript{v}1.2 reconstituted in liposomes. (A) Representative single-channel currents recorded at +100 mV in control long NT isoform of Ca\textsubscript{v}1.2 protein in room oxygen (upper trace) and during exposure to hypoxia (pO\textsubscript{2} 15 mmHg) (lower trace). External and pipette solutions contained 100 mM BaCl\textsubscript{2}, 50 mM NaCl, 10 mM Hepes, 2 µM BayK8644, pH 7.4 at 22 °C. (B) Mean ±SE of alterations in P\textsubscript{o} during hypoxia relative to P\textsubscript{o} recorded in control long NT isoform and short NT isoform as indicated (at left) and I–V relationship.
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**Figure 5.** Exposure of guinea-pig ventricular myocytes to hypoxia induces a rapid decrease in basal macroscopic L-type Ca\textsuperscript{2+} current. (A) Time course of changes in membrane current recorded during exposure to hypoxic Tyrode’s solution (pO\textsubscript{2} of 17 mm Hg). a, b, and c refer to time points at which membrane currents (shown in B) were recorded. (B) Membrane currents recorded at time points in protocol illustrated in panel A. (C) Mean±SE of current-voltage (I\textendash-V) relationships measured in 9 cells during voltage steps from -60 mV to +80 mV. I\textendash-Vs were recorded in 2 mM nisoldipine (nisol) to indicate that calcium currents recorded were L-type. Reproduced with permission from Ref. 74.

**Figure 6. Schematic representing positions of cysteines on the long NT isoform of Ca\textsubscript{v}1.2 channel protein.** Cysteines are shown as black dots. Ca\textsubscript{v}1.2 protein (\(\alpha\) subunit) is comprised of 4 heterotetramers (I – IV) each with 6 transmembrane spanning regions (S1-S6). P= ion conducting pore region. Voltage sensing region S4 is denoted with +++

**Figure 7.** Modification of thiol groups on the human long NT isoform of Ca\textsubscript{v}1.2 alters function. (A) Representative single-channel currents recorded at -100 mV in the absence and presence of 200 \(\mu\)M DTNB followed by 1 mM DTT and then 2 \(\mu\)M nisoldipine, including the \(P_o\) for each treatment as shown. (B) Means±SE of channel open probability for all patches exposed to DTNB,
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Figure 8. Adult guinea pig cardiac myocytes express functional NAD(P)H oxidase but cannot increase superoxide in response to stimulation by angiotensin II. (A) NADPH consumption in the presence of 100 µM DPI and 500 µM apocynin (Apo) assessed by spectrophotometry from 5 hearts. (B). Hypoxia is associated with a decrease in cellular superoxide. Dihydroethidium (DHE) fluorescence recorded from a cell exposed to normoxia only and in another cell exposed to normoxia followed by hypoxia (Normoxia/hypoxia); a.u.=arbitrary units. The superoxide scavenger N-tert-butyl-alpha-phenyl-nitrone (PBN) caused 90±3% decrease in the rate of DHE signal (inset right). (C) Ratio of fluorescence for cells exposed to normoxia only and for cells exposed to normoxia followed by hypoxia (Normoxia/hypoxia). (D) DHE fluorescence recorded from a cell exposed to hypoxia only and in another cell exposed to hypoxia followed by 5 µM angiotensin II (Ang II) (Hypoxia/Ang II). Ratio of fluorescence for cells exposed to hypoxia only and for cells exposed to hypoxia followed by Ang II (Hypoxia/Ang II) is shown inset right. (E). Ang II does not alter superoxide during exposure to room oxygen in cardiac myocytes. DHE fluorescence recorded from a cell exposed to normoxia only and in another cell exposed to normoxia followed by addition of 5 µM Ang II at 30 min (Normoxia/Ang II). Ratio of fluorescence for cells exposed to normoxia only and for cells exposed to normoxia followed by Ang II (Normoxia/Ang II) is shown inset right. (F) Ang II increases cellular superoxide in embryonic cortical neurons. DHE
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**Figure 9.** Representative experimental traces demonstrating the effects of hypoxia and β-adrenergic receptor stimulation on action potentials (AP) paced at 1000-ms cycle length in a guinea pig ventricular myocyte. (A) Effect of reducing pO2 from 150 mm Hg (normoxia) to 17 mm Hg (hypoxia) on the AP. (B) Effect of hypoxia + 3 nM isoproterenol (β-adrenergic receptor agonist) on the AP. (C) Progression to early afterdepolarization formation in the same cell. (D) Progression to sustained activity in the same cell. In D, the myocyte was not stimulated and APs were spontaneous. See text for further detail. Reproduced with permission from Ref. 129.
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