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Regulation of hypoxic pulmonary vasoconstriction: basic mechanisms

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ABSTRACT: Hypoxic pulmonary vasoconstriction (HPV), also known as the von Euler–Liljestrand mechanism, is a physiological response to alveolar hypoxia which distributes pulmonary capillary blood flow to alveolar areas of high oxygen partial pressure.

Impairment of this mechanism may result in hypoxaemia. Under conditions of chronic hypoxia generalised vasoconstriction of the pulmonary vasculature in concert with hypoxia-induced vascular remodelling leads to pulmonary hypertension. Although the principle of HPV was recognised decades ago, its exact pathway still remains elusive. Neither the oxygen sensing process nor the exact pathway underlying HPV is fully deciphered yet. The effector pathway is suggested to include L-type calcium channels, nonspecific cation channels and voltage-dependent potassium channels, whereas mitochondria and nicotinamide adenine dinucleotide phosphate oxidases are discussed as oxygen sensors. Reactive oxygen species, redox couples and adenosine monophosphate-activated kinases are under investigation as mediators of hypoxic pulmonary vasoconstriction. Moreover, the role of calcium sensitisation, intracellular calcium stores and direction of change of reactive oxygen species is still under debate.

In this context the present article focuses on the basic mechanisms of hypoxic pulmonary vasoconstriction and also outlines differences in current concepts that have been suggested for the regulation of hypoxic pulmonary vasoconstriction.

KEYWORDS: Hypoxia, hypoxic pulmonary vasoconstriction, lung, oxygen, oxygen sensing

Hypoxic pulmonary vasoconstriction (HPV) is a physiological self-regulatory response to alveolar hypoxia that distributes pulmonary capillary blood flow to areas of high oxygen availability. This principle, also known as the von Euler–Liljestrand mechanism, thereby optimises gas exchange at the blood–air interface [1, 2].

Impairment of this mechanism during pathological situations in lung or systemic disease (for example, adult respiratory distress syndrome [3] or hepatopulmonary syndrome [4]) or during anaesthesia [5], may result in insufficient oxygenation of arterial blood and poor oxygen supply to the body. Chronic hypoxia, as it occurs at high altitude or during respiratory diseases (including chronic obstructive pulmonary disease, sleep apnoea, fibrosis, failure of ventilation due to neurological diseases), may lead to general vasoconstriction of the pulmonary vasculature inducing vascular remodelling processes with subsequent right heart hypertrophy and cor pulmonale.

Due to the opposite functions of lung vessels and systemic vessels, collecting and distributing oxygen, respectively, different reactions to hypoxia have developed. Whereas most systemic vessels of adult organisms dilate during hypoxia, pulmonary vessels constrict. During embryonic development lung vessels exhibit pronounced

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vasoconstriction, in order to minimise blood flow through the noninflated lungs, and to preserve the foetal circulation. After birth, pulmonary oxygenation, in concert with lung inflation, leads to vasodilation and perfusion of the lung. From the ontogenic point of view, therefore, HPV also can be seen as ''normoxic pulmonary vasodilation'' [6].

The present article focuses on the basic mechanisms and features of HPV, and also outlines differences in current concepts that have been suggested for the regulation of HPV. Further research will be necessary to completely elucidate the molecular pathways underlying HPV, which may serve as a basis for the development of new therapeutic strategies to treat disturbances in gas exchange caused by impaired HPV and pulmonary hypertension caused by exacerbated HPV.

CHARACTERISTICS AND KINETICS OF HPV

HPV is a highly conserved mechanism, present in most mammals [7–10], partially in reptiles [11] and probably even in fish [12]. Although an increase in pulmonary arterial pressure in response to alveolar hypoxia was recognised early [13, 14], it was in 1946 that von EULER and LILJESTRAND [15] suggested that ventilation-perfusion matching was the purpose of this response. Subsequently HPV was demonstrated to be present in humans, determined by a 50% increase in pulmonary arterial resistance to an alveolar oxygen partial pressure (P_{A,O_2}) <50 mmHg (<6.66 kPa) [16]. Since then, factors modulating HPV have been identified by dissecting the mechanism of HPV using a variety of approaches including isolated organs, tissues and single cells. Modulating factors include sex, local and circulating vasoactive substances, pH, partial pressure of carbon dioxide [17, 18] and red blood cells, although there was an early consensus that the mechanism itself was independent from neural [19] or humoral [20, 21] triggers.

In the isolated, buffer-perfused lung, HPV is activated at a partial pressure of oxygen (P_{O_2}) <100 mmHg (<13.3 kPa) [8, 10]. The precapillary smooth muscle layer of the resistance vessels, located at the entrance of the acinus has been identified as the effector cell-type for HPV [22–25]. Pulmonary arterial smooth muscle cells (PASMC) contract in response to hypoxia at a P_{O_2} of 25–50 mmHg (3.33–6.66 kPa), particularly in resistance arteries, whereas smooth muscle cells from isolated cerebral arteries dilate [26, 27]. At least for acute HPV, the sensor cell is most probably also the effector cell, although some necessity for preconditioning of these cells for contraction has been suggested. The pulmonary vasculature responds to changes in P_{A,O_2} within seconds, as is assumed from a teleological point of view, since HPV has to adapt perfusion to ventilation ''breath-to-breath'' [8]. For sustained hypoxia (lasting several minutes to hours and sometimes in contrast to chronic hypoxic reactions in the range of days also referred to as ''acute or sub-acute HPV'') a temporary vasodilation has been described, followed by a secondary vasoconstrictor response. However, these kinetics are not observed in all investigations and a contribution of endothelial cells must also be taken into account [28, 29]. Sustained HPV may be of major relevance for continuous ventilation-perfusion matching and under conditions of generalised hypoxia, which results in pulmonary hypertension.

SIGNAL TRANSDUCTION AND EFFECTOR MECHANISM: ABOUT CONTRACTION AND CALCIUM

The pathways leading to contraction of the PASMCs converge in an intracellular calcium increase and include the influx of extracellular calcium and release of intracellularly stored calcium [30].

For calcium influx from the extracellular space there is evidence that both, L-type calcium channels [31, 32] and nonspecific cation channels (NSCC) are involved. In line with this suggestion, inhibition of L-type calcium channels only partially abolished HPV [33, 34], whereas inhibition of NSCC completely inhibited HPV [35–37]. Hypoxic regulation of the L-type calcium channels may be achieved by direct modulation *via* reactive oxygen species (ROS) or membrane depolarisation *via* potassium channels [38–40] and NSCCs.

Different types of potassium channels have been demonstrated to be involved in hypoxia-associated membrane depolarisation, particularly the voltage-gated potassium (K_v) channels, $K_v2.1$, $K_v1.5$, $K_v9.3$ [41–43], which have been suggested to be the ''first step'' in HPV. However, only partial inhibition of HPV in knockout animals suggests this may not be the sole mechanism of HPV [44]. The concept of K_v channels being directly regulated by redox-changes has been proposed by POST *et al.* [39]. The K_v channels may be regulated by: 1) reducing agents [45–48], such as glutathione (GSH) [49]; 2) an associated haemoprotein; 3) membrane depolarisation; or 4) a calcium increase triggered by intracellular calcium release from intracellular stores or *via* NSCCs. Thus NSCCs may be key channels for hypoxic signalling, for example, in the regulation of potassium channels, of L-type calcium channels or the calcium level. The NSCCs have recently been emphasised as potential key regulators in HPV [37]. They consist of a group of store-operated and receptor-operated calcium channels (SOCs and ROCs, respectively), and their molecular identity has been revealed as transient receptor potential (TRP) channels. Certain subtypes of the TRP channels have been demonstrated to be increased in rat pulmonary arteries (PAs) after exposure to chronic hypoxia and human PASMCs in idiopathic pulmonary hypertension, suggesting a pathogenetic importance of TRP canonical (TRPC)1 and TRPC6 [50, 51]. The SOCs play a role in capacitative calcium entry (CCE), a calcium influx from extracellular space that can be induced by emptying intracellular stores, and has been shown to be activated in HPV [37, 52]. While CCE is a phenomenon observed in several types of systemic arteries, its coupling to contraction appears to be of particular importance in PAs [53], but its exact impact on HPV has not yet been resolved, especially in the face of a lack of specific experimental manipulation methods [37]. The ROCs can be activated by protein kinases and diacylglycerol. Recently, the present authors have demonstrated that this mechanism is indispensable for acute HPV, occurring within seconds to minutes, as: 1) TRPC6 knockout mice have no pulmonary vascular reactivity to hypoxia although they fully respond to non-hypoxia induced vasoconstriction; and 2) PASMCs isolated from TRPC6-deficient mice exhibit no hypoxic calcium increase and membrane current when exposed to hypoxia, in contrast to wild-type PASMCs [54]. TRPC6 may modulate intracellular calcium and membrane potential by subsequent gating of K_v and L-type calcium channels.

There is evidence that internal calcium release plays an obligatory role in hypoxic inhibition of potassium currents [55, 56]. Although challenged by a recent study [54], an initial release of calcium from intracellular stores was suggested as a key trigger for HPV. This concept is based on studies that demonstrate an increase in intracellular calcium even after removal of extracellular calcium and release of calcium from internal stores *via* ryanodine-sensitive calcium stores [57] with calcium buffering of inositol triphosphate (IP_3) sensitive stores [58–60]. Debate continues regarding how they might be regulated and the observed increase of intracellular released calcium may be restricted to PASMCs of nonresistance PAs with a large diameter [54]. In other cell types, intracellular calcium stores are susceptible to redox modulation *via* ryanodine-sensitive receptor channels that are modulated by redox-regulating systems [61], ROS [62, 63] and cyclic adenosine diphosphate-ribose [64], and IP_3 receptor channels modulated *via* cyclic guanosine monophosphate and reduced nicotinamide adenine dinucleotide (NADH) [65].

For sustained HPV in particular, a Ca^{2+} sensitisation, in addition to an increase in calcium, possibly *via* activation of Rho-kinase, has been suggested [66–68]. A recent point-counterpoint discussion summarised the debate regarding Ca^{2+} sensitisation as a further possible mechanism for HPV [69]. For sustained HPV in particular, the importance of the endothelium, the lack of correlation between tension and internal calcium concentration and the effect of inhibition of RhoA/Rho kinase are good arguments for Ca^{2+} sensitisation. For acute HPV, the sensitising effects of endothelium are considered to be negligible, as acute HPV could be detected in the absence of endothelium [70–72], but Rho-kinase could also be activated in an endothelium-independent fashion [67]. However, the debate is obscured by an absent systemic definition of preconditioning, and the lack of consensus regarding the characteristics and existence of sustained HPV in different models, and data from different laboratories. Therefore, it is not yet clear if Rho-kinase and/or other protein kinases play only a modulating, or an indispensible, role in HPV [73].

THE ROLE OF ROS IN HPV

As the modulation of the cellular redox state and alteration of ROS releasing systems have been demonstrated to affect the strength of HPV, it has been concluded that ROS or the cellular redox state may play a role in the hypoxic signalling pathway. The L-type calcium channels and intracellular calcium channels can be activated by ROS [74], and K_v channels can be inhibited by reducing equivalents. Whereas there is wide consensus regarding the importance of ROS, two opposing scenarios are offered for ROS regulation during HPV. One concept favours K_v channel modification by a reduced level of ROS as a trigger for contraction. The second concept proposes an increase in ROS during hypoxia from mitochondria or reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases (for review see [75–79]).

The application of oxidants and antioxidants has been helpful in understanding the reactivity of the pulmonary vascular system with regard to HPV. Hydrogen peroxide induces pulmonary vasoconstriction during normoxia [80–82], but antioxidants also cause constriction of isolated vessels by inhibition of K_v channels [48]. Such discrepancies may result from the possibility that the applied agents reach only parts of the vasoreactive pathways without necessarily affecting the physiological pathway activated during hypoxia. This view is supported by the observation that oxidants [48, 82, 83] as well as antioxidants [84] inhibited HPV. Detailed pharmacological interventions provided evidence for both an upregulation as well as for a downregulation of superoxide, and subsequently H_2O_2, as the underlying pathway of HPV [85, 86]. An elegant study demonstrated that overexpression of glutathione peroxidase, cytosolic or mitochondrial catalase attenuated the hypoxia-induced increase in ROS signalling and intracellular calcium, whereas mitochondrial matrix-targeted manganese superoxide dismutase (which decreases superoxide, but increases H_2O_2) augmented intracellular calcium [87]. This suggests that superoxide production leading to H_2O_2 being released from mitochondria, contributes to the hypoxia-induced increase in intracellular calcium. Application of the thiol-reducing agent dithiothreitol during the sustained phase of HPV reversed hypoxic vasoconstriction, whereas the superoxide scavenger nitroblue tetrazolium prevented further hypoxic pulmonary vasoconstriction during the sustained phase of HPV, but did not reverse it [62].

As summarised in table 1, measurement of ROS has been performed by different methods, but no consensus regarding an up or downregulation could be achieved up to now. The lack of valid techniques for ROS measurement may be one reason for the uncertainty with regard to their role in HPV. Technical limitations of different fluorescent probes with regard to their lack of specificity and auto-oxidation [100] have been the major drawbacks in the past. In addition, artefacts due to a nonphysiological environment of isolated arteries and PASMC have to be considered. For example, pretone may influence ROS production by activation of stretch-sensitive NADPH oxidase [101] and some investigators believe that healthy mitochondria do not release ROS under baseline conditions at all [102]. However, it is still curious why different groups have obtained different results with similar techniques. Apart from artefacts related to the ROS detection methodology and the experimental models employed (isolated lungs, vessels or cells), the kinetics and time-frame of the measurements (related to the kinetics of HPV) must also be taken into account as potential sources for discordant data. The question of the location of the ROS in particular must also be considered. Therefore, the apparently conflicting conclusions concerning ROS in HPV may be explained by the hypothesis that a local, subcellular and compartmentalised regulation of ROS triggers HPV. This may also be true for the intact organ where a clear majority of investigations demonstrate a decrease in ROS, measured with different methods, although evidence for increased ROS was obtained when some of the ROS-generating systems were blocked (table 1) [103]. Moreover, recent findings of enrichment of ion channels in membrane microdomains may also support the theory of compartimentalisation [104].

THE OXYGEN SENSOR OF HPV

To identify the triggering mechanism of HPV, two main assumptions have to be made: 1) the sensor has to be an oxygen consuming or binding protein/organelle with sensitivity also in the range of mild hypoxia; and 2) the sensor has to communicate with the PAMSC, the effector cell of HPV. This underscores the

TABLE 1 Hypoxia induced changes of reactive oxygen species (ROS)

	Increase of ROS	Decrease of ROS	Grade of hypoxia, time kinetic	[Ref.]
Isolated perfused rat lung		Chemiluminescence (luminol and lucigenin), surface of lung	0, 1, 2.5, 10 and 95% O_2 decrease of signal within seconds	[88]
Isolated perfused rabbit lung		Chemiluminescence (lucigenin)	0% O_2	[86]
Isolated perfused rat lung		Chemiluminescence, hypoxia reduced luminol, not lucigenin, surface of lung	2.5% O_2, decrease of signal before increase of PAP	[89]
Isolated perfused rabbit lung, intubated animals and animals with breathing masks		H_2O_2 in exhaled air	O_2 concentrations between 21 and 1%, hypoxia for 50 min	[90]
Isolated perfused rabbit and mouse lung	ESR with PMA stimulation and hypoxia	ESR, in buffer fluid	21, 16, 10, 5, 2.5 or 1 % O_2, max. decrease at 1%/2.5%, max. increase with PMA 5%, sample collection each 1 or 2 min	[91]
Isolated perfused mouse lung	In $p47^{phox}$-deficient mice, ESR	ESR, in buffer fluid	1% O_2 sample collection each 2 min, HPV decreased in $p47^{phox}$ knockout	[92]
Isolated PAs endothelium denuded		Amplex Red, DCFH and lucigenin	Hypoxia *via* bubbling with a hypoxic gas (P_{O_2} 40 mmHg)	[92]
Murine precision-cut lung slices	DCFH, number of ROS producing cells increases		1% O_2, exposed for 3 h	[93]
Isolated porcine PA with endothelium	Lucigenin derived chemiluminescence, ESR, increase of hydroxyl and alkyl radicals during hypoxia in some preparations		O_2 concentrations from 16 to 4%, oxygen tensions at the vessel from 114 to 29 mmHg; samples taken after 30 min or 1 h of exposure to hypoxia	[94]
Isolated PA of rat		L-012 (luminol analogue) and Amplex Red		[95]
Microsomal preparation of calf PAs		Lucigenin	P_{O_2} 8–10 mmHg	[96]
Calf PASMCs	Lucigenin		P_{O_2} 40 mmHg	[97]
Rat PASMCs	DCFH		P_{O_2} 25 mmHg, start at 10 min, (but P_{O_2} after 5 min >100 mmHg)	[98]
Rat PASMCs	DCFH		2% O_2, immediate increase	[84]
Porcine PASMCs	DCFH		4% O_2, increase after 2 min first sample)	[94]
Mouse PASMCs	DCFH		1% O_2, PASMCs exposed to hypoxia for 1 h	[93]
Mouse PASMCs	FRET		1.5% O_2; simultaneous ROS and calcium increase (after 3–5 min)	[87]
Human PASMCs		DCFH, DHE, Amplex Red	5% O_2	[99]

PA: pulmonary artery; PASMC: pulmonary artery smooth muscle cell; ESR: electron spin resonance; PMA: phorbol-12-myristate-13-acetate; DCFH: 2', 7'-dichlorfluorescein-diacetate; FRET: fluorescence resonance energy transfer; DHE: dihydroethidium; PAP: pulmonary arterial pressure; max: maximum; HPV: hypoxic pulmonary vasoconstriction; P_{O_2}: partial pressure of oxygen. 1 mmHg=0.133 kPa.

problem of defining hypoxia. HPV in the isolated lung exhibits a sigmoidal stimulus–response relationship to varying degrees of alveolar hypoxia in a portion of investigations, with an onset at 50–70 mmHg (6.66–9.33 kPa) and half-maximal response at 30–50 mmHg (3.99–6.66 kPa) [8]. For sustained hypoxia, lasting for 20–40 min, a biphasic stimulus–response relationship with a maximal vasoconstriction at an average P_{O_2} of 25 mmHg (3.33 kPa) and decreasing vasoconstriction [10] or even vasodilatation [105] at lower oxygen concentrations was demonstrated, but not all investigations reported biphasic kinetics of HPV. Isolated cells were reported to contract in response to a P_{O_2} of 25–50 mmHg (3.33–6.66 kPa) [26, 27], whereas studies with measurement of intracellular calcium were usually performed at P_{O_2} levels <30 mmHg (3.99 kPa) [36, 106, 107]. Integrating these data, it is clear that a sensor must exhibit sensitivity to at least 50 mmHg (6.66 kPa) and below. Regardless of the debate on the dependence of HPV on priming factors, the primary oxygen sensor is obviously located in the PASMC as isolated PASMC contract in response to hypoxia and increase their intracellular Ca^{2+}.

MITOCHONDRIA IN THE CELL

The importance of mitochondria in HPV signalling pathways is undisputable, since their exact role has been the basis of debate for many years. Mitochondria are the main oxygen consuming cell organelles and the application of inhibitors has been used to elucidate the mechanism of HPV [84, 108–110].

Mitochondria may regulate or trigger HPV by hypoxic inhibition of complex IV, regulation of ROS metabolism or controlling calcium homeostasis in the cell. In regard to the general requirement of oxygen sensors in HPV, which is sensitivity to mild hypoxia, high oxygen affinity of the key enzyme in complex IV seemed to prevent a decrease of adenosine triphosphate (ATP) under conditions of mild hypoxia [111]. In line with this notion, no ATP depletion [112, 113] was evident in the whole lung during hypoxia, although this might be due to a shift from oxidative phosphorylation to glycolytic ATP production [114]. This mechanism is further supported by tissue-specific modulation of oxygen affinity of cytochrome c oxidase of complex IV, for example, by NO, CO or ROS, and may affect the adenosine monophosphate/ATP ratio to induce calcium release from intracellular stores *via* cyclic adenosine diphosphate ribose and not necessarily by ATP depletion [115]. In contrast to observations on acute HPV, there is evidence that oxidative ATP generation is impaired in isolated PAs during sustained HPV [116], in salt perfused PAs (even after 4 min [117]) and in intact lung during severe hypoxia (<10 mmHg (<1.33 kPa)) [118]. During sustained hypoxia the cellular energy state is balanced by an upregulation of glycolysis [112, 116, 119]. However, since exposure of PAs to prolonged hypoxia may cause ATP-sensitive potassium channels to open due to ATP depletion, the balance of energy state may be incompletely achieved [120]. This may explain the higher sensitivity of HPV to mitochondrial inhibition during prolonged hypoxia [103]. A hint at some potential inhibition of the mitochondrial respiratory chain during hypoxia is the high NADH/nicotinamide adenine dinulceotide (NAD) ratio during acute hypoxia (15–18 mmHg (1.99–2.39 kPa)) [119], but not the NADPH/NADP ratio (35–60 mmHg (4.66–7.99 kPa)) [117] in isolated PAs. Although the redox state is an important determinant for the gating of redox-sensitive ion channels, no correlation with HPV in isolated PAs could be demonstrated during application of mitochondrial inhibitors [119]. Nevertheless the failure of inhibiting HPV by inhibition of complex IV with cyanide in some investigations (table 2) questioned the general importance of hypoxic inhibition of complex IV. For this reason, and the potentially ''wrong'' reaction range of complex IV, studies focused on an oxygen sensing mechanism *via* mitochondrial ROS production independent of complex IV. Again, different concepts with regard to ROS production have emerged. The ROS production by mitochondria has been suggested to be decreased or increased by direct measurement (table 1) and to originate from complex I, II or III of the respiratory chain [98, 110, 122]. The original redox hypothesis was proposed by WEIR and ARCHER [123] and ARCHER *et al.* [89] that assumed a decrease in the production of mitochondrial ROS, resulting in the inhibition of K_v channels mediated by redox couples, such as GSH/GSSG and NADH/NAD ratio. The inhibitors of complex I and III, respectively, decreased ROS, caused vasoconstriction during normoxia, and inhibited HPV in isolated lungs, PAs and PASMCs. At the same time they inhibited K_v channels. Conversely, cyanide increased ROS, and also caused vasoconstriction during normoxia, but did not inhibit HPV [89, 92]. From these experiments, the conclusion was drawn that decreased ROS production of the mitochondrial respiratory chain upstream of complex IV is necessary for HPV. In contrast, WAYPA and coworkers [77, 84] suggested that an increase in ROS production during hypoxia triggers subsequent intracellular calcium release and thus HPV. Arguments of increased ROS from the semiubiquinone binding site in mitochondrial complex III are mainly related to rotenone (inhibition of complex I) and myxothiazol (inhibition of complex III proximal to the suggested superoxide production site) attenuating the hypoxia-induced increase in intracellular Ca^{2+} in PASMCs and HPV and myxothiazol inhibiting the hypoxic ROS production. In contrast, antimycin A (inhibition of complex III distal to the suggested superoxide production site), which is assumed to increase hypoxic ROS production, did not attenuate the hypoxia-induced Ca^{2+} increase and had no effect on HPV. In line with these observations, cyanide (inhibition of complex IV) did not inhibit HPV. Direct measurements with a new fluorescence resonance energy transfer method support the theory of ROS increase during HPV [87]. An elegant extension of the experiments is the application of succinate, which restores rotenone-inhibited effects by restoring function of complex III [119]. Effects of mitochondrial inhibitors are summarised in table 2, and provide arguments for both concepts, although generally their specificity should be considered carefully [109]. Both mechanisms could cause vasoconstricton by modulating ion channels and could be triggered or modulated by hypoxia. Generally, mitochondrial ROS can be increased by unspecific interference of oxygen with the mitochondrial membrane [124], increased time life of ubisemiquinone, *e.g.* by peripheral inhibition, electron backflow through complex II [93], mitochondrial calcium influx [125] or activation of mitochondrial potassium channels [126]. A decrease in the ROS level can be caused by a decreased P_{O_2}, electron flux through respiratory chain, or membrane potential. Besides being a possible trigger organelle, mitochondria can be a modulator of HPV. In particular, calcium homeostasis of the cell is regulated by mitochondria, by calcium buffering [127–129] and regulating capacitative calcium entry [130].

NADPH OXIDASE

The NADPH oxidases are superoxide/producing multiprotein complexes consisting of the transmembrane superoxide producing subunits and cytosolic subunits. The classical leukocyte NADPH oxidase is composed of the transmembrane subunits gp91 phox (now also termed NOX2) and p22, which comprises of cytochrome b558, and the cytosolic subunits p47phox, p67phox and p40phox. Compared with the leukocytic NADPH oxidase, which is activated by an assembly of the cytosolic and the membrane-bound subunits, certain nonphagocytic NADPH oxidases that substitute gp91 (NOX2), *e.g.* by the newly identified isoforms NOX1 and NOX4 [131], constitutively produce low amounts of superoxide in the intracellular compartment, probably by assembly of NOX1 with subunit p47 homologue NOX-O1 [131, 132]. Activation by assembly can be induced by phosphorylation of the p47phox subunit and Rac GTPase, for example [133]. Such regulation of NADPH

TABLE 2 Influence of mitochondrial inhibition on hypoxic pulmonary vasoconstriction (HPV) and normoxic vessel tone

	ROS	Normoxic tone	HPV	Model	[Ref.]
Rotenone	↓	↑	Inhibited	Whole lung and PASMCs	[89]
	↓	↑	Inhibited	PA and PASMCs, opposite in renal tissue	[92]
		↑	Inhibited, unspecifically	Isolated lung	[108]
	↓	↑		Vessel wall	[93]
		↑	Inhibited	Buffer perfused rat lungs, PASMCs	[84]
		No change with inhibited NO	Inhibited, specificity pronounced under blocked NO	Isolated rabbit lung	[109]
		No change	Inhibited	PAs	[119]
3-NPA	Prevents hypoxia induced increase	↑	Inhibited	Intraacinar arteries	[121]
		No change	Inhibited	Isolated lung	[109]
Succinate		No change	Reverses effect of rotenone	PAs	[119]
	SDH activity decreased during hypoxia	No change	Inhibited (unspecifically), fumarate delays beginning, malate prevents unspecifically	Intraacinar arteries	[121]
Antimycin A	↓ in normoxia and hypoxia	↑	Inhibited	Whole lung	[89]
	↓	↑	Inhibited	PA and PASMCs opposite in renal tissue	[92]
		↑	Inhibited, unspecifically	Isolated rat lung	[108]
		↑	Inhibited unspecifically in high concentrations, no change in low concentrations	Buffer perfused lung	[84]
	↓ in normoxia and hypoxia	↑	Pronounced vasodilation	PA	[121]
		No change with inhibited NO	Inhibited unspecifically under blocked NO	Isolated lung	[109]
HQNO		↑	Inhibited specifically	Isolated lung	[109]
Myxothiazol	↓ during hypoxia		Decreased intracellular Ca^{2+} during hypoxia	PASMC	[87]
	↓ during hypoxia	No change	Inhibited	Buffer perfused rat lungs, PASMCs	[84]
		No change	Inhibited	IPA	[119]
		No change with inhibited NO	HPV inhibited unspecifically, especially under preblocked NO	Isolated lung	[109]
Cyanide	↑	↑, inhibited by SOD	No change	Whole lung PASMCs	[89]
	No change during hypoxia		Increased intracellular Ca^{2+} during hypoxia	PASMCs	[87]
		↑, inhibited by ebselen and myxo-thiazol	↑	Buffer perfused lung	[84]
		No change	No effect on phase 1, potentiates phase 2	PAs	[119]
		↑	Inhibited, unspecifically	Isolated rat lung	[108]
		↑	Inhibited, especially phase 2	Rabbit lung	[92]
Azide		↑	Inhibited, unspecifically	Isolated rat lung	[108]
	No change		Inhibited, unspecifically	PA	[121]

Vasoconstriction during normoxia when applying the inhibitor is usually smaller than HPV and of transient character. ROS: reactive oxygen specificity; 3-NPA: 3-nitropropionic acid; HQNO: 2n-heptyl-4-hydroxyquinoline-N-oxide; SDH: succinate dehydrogenase; No: nitric oxide; SOD: superoxide dismutase; PASMC: pulmonary arterial smooth muscle cells; PA: pulmonary arteries; IPA: intrapulmonary arteries. ↓: decrease; ↑: increase.

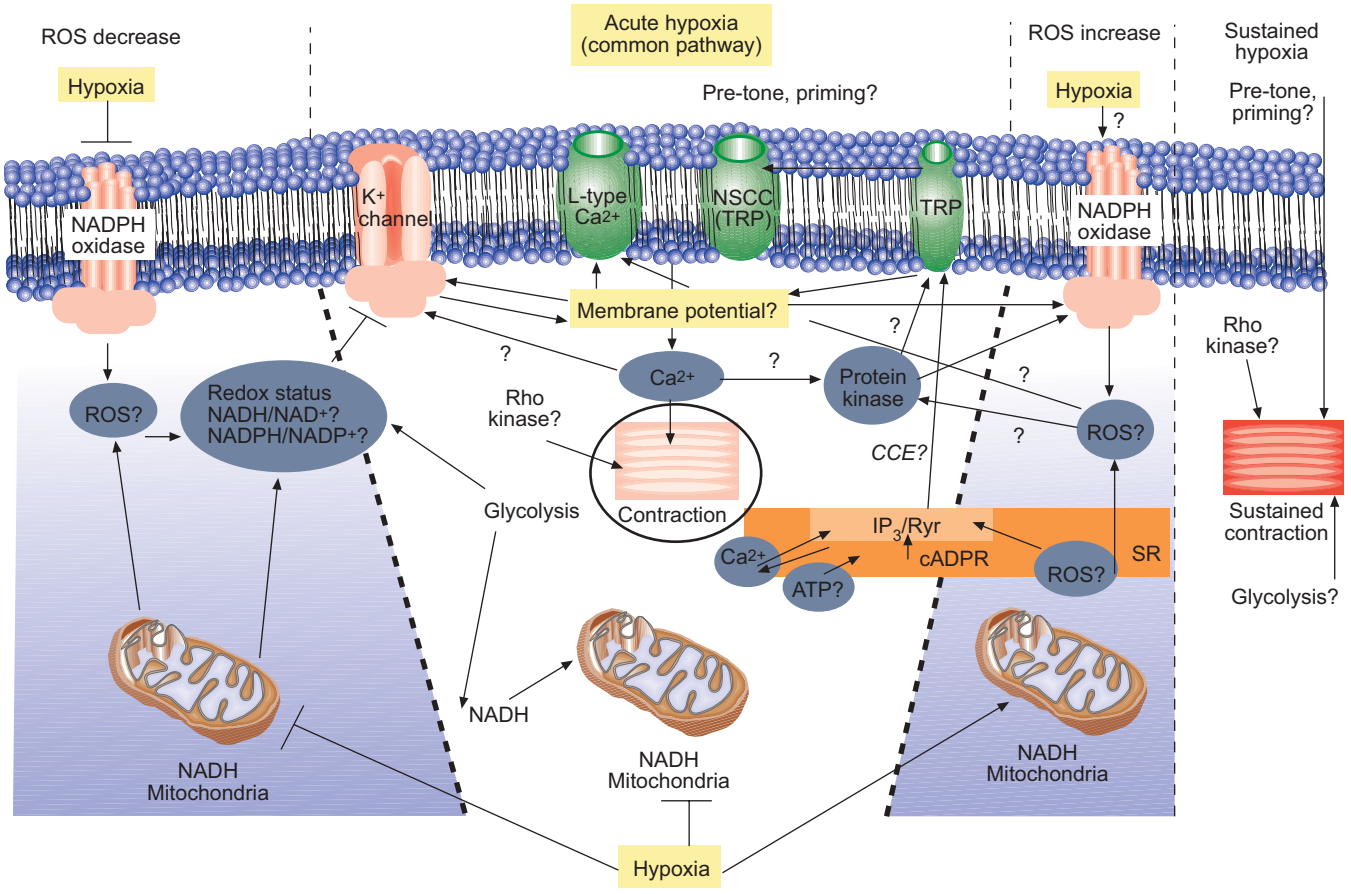

FIGURE 1. Pathways involved in hypoxic pulmonary vasoconstriction. Acute hypoxia results in an increase of intracellular calcium in pulmonary arterial smooth muscle cells and thus contraction. This increase in calcium is achieved by inflow of extracellular calcium through plasmalemnal calcium channels and release of intracellularly stored calcium. Hypoxic effects could be mediated or modulated by a decrease (left side) or increase (right side) of reactive oxygen species (ROS). NADPH: reduced nicotinamide adenine dinucleotide phosphate; NSCC: nonspecific cation channels; TRP: transient receptor potential; NADH: reduced nicotinamide adenine dinucleotide; NAD: nicotinamide adenine dinulceotide; NADP: nicotinamide adenine dinucleotide phosphate; CCE: capacitative calcium entry; ATP: adenosine triphosphate; IP_3: inositol triphosphate; cADPR: cyclic ADP-ribose; SR: sarcoplasmatic reticulum.

oxidase activity may involve phospholipase A2 and protein kinase C (PKC) [134, 135]. Classical NADPH oxidase activity depends on membrane potential in the physiological range and the NADPH concentration [136, 137]. In leukocytic cells, activation of NADPH oxidase leads to rapid depolarisation of the cell, because of outward movements of electrons [138, 139].

In vascular smooth muscle cells, NADPH-dependent ROS production occurs mainly intracellularly [131] and has been proposed to be the main ROS producing system compared with other cellular systems [140]. Leukocytic NADPH oxidase superoxide production was shown to be dependent on P_{O_2}, with a slow decrease of ROS release with decreasing P_{O_2} and a sharp decline at an O_2 concentration <1% [141].

However, in isolated perfused lungs, intravascular NADPH oxidase dependent superoxide concentration increased during graded hypoxia peaking at 5% O_2, when NADPH oxidase was maximally stimulated with phorbol-12-myristate-13-acetate; however, under nonstimulated conditions superoxide release decreased with hypoxia. It was concluded that regulation of electron flux rather than oxygen may be the rate-limiting step in superoxide formation, from nonphagocytic NADPH oxidase [91].

Regarding its role in HPV, MARSHALL *et al.* [97] first described an NAD(P)H oxidase in PAs with an unusually low redox potential. Isolated PASMCs demonstrated an increase in superoxide production under hypoxic conditions, which was suggested to be derived from NADPH oxidase. Several studies showed inhibition of HPV by diphenyleneiodonium (DPI) [142, 143]. However, DPI was proven to inhibit flavin adenine dinucleotide-dependent enzymes in general and thus also those of the mitochondrial chain [144, 145]. To overcome this problem, different NADPH oxidase inhibitors were investigated as follows: apocynin, which, 1) interfered with vascular tone in general in isolated rabbit lung studies; and 2) 4-(2-aminoethyl)benzenesulfonyl fluoride, which selectively inhibited HPV in isolated rabbit lungs [143]. Recently, cadmium sulphate, which is thought to be more specific than DPI, also showed inhibition of HPV in isolated rat PAs [146]. The current authors' investigations suggested an activation of a NAD(P)H oxidase during hypoxia, as all the blockers inhibited, but did not mimic HPV [147].

As an extension of these studies PKC, a possible activator of the NADPH oxidase, has been suggested to regulate HPV *via*

NADPH oxidases [135] (although PKC may also affect HPV without interaction with an NADPH oxidase [73]), and a phospholipase A_2 knockout mouse exhibited reduced HPV, which may also interfere with the NADPH oxidase pathway [134, 148]. However, the NADPH oxidase concept of oxygen sensing then seemed to be rejected in study by ARCHER *et al.* [149], who demonstrated that gp91phox-deficent mice fully responded to acute hypoxia. Although this clearly excludes the role of the phagocytic NADPH oxidase type in HPV, the present authors recently demonstrated that mice deficient of the cytosolic NADPH oxidase subunit p47 had a ~25% reduced acute, but unchanged sustained HPV [92]. This supports the concept that isoforms of the leukocytic NADPH oxidase may, at least in part, function as oxygen sensors in HPV.

CONCLUSION: INTEGRATING MULTIPLE EFFECTS

Although understanding the precise sequence and interdependence of events leading to HPV is still far away, a more detailed picture of the pathways involved is being created. Hypothetically, the findings summarised in this review may be integrated into a complex model (fig. 1). There are multiple components (L-type calcium channels, K_v channels, NSCC (*e.g.* TRP channels) and Rho-kinase) involved that could be activated sequentially or in parallel during HPV and lead to an intracellular calcium increase and/or calcium sensitisation and thus HPV. The contribution of these mechanisms is generally accepted; however, there are opposing theories regarding the contribution of ROS. One theory favours an increase of ROS for the signal transduction underlying HPV and others emphasise a decrease of ROS.

Assuming an increase of ROS underlying the signal transduction of HPV, it was suggested that hypoxia: 1) interferes with mitochondrial complex I/III or inhibits complex IV, above the range of changing the cellular energy status, to increase ROS; or 2) that an activation of NADPH oxidases by a yet unknown mechanism may lead to an increase of ROS.

A decrease of ROS has been attributed to a decreased mitochondrial ROS release (from complex I and III). This decrease then may lead to a more reduced cellular redox status, which inhibits K_v channels. Inhibition of K_v channels results in membrane depolarisation and activation of L-type calcium channels [89, 110, 150, 151]. Moreover, K_v channels have been shown to be inhibited by antioxidants [45]. However, even the importance of ROS itself was questioned [152] and cADP-ribose, for example, has been suggested as another signalling molecule [115]. Exact P_{O_2} dependence and localisation of subcellular ROS production may resolve the currently contradictory findings with regard to the contribution of ROS to HPV.

Interfering effects between the different pathways suggested to contribute to HPV may further complicate the issue. For example, NADPH oxidases can be activated by protein kinases, which have been shown to be essential in HPV [73], and mitochondrial ROS production can be influenced by cellular Ca^{2+} levels [153]. Moreover, mitochondria play an important role in regulation of cellular Ca^{2+} homeostasis [129] and capacitative Ca^{2+} entry [154], which has been shown to be activated in HPV.

Thus, although a portion of the pathways essential for hypoxia pulmonary vasoconstriction have been identified there are still plenty of open questions to solve before a complete picture about the oxygen sensing and signal transduction mechanisms of this important physiological response can be achieved.

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