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Hypoxic Pulmonary Vasoconstriction: mechanisms of oxygen-sensing

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Abstract

Purpose of the review—Hypoxic pulmonary vasoconstriction (HPV) is driven by the intrinsic response to hypoxia of pulmonary arterial smooth muscle and endothelial cells. These are representatives of a group of specialised O_2-sensing cells, defined by their acute sensitivity to relatively small changes in pO_2, which have evolved to modulate respiratory and circulatory function in order to maintain O_2 supply within physiological limits. The aim of this article is to discuss recent investigations into the mechanism(s) of hypoxia-response coupling and, in light of these, provide a critical assessment of current working hypotheses.

Recent Findings—Upon exposure to hypoxia state-of-the-art technologies have now confirmed that mitochondrial oxidative phosphorylation is inhibited in all O_2-sensing cells, including pulmonary arterial smooth muscle cells. Thereafter, evidence has been presented to indicate a role as principal effector for the “gasotransmitters” carbon monoxide and hydrogen sulphide, reactive oxygen species or, in marked contrast, reduced cellular redox couples. Considering recent evidence in favour and against these proposals we suggest that an alternative mechanism may be key, namely the activation of AMP-activated protein kinase (AMPK) consequent to inhibition of mitochondrial oxidative phosphorylation.

Summary—HPV supports ventilation-perfusion matching in the lung by diverting blood flow away from oxygen-deprived areas towards regions rich in O_2. However, in diseases such as emphysema and cystic fibrosis, widespread HPV leads to hypoxic pulmonary hypertension and ultimately right heart failure. Determining the precise mechanism(s) that underpins hypoxia-response coupling will therefore advance understanding of the fundamental processes contributing to related pathophysiology and provide for improved therapeutics.

Keywords

hypoxia; pulmonary artery; AMPK; ROS; redox; H_2S; CO; HO-2

Introduction

The process of hypoxic pulmonary vasoconstriction (HPV) was first identified in 1894, as a rise in pulmonary arterial pressure upon asphyxia[1]. Fifty years on it was demonstrated that hypoxia without hypercapnia induced constriction within the pulmonary circulation, and the

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hypothesis proposed that HPV may assist ventilation-perfusion matching in the lung[2]. Thus, HPV was recognised as the critical and distinguishing characteristic of pulmonary arteries. In contrast, systemic arteries dilate in response to tissue hypoxemia, in order to match local perfusion to local metabolism[3].

Early studies showed that gross excitation of the spinal cord caused vasorelaxation within the systemic circulation, without effect on the pulmonary circulation[1]. Thereafter it was confirmed that HPV was a local response largely, or entirely, independent of the autonomic nervous system[4], being evident after chemical sympathectomy, surgical denervation of the carotid and aortic chemoreceptors or after bilateral cervical vagotomy[5, 6]. Most significantly, bilateral lung transplants established that HPV remained unaffected following denervation in man[7]. Therefore, neither central nor local regulation of the autonomic nervous system contributes to HPV.

HPV is not induced when the lung is perfused with hypoxic blood at a constant, normoxic alveolar oxygen tension[8], but by a fall in airway / alveolar pO_2[9]. Moreover, precapillary resistance arteries contribute most to the increase in pulmonary vascular perfusion pressure during alveolar hypoxia, the magnitude of HPV being inversely related to pulmonary artery diameter[10]. The threshold for HPV is ~60 mmHg and thereafter HPV increases in magnitude in a manner proportional to the degree of hypoxia[11], but it fails under near anoxic conditions(~5 mmHg)[11]. In the perfused and ventilated rat lung a monophasic and sustained increase in perfusion pressure is induced by hypoxia[11, 12], while HPV appears biphasic in isolated pulmonary arteries (Fig. 1) comprising a discrete transient constriction (Phase 1; 5-10 min) and a concomitant slow tonic constriction (Phase 2; peak after 30-40 min)[13, 14].

Mechanisms intrinsic to the smooth muscle and endothelial cells mediate HPV (Fig. 1), with calcium release from the smooth muscle sarcoplasmic reticulum via ryanodine receptors pivotal[11, 12, 14] and subsequent constriction augmented, via myofilament calcium sensitisation, by the release of an as yet unidentified vasoconstrictor from the endothelium[14, 15]. Hypoxia also modulates the activity of voltage-gated potassium channels (K_v) in the plasma membrane of the smooth muscle cells[16-18], although the functional consequence remains contentious[19]. More contentious still, is the mechanism of hypoxia-response coupling, which is likely common to all O_2-sensing cells, including pulmonary arterial smooth muscle and endothelial cells, carotid body type I cells and neonatal adrenomedullary chromaffin cells. These cells have been defined by their acute sensitivity to “activation” by relatively small changes in pO_2 and consideration of recent observations on hypoxia-response coupling in all representatives of this group is most revealing.

Heme oxygenase-2 as an O_2 sensor

Heme oxygenase-2 (HO-2) has been implicated in both pulmonary artery constriction[20] and carotid body activation by hypoxia[21, 22]. This was intriguing given the requirement of this enzyme for NADPH and O_2 as co-factors for the generation of carbon monoxide (CO), biliverdin and Fe^{2+} by catabolism of heme. Thus, it was proposed that under normoxic conditions, HO-2 controlled targets within the signal transduction cascade tonically through the production of CO (Fig. 2D) and that the activity of protein targets would be modulated by hypoxia because the co-factor for CO production, O_2, was limiting and / or through modulation by accumulating heme (due to the lack of its degradation by HO-2[22]). However, data from transgenic mice lacking HO-2 are variable. Some report that hypoxia-response coupling in HO-$2^{-/-}$ mice is blunted[20], while others suggest that hypoxia-response coupling remains unaltered[23-25]. Thus, it seems unlikely that HO-2 is physiologically important to hypoxia-response coupling in O_2 sensing cells.

Hydrogen sulphide and hypoxia-response coupling

Another gasotransmitter, H_2S, has been implicated in HPV[26] and carotid body activation by hypoxia[27]. Thus, hypoxia has been proposed to promote, indirectly, H_2S accumulation via cystathionine γ-lyase (which generates H_2S in peripheral systems). Two alternative mechanisms have, however, been considered. One suggestion is that under normoxic conditions HO-2 derived CO suppresses H_2S production (Fig. 2D), an effect which is depressed by hypoxia because of the requirement of HO-2 for O_2 to generate CO[27]. The alternative proposal is that hypoxia suppresses "normal" mitochondrial H_2S metabolism in an O_2-dependent manner[26]. However, recent studies have cast doubt on these hypotheses[28, 29], and it is clear that the ability of H_2S to mimic HPV may be due to the previously noted inhibition by H_2S of mitochondrial oxidative phosphorylation[28, 30].

That altered production of any "gasotransmitter" is of central importance to O_2 sensing therefore remains open to question, although H_2S may contribute to the inhibition of mitochondrial oxidative phosphorylation by hypoxia (see below) and, thereby, CO may influence this process. It should noted, however, that nitric oxide does exert an influence, since inhibitors of nitric oxide synthase potentiate HPV[31] and increase carotid body afferent fibre discharge[32].

An emerging role for AMP-activated protein kinase in O_2 sensing

The ubiquitously expressed AMP-activated protein kinase (AMPK) is a heterotrimer comprising catalytic α and regulatory β and γ subunits, of which there are multiple isoforms[33]. In response to metabolic stress, AMPK is activated by an increase in the ADP/ATP ratio, which is amplified by adenylate kinase into a greater increase in the AMP/ATP ratio[34]. Activation of AMPK (>100-fold) is conferred by phosphorylation at Thr-172 within the α subunit by upstream kinases, of which the most important is the tumor suppressor, LKB1. LKB1 appears to phosphorylate Thr-172 constitutively, with binding of AMP to the two exchangeable sites on the γ subunit inhibiting dephosphorylation and yielding the active, phosphorylated form. Binding of AMP to the γ subunit also causes allosteric activation by ~10-fold; the combinatorial effect causing >1000-fold activation. This ensures great sensitivity, with basal AMPK activity kept low in unstressed cells by ATP and ADP competing with AMP for binding at the γ subunit sites. Thus, AMPK can be activated within seconds[35] and serves to maintain ATP supply by upregulating catabolic processes, such as β-oxidation of fatty acids, and suppressing non-essential ATP-consuming reactions[33]. Pertinent to this review, is the new concept that AMPK may contribute to the regulation of O_2 and thereby energy (ATP) supply at the whole-body level and in doing so provide for a universal mechanism of hypoxia-response coupling[19, 36]. That AMPK may regulate aspects of cell function other than metabolism brings us to the mitochondrial hypothesis for O_2 sensing.

The first direct indication of a role for mitochondria was provided by studies on the carotid body, in which spectrophotometric analysis of the respiratory chain redox status and fluorometric measurement of the $NAD(P)H/NAD(P)^+$ ratio were related to afferent sinus nerve discharge during hypoxia[37]. An increase in the $NAD(P)H/NAD(P)^+$ ratio was observed, which correlated with afferent nerve activity over the physiological range of O_2 levels. It was suggested that mitochondria of most cells may utilise a high affinity (i.e. normal) cytochrome a_3, while the cytochrome a_3 incorporated in mitochondria of O_2 sensing cells may have a low affinity for O_2. However, evidence now points not to the mitochondria themselves, but to their local environment. Firstly, intracellular O_2 gradients, and possibly ATP gradients, occur at the cellular level[38] and such gradients may vary between tissues. Moreover, there are tissue-specific differences with respect to mitochondrial function that have been attributed to tissue-specific O_2 supply, substrate availability and other

intracellular variables (including ADP and ATP demand). Such variables can regulate many aspects of mitochondrial function, including the affinity of cytochrome c oxidase for O_2[39]. In this respect, it is also notable that recent studies suggest that PKC delta may modulate the rate of O_2 consumption and ATP generation by mitochondria[40]. Therefore, the exquisite sensitivity of O_2 sensing cells to a fall in O_2 levels could be attributed to a high rate of O_2 consumption, as has long been claimed[41-43].

The strongest evidence in favour of a requirement for functional mitochondria in O_2 sensing comes from recent studies on immortalised neonatal adrenomedullary chromaffin cells that incorporate or lack functional mitochondria. Those with functional mitochondria were found to respond to hypoxia and to inhibitors of mitochondrial oxidative phosphorylation. By contrast those cells lacking functional mitochondria failed to respond to either stimulus[44, 45]. Moreover, recent studies on the pulmonary vasculature have provided new spectrophotometric evidence that mitochondrial respiration in pulmonary arterial smooth muscle is indeed inhibited by hypoxia over the physiological range, and is particularly sensitive to changes in pO_2 when compared with systemic arterial smooth muscle[46]; that hypoxia increases the NAD(P)H / $NAD(P)^+$ ratio in pulmonary arterial smooth muscle[47] supports this view. Significantly, the threshold for inhibition of mitochondrial oxidative phosphorylation was ~60mmHg and inhibition increased in a manner related to the degree of hypoxia, as is the case with HPV[11] and carotid body activation by hypoxia[48].

Consistent with these findings, comprehensive data indicate that inhibitors of mitochondria (either uncouplers or blockers of specific respiratory chain complexes) mimic hypoxia in their ability to inhibit leak K^+ currents and thereby induce voltage-gated Ca^{2+} entry into carotid body type I cells[49]. Clear parallels exist with similar studies on the pulmonary vasculature, although there is one twist in the tale. All mitochondrial inhibitors tested thus far mimic the effects of hypoxia at the level of the O_2-sensitive K_v current[17]. Yet only some mitochondrial inhibitors mimic and occlude HPV in the perfused lung[50], while others have been shown to block but not mimic HPV in the perfused lung and isolated pulmonary arteries[47, 50, 51]. This has been a bone of contention in the field and has been cast as inconsistent with the view that HPV, at least, may be triggered by inhibition of mitochondrial oxidative phosphorylation.

However, it has been noted that HPV fails under near anoxic conditions (< 2% O_2), i.e. there is a pO_2 window within which hypoxia may trigger pulmonary artery constriction[52]. Moreover, early studies on the carotid body showed that afferent fibre discharge is depressed under anoxic conditions, and so too is the response of the carotid body to mitochondrial inhibition[53]. It is notable, therefore, that in dorsal root ganglion neurones, which do not serve to monitor O_2 supply, no shift in the NAD(P)H/$NAD(P)^+$ ratio is observed until the pO_2 falls to ~5 mmHg, at which point HPV and carotid body discharge begin to fail. Why might this be significant? Strictly speaking, it is the “anoxic” and not the “hypoxic” condition that mitochondrial inhibitors would mimic at concentrations that ablate oxidative phosphorylation. Therefore, an explanation for the inconsistency of outcome with respect to the effects of mitochondrial inhibitors on HPV and the pO_2 window within which HPV is triggered, may ultimately be provided by a greater understanding of the impact of degrees of metabolic stress. A case in point with respect to mitochondrial inhibitors may be that one such agent, metformin, provides for effective therapy of type II diabetes via AMPK activation, whereas a more potent analogue, phenformin, is no longer prescribed because of related contraindications[54].

These considerations bring us back nicely to AMPK, which is activated by all mitochondrial inhibitors in a manner dependent on the degree of inhibition of mitochondrial oxidative phosphorylation. Consider the possibility, therefore, that physiological levels of hypoxia

may activate AMPK and thereby precipitate, for example, HPV. It is quite possible that during more extreme metabolic stress, such as anoxia, AMPK may revert to its now classical role and "switch off" non-essential ATP-consuming processes in order to ensure cell survival. In other words, when smooth muscle "energy reserves" fail to maintain a desired level of ATP supply via, for example, β-oxidation of fatty acids[55], AMPK may not function itself to drive pulmonary artery constriction. After all, dilating pulmonary arteries in response to anoxia might be a logical "last gasp" in terms of achieving optimal gaseous exchange within the lungs

What then of the evidence in support of a role for AMPK in O_2 sensing? Perhaps the most detailed information to date comes from investigations on pulmonary arteries[56]. Hypoxia precipitates an increase in the AMP/ATP ratio in pulmonary arterial smooth muscle, concomitant activation of AMPK (Fig. 2A) and phosphorylation of acetyl CoA carboxylase (an established marker for AMPK action), despite the fact that cellular ATP levels remain remarkably stable in the presence of hypoxia[57]. Moreover, AMPK activation is induced in pulmonary arterial smooth muscle both by the mitochondrial inhibitor phenformin and by AICAR, which activates AMPK not by altering the AMP/ATP ratio but by uptake and subsequent metabolism to the AMP mimetic, ZMP[56]. Each agent also induced an increase in the intracellular Ca^{2+} concentration in acutely isolated pulmonary arterial smooth muscle cells, by mobilising sarcoplasmic reticulum stores via ryanodine receptors as does hypoxia. As expected, however, only phenformin increased the cellular NAD(P)H autofluorescence. Most significantly, AMPK activation by AICAR evoked a slow, sustained and reversible constriction of pulmonary artery rings that exhibits all the primary characteristics of HPV, namely a requirement for smooth muscle SR Ca^{2+} release via ryanodine receptors, and Ca^{2+} influx into and vasoconstrictor release from the endothelium. Moreover, preliminary data also suggest that AMPK modulates plasmalemmal K_v channels in a similar manner to hypoxia[58]. Consistent with these findings the non-selective AMPK antagonist, compound C, blocks HPV[59].

The proposal that AMPK may be of general importance to hypoxia-response coupling in all O_2 sensing cells gained notable support from studies on carotid body type I cells[56, 60]. Firstly, AICAR was shown to elicit a rise in $[Ca^{2+}]_i$ in isolated rat type I cells, and increased afferent sensory nerve activity recorded from the carotid sinus nerve - effectively mimicking hypoxia. Importantly, AMPK activation, like hypoxia, triggered these events by selectively inhibiting both BK_{Ca} and leak K^+ currents in rat type I cells, and thereby causing voltage-gated Ca^{2+} entry. Furthermore, emerging data from mice lacking the α2 subunit of AMPK indicate an important role for AMPK in the carotid body-mediated ventilatory response to hypoxia[19]. These observations suggest that hypoxia-response coupling may occur by direct phosphorylation and regulation of O_2 sensitive ion channels by AMPK, as AMPK phosphorylates and regulates, for example, recombinant BK_{Ca} channels in an AMP-dependent manner[60]. Thus, this mechanism provides a simple and conceptually satisfying link between the mitochondrial and membrane hypotheses for O_2 sensing in both the pulmonary artery and the carotid body.

Mitochondria, hypoxia and reactive oxygen species (ROS)

Schumacker, Chandel and colleagues were the first to propose that hypoxia may trigger a paradoxical increase in ROS production at complex III of the electron transport chain[61, 62] (Fig. 2B). That this may mediate HPV has gained significant prominence and support over the last 10 years[47, 63]. However, all measures of ROS production have been carried out under relatively severe hypoxia (≤ 5mm Hg). Moreover, it is notable that measured ROS generation occurred over the same range of pO_2 in (cultured) pulmonary arterial smooth muscle[62] and a wide range of other cultured cell types[64-67] that do not serve to monitor / sense O_2 supply. At face value this would not allow for selective regulation by hypoxia of

O_2 sensing cells. Moreover, both HPV and carotid body discharge are depressed by the degree of hypoxia utilised.

Most recently, it was suggested that mitochondrial ROS can lead to activation of AMPK without a shift in the AMP/ATP ratio[68, 69]. However, these studies were carried out on embryonic fibroblasts and osteosarcoma cells, which again are not recognised O_2 sensors. Therefore, outcomes most likely reflect a generalised cellular response to severe hypoxia. Nonetheless, it was reported that AMPK was activated via LKB1 only when functional mitochondria were present, with no change in the AMP/ATP ratio, and that AMPK activation was blocked by an antioxidant. Moreover, in mitochondria-deficient cells AMPK was activated by exogenous application of H_2O_2 and in cells lacking mitochondrial cytochrome b (which would not be expected to consume O_2, while allowing ROS generation at complex III) AMPK was activated by hypoxia. Importantly, however, in LKB1 deficient cells the response to H_2O_2 was markedly attenuated, suggesting that H_2O_2 must necessarily, like AMP, inhibit dephosphorylation of AMPK at Thr-172. Collectively, therefore, it may be reasonable to conclude that hypoxia could activate O_2 sensing cells through ROS-dependent activation of AMPK, but available evidence suggests this scenario is unlikely. Thus, Hawley et al. (2010) have demonstrated conclusively, using HEK293 cells expressing either wild-type AMPK or an AMP-insensitive mutant, that H_2O_2 activates AMPK indirectly and in an LKB1-dependent manner, by inhibiting mitochondrial function and increasing the AMP/ATP ratio[70]. Therefore, if mitochondrial ROS were to contribute to AMPK activation by hypoxia this would likely be by way of facilitating mitochondrial inhibition.

There are, however, other issues that remain to be accounted for with respect to the ROS hypothesis:

1. Antimycin A increases mitochondrial ROS production[71] and ablates but does not mimic HPV[50], whilst mitochondrial inhibitors that do not increase ROS also mimic and / or occlude HPV[47, 50]. By contrast all mitochondrial inhibitors examined, antimycin A included, modulate in a manner consistent with the effects of hypoxia all O_2 sensitive K channels in pulmonary arterial smooth muscle[17], carotid body type I cells[49] and neonatal adrenomedullary chromaffin cells[45].
2. In carotid body type I cells H_2O_2 does not increase $[Ca^{2+}]_i$, nor does it interfere with the hypoxic response of type I cells. Furthermore, in neonatal adrenomeduallary chromaffin cells H_2O_2 *opposes* hypoxia-response coupling[72].
3. Hyperoxia, which precipitates ROS formation in all cell types[73], is without significant effect on the pulmonary vasculature[74] and attenuates carotid body output[75].
4. Application of oxidising and reducing agents provides inconsistent outcomes in terms of the functional response to hypoxia of pulmonary arteries[63, 76-79] and carotid body type I cells[80, 81].
5. In studies on isolated mitochondria an increase in mitochondrial ROS has been observed under hyperoxia, but not in response to hypoxia[82, 83]. So, can mitochondria *per se* provide for both increased ROS generation and release in response to hypoxia in O_2 sensing cells[46, 84]?

Finally, it remains a concern that some laboratories consistently measure an increase in mitochondrial ROS in response to hypoxia irrespective of the cell type[46, 62, 64, 65], while others observe either no change or the exact opposite in all O_2 sensing cells[16, 72, 80, 85]. Either way, experimental observations are less than robust and likely provide as much evidence against as for the proposal that changes in the cellular redox status (Fig. 2C) or ROS *per se* underpin hypoxia-response coupling. Nonetheless, it is clear that physiological

levels of hypoxia increase the NAD(P)H / $NAD(P)^+$ ratio, and this may contribute to hypoxia-response coupling(see for example[86]) in addition to the regulation of AMPK by the AMP/ATP ratio.

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Key Points

- It is unlikely that CO / H_2S and ROS contribute to hypoxia-response coupling other than by inhibiting mitochondrial oxidative phosphorylation.
- Accumulating evidence supports a central role for AMPK, which is likely necessary and sufficient for hypoxia-response coupling in O_2 sensing cells.
- The precise nature of the functional outcome triggered by AMPK activation will depend not only on the AMPK heterotrimers present, but on the types of AMPK-sensitive and/or AMPK-insensitive ion channels (or other protein targets) expressed by a given cell type. Thereby AMPK may also differentially regulate a variety of cells that respond to metabolic signals other than hypoxia, such as glucose.
- Identification of the ion channels and transporters regulated by AMPK will account for cell- and system-specific responses vital to whole body energy homeostasis.
- It is our view, therefore, that AMPK regulates O_2 and energy (ATP) supply at the whole body level.

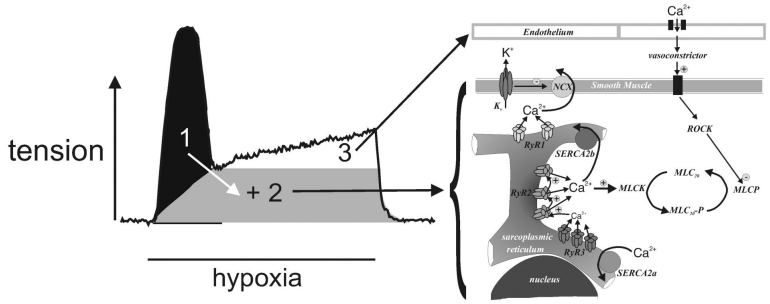

Figure 1. Hypoxic pulmonary vasoconstriction

Experimental record (left) highlights three identified components that underpin the transient and tonic phases of hypoxia-induced constriction of an isolated pulmonary artery ring. In sequence, two discrete mechanisms enhance calcium release from the smooth muscle sarcoplasmic reticulum (1, black; 2, grey) with a third component underpinned by the release of a vasoconstrictor from the endothelium (3, white). The mechanisms involved are described in further detail in the associated schematic (right): K_V, voltage-gated potassium current; NCX, sodium / calcium exchanger; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; MLCK, myosin light chain kinase; MLC_{20}, myosin light chain-20; MLCP, myosin phosphatase; ROCK, Rho associated kinase.

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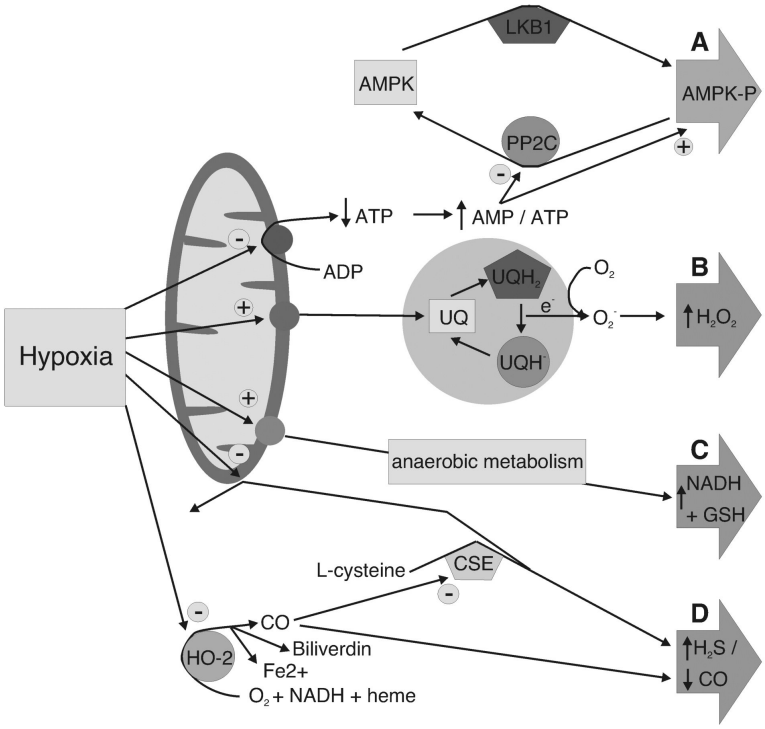

Figure 2. Proposed mechanisms of hypoxia-response coupling

A, activation of AMP-activated protein kinase (AMPK) initiated by an increase in the cellular AMP / ATP ratio: PP2C, protein phosphatase 2C. **B**, Electron transfer and ROS production via complex III of the mitochondrial electron transport chain: UQ, ubiquinol; UQH_2, reduced ubisemiquinone; $UQH^{\cdot}$, usemibiquinone radical. **C,** Reduction in the cellular redox status: NADH, reduced β-nicotinamide adenine dinucleotide; GSH, reduced glutathione. **D**, increased H_2S and / or decreased CO: HO-2, heme oxygenase-2; CSE, cystathionine γ-lyase.