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Hypoxia and Anoxia

Edited by Kusal K. Das
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HYPOXIA AND ANOXIA

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Meet the editors

Kusal K. Das (b. 1962) is Professor of Physiology and Director at the Laboratory of Vascular Physiology and Medicine, Shri B.M. Patil Medical College, BLDE (Deemed to be University), India. He was also Visiting Professor of Physiology at the Hypertension and Renal Centre of Excellence, Tulane University School of Medicine, New Orleans, USA (2017) and Visiting Professor of Medicine at the School of Medicine, University of Leeds, UK (2014–2016). Prof. Das did his PhD and Master's in Physiology at University Calcutta. His area of research is experimental hypoxia and cell signalling mechanisms during heavy metal-induced stress and alteration of vascular integrity in both experimental and human models. He invented a modified method of determining serum vitamin E. He is a Member of the Education Committee of the International Union of Physiological Sciences and Vice President of the Physiological Society of India. He was admitted as a Fellow of the Royal Society of Biology, London, on January 1, 2018.

Mallanagouda Shivanagouda (b. 1955) is Vice Chancellor and Professor of Medicine of BLDE (Deemed to be University), Vijayapura, Karnataka, India. He did his MD with gold medal in General Medicine and entirely devoted himself for medical teaching, research, and administration.

Prof. Biradar's greatest contribution to the field of medicine was to bring a dynamic, purposeful, meaningful, and object-oriented medical sciences program that rediscovered the undergraduate, postgraduate, and doctoral medical curriculum under the university. He has also contributed immensely to the area of medical research ethics and practices through his numerous publications, including books and book chapters. For his immense contribution to the field of medical administration, the Physiological Society of India conferred on him "Prof. K. Raghottam Rao Oration 2016." Prof. Biradar was also a visiting professor at Tulane University School of Medicine, New Orleans, USA.

Contents

Preface

Hypoxia is a pathological condition in which the body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) is deprived of adequate oxygen supply. Variations in arterial oxygen concentrations can be part of the normal physiology, for example, during strenuous physical exercise. A mismatch between oxygen supply and its demand at the cellular level may result in a hypoxic condition. Hypoxia, in which there is complete deprivation of oxygen supply, is referred to as anoxia**.** Hypoxia belongs to the most serious of factors that can directly impair the function of metabolic pathways in the animal cell. Exposure of experimental animals to hypoxia has been widely used in many morphological and physiological studies [1]. The decrease in tissue oxygenation induced by hypoxia alters many physiological and psychological processes in an elevation- and duration-dependent fashion. Exposure of an organism to transient hypoxic stress activates respiratory and circulatory systems and adrenal glands, and affects neurotransmitter release and action in the central nervous system [2]. Another study had found that short exposure (5 days) to an altitude of 7576 m caused increased lipid peroxidation levels in the plasma of rats. This result was confirmed by the same experimental protocol by adding vitamin E supplemented groups [3]. Defining the mechanisms by which mammalian cells and organisms adapt to acute and chronic perturbations in ambient oxygen tension is critical to the understanding of maintenance of homeostasis and consequently the development of therapeutic strategies to counteract hypoxia-induced cell damage. Deprivation of oxygen molecules in physiological microenvironments greatly affects development and growth of multicellular organisms.

This book addresses hypoxia pathophysiology and the discovery of HIF-1α in its introductory chapter in the first section entitled "Introduction to Hypoxia and Anoxia." The introductory chapter (Chapter 1) also explains hypoxia and oxidative stress especially on hypoxia and mitochondrial reactive oxygen species (ROS) generation. The chapter concludes that hypoxia exposure induces the generation of ROS and increases expression of p53, NF-kβ, AP-1, MAPK, and HIF-1α. The increased expression of all these transcription factors leads to either cellular adaptation or cell death [1]. The second section is entitled **"**Hypoxia Cell Signaling" and consists of two chapters. The chapter entitled "Mitochondrial KATP channel functions under hypoxia" (Chapter 2) elaborately analyzes the impact of hypoxia on mitochondrial functions and metabolism with detailed analysis of respiratory chain functions and hypoxia-induced alteration of mitochondrial morphology and subcellular distribution. The chapter also explains the impact of hypoxia on mitochondrial cell signaling and potassium transportation, including direct bioenergetic consequences of mKATP channel opening and subsequently F_0F_1 ATP synthase activity. The chapter concludes that several mechanisms of hypoxia are brought into action to reduce oxygen consumption by mitochondria by downregulation and nitrosylation of respiratory complexes, by producing H_2S to substitute

oxygen as electron donor to the respiratory chain, by downregulation of the OxPhos, and by the activation of mitochondrial ATP-sensitive K^+ transport to reduce ATP synthesis and oxygen expenses for one of the most oxygen-consuming mitochondrial functions along with upregulation of glycolysis. This saves oxygen and preserves cellular ATP needed for energy-consuming processes [4]. The chapter entitled "Hypoxia signaling in cardiovascular diseases" (Chapter 3) explains hypoxia-induced alteration of transcriptional factors like NF-kβ, HIF-1α, etc. This chapter also explains hypoxia in relation to obstructive sleep apnea syndrome, venous thrombosis, ischemia-associated thrombosis along with hypoxia signaling in inflammation and tissue regeneration, and chronic obstructive pulmonary diseases. It also elaborately describes hypoxia in cardiac dysfunction atherosclerosis. The chapter concludes with an in-depth understanding of hypoxia-induced alteration of transcription factors and their association with various cardiovascular diseases [5].

The third section is entitled "Hypoxia Biochemistry" and it has only one chapter entitled "Glycolysis fermentative by-products and secondary metabolites involved in plant adaptation under hypoxia during pre- and postharvest" (Chapter 4). This chapter elaborately describes plant biochemistry in low oxygen microenvironments. It also explains how metabolic adaptation takes place under hypoxia and how growth of the plant and fruit development take place under hypoxia. There are several interesting case studies incorporated in this chapter [6]

The fourth section entitled "Hypoxia, Ischemia and Hypoxic Preconditioning" has two chapters. The chapter entitled "Perinatal and neonatal hypoxia ischemia: the unique challenges of treating the infant brain" (Chapter 5) explains the unique molecular landscape of the infant brain and how hypoxia exacts damage on the neonatal brain. The chapter also describes how hypoxia ischemia mediates neonatal brain damage through three overlapping molecular cell death cascades: excitotoxicity, oxidative stress, and brain inflammation. The chapter also elaborately informs on the therapeutic angles of neonatal hypoxia ischemia. The chapter concludes with suggestions of some promising neuroprotective treatments, such as erythropoietin and tissue plasminogen activator, and future approaches to more novel therapeutic treatment on neonatal hypoxic ischemia [7]. The final chapter entitled "Hypoxic preconditioning: the multiplicity of its mechanisms and a method for preconditioning of effectiveness" (Chapter 6) explains how a hypoxia precondition prevents many hypoxia-induced injuries. This chapter addresses systemic reaction of autonomic systems to hypoxia, the opioid system, serotoninergic and GABAergic systems, etc. on hypoxia. The chapter also discusses preconditioning effects on the resistance to severe hypoxia and the synaptic pool of the caudal brainstem, cortex, and hippocampus. The chapter concludes with certain experimental models to demonstrate the beneficial role of hypoxic preconditioning through neuronal adaptive pathways [8].

Overall this book will enrich the understanding of hypoxia biology for both basic scientists and clinical medicine experts and will further advance our knowledge of the sciences.

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Section 1

Introduction to Hypoxia and Anoxia

Chapter 1

Introductory Chapter: Primary Concept of Hypoxia and Anoxia

Shrilaxmi Bagali, Gavishsidappa A. Hadimani, Mallanagouda S. Biradar and Kusal K. Das

Additional information is available at the end of the chapter

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1. Introduction

Hypoxia is a pathological condition classified as generalized hypoxia and tissue hypoxia. It is well known that arterial oxygen saturation is a part of the normal physiology. A disparity between oxygen supply and its demand at the cellular level may result in a hypoxic condition. Hypoxia in which there is complete deprivation of oxygen supply is referred to as anoxia. Hypoxia belongs to the most grave factors that can directly impair the function of metabolic pathways in the animal cell. These studies dealt mostly with changes in the structure of pulmonary vessels. Normally, PO2 values of −100 Torr in the alveoli of the lungs to less than 10 Torr in tissues such as the medulla of the kidney and the retina in healthy humans are considered as the range of physiological oxygen levels within the tissues of the body [1]. Hypoxia, which occurs when oxygen levels in the microenvironment of a cell, tissue, or organism are reduced relative to the normal physiological state, is associated with a range of physiological and pathophysiological processes [2].

Hypoxia may limit the energy reserves or scope for augmentation and bustle of an organism, it may cause an organism to alter its behavior and/or it may limit the tolerance of an organism to other environmental challenges [3].

2. Hypoxia pathophysiology

Tissue hypoxia is also associated with a diverse and wide range of pathophysiological processes including (but not limited to) vascular disease, chronic inflammation, and cancer [3]. In vascular

diseases such as atherosclerosis and stroke, vascular occlusion leads to acute or chronic tissue ischemia with resultant hypoxia [4]. In cancer, the growth of a tumor away from the local blood supply eventually leads to tumor hypoxia [2]. Hypoxia induces ischemia, hemorrhage, and stroke and found to cause systemic inflammation response syndrome (SIRS), multiple organ dysfunctions (MOD), and multiple organ failure [5].

Hypoxia has been shown to lead to increases in intracellular free calcium concentration (Ca^{2+}), 5-lipoxygenase, lipid peroxidation, cyclooxygenase (COX), constitutive nitric oxide synthase (cNOS), leukotriene B4 (LTB4), prostaglandin E2 (PGE2), interleukins, tumor necrosis factor-α (TNF-α), caspases, complement activation, Kruppel-like factor 6 (KLF6), inducible nitric oxide synthase (iNOS), heat shock protein 70 kDa (HSP-70), and hypoxia-inducible factor-1α (HIF-1α). The sequence of their occurrence provides the useful information for studying the mechanisms underlying the hypoxia-induced injury as well as therapeutic targets to prevent or ameliorate the injury [6, 7].

3. Discovery of HIF-1

Before HIF-1 was found, HRE had been identified in the 3′-enhancer region of the erythropoietin gene, whose transcription is up-regulated by more than 100-fold by severe hypoxia. Hypoxia, or

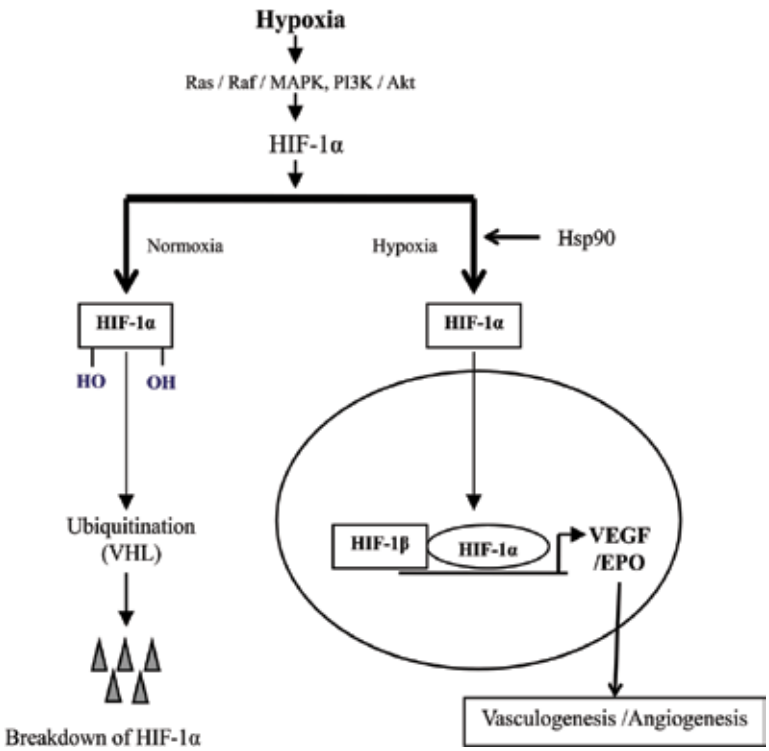

Figure 1. Schematic representation of the cell signaling events leading to ubiquitin-mediated degradation of hypoxia-inducible factor 1α (HIF-1α) and hypoxia-mediated expression of VEGF and EPO protein. VEGF, vascular endothelial growth factor; EPO, erythropoietin; MAPK, mitogen-activated protein kinase.

inadequate oxygenation, causes various responses within the body. Its effects are usually mediated via the activation of hypoxia inducible factor 1 (HIF-1). HIF-1 activation can lead to upregulation of various genes such as erythropoietin and growth factors that help tissues adjust to the decreasing oxygen availability. Another key molecule within this hypoxia-induced response is the presence of nitric oxide [NO]. NO is a ubiquitous gaseous molecule within our body. It is synthesized by nitric oxide synthases (NOS) and its release can be stimulated as a result of inflammatory responses, sympathetic activation and drop in oxygen levels [5]. Increase of HIF-1 translocates to the nucleus, dimerizes with the alpha subunit, and activates the transcription of a number of target genes displaying an HRE shape. Nuclear localization signal (NLS) domains in the alpha and beta subunit confer autonomous translocation into the nucleus 248 [8–10].

One group of HIF-1 target genes is involved in the adaptive response facilitating oxygen delivery to oxygen-deprived tissues. It includes, for example, genes coding for erythropoietin, VEGF-A, and inducible NOS (iNOS) [11, 12] (**Figure 1**).

4. Hypoxia and oxidative stress

During normoxia, about 2–3% of oxygen consumed by the mitochondria is incompletely reduced yielding reactive oxygen species (ROS) [13]. The mitochondrial ROS route to the cytosol and at low to moderate concentrations act as signaling molecules for a number of biological functions like cell growth, differentiation and metabolism and immune functions. Although at high concentrations, they can adversely modify the cell components like lipids, proteins, and DNA. However, the cells are well equipped with antioxidants that are capable of mounting an adequate antioxidant defense against ROS. Oxidative stress results when there is a shift in the balance between the oxidants and antioxidants in favor of oxidants disrupting redox signaling and control and/or inducing molecular damage [14]. In addition to a host of factors, both low and high oxygen levels are capable of inducing increased ROS formation and ultimately oxidative stress. Since oxygen is essential for formation of all ROS several controversies exist, it appears paradoxical for increased ROS formation in low oxygen microenvironment like hypoxia. However, there is enough evidence in support of increased ROS formation and oxidative stress in hypoxia.

4.1. Hypoxia and mitochondrial ROS generation

Mitochondria have been considered the main source of ROS generation (particularly H_2O_2) during hypoxia. Bell et al. have demonstrated a dose-dependent relationship between ROS and available oxygen levels with an increase in intracellular ROS with increasing severity of hypoxia [15]. During mitochondrial respiration, electrons from NADH and $FADH_2$ are transferred successively through several electron carrier molecules of the electron transport chain (respiratory chain). The electron transport chain consists of series of proteins and organic molecules located in the inner mitochondrial membrane (IMM) and organized as four membrane bound complexes (complex I–IV) which generate a proton gradient across the inner mitochondrial membrane and two mobile carriers—cytochrome c and Ubiquinone (coenzyme Q) and a ATP synthase (complex V, F1F0-ATPase) that uses the proton gradient for ATP synthesis. Complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) transfer electrons from

NADH and FADH2, respectively, to coenzyme Q (ubiquinone). Ubiquinone can accept one electron to form ubisemiquinone or two electrons to form ubiquinol. Because of its lipid solubility and small size, it is freely mobile within the lipid bilayer of the inner mitochondrial membrane (IMM). Reduced coenzyme Q further feeds the electrons to complex III of the electron transport chain (ETC). Complex III pushes the electrons to complex IV (cytochrome oxidase) via cytochrome c, second mobile carrier of the ETC. At this stage, complex III plays a crucial role of transferring single electrons sequentially to cytochrome c and complex IV since they can accept single electrons at a time unlike complex I and complex II [16]. Complex IV transfers the electrons from cytochrome c to final electron acceptor oxygen which is reduced to water in the process. As the electrons travel through ETC, they move downhill from a higher to lower energy level. The energy released in this downhill movement of electrons is used to pump protons (H^+) (by complexes I, III, and IV) from the mitochondrial matrix to the inter membrane space creating a proton gradient. The generated proton gradient drives formation of energy in the form of ATP from ADP and Pi by ATP synthase. During the ETC, ROS are produced at complex I, II, and III. Complex I and II produce ROS only into the matrix, while complex III produces ROS on both sides of the inner mitochondrial membrane [13]. Complex III is considered as an important source of ROS during hypoxia [17]. Crucial role of complex III in ROS formation in hypoxia necessitates a greater understanding of its role in ETC. Mitochondrial complex III also referred to as cytochrome bc1 complex (cyt bc_1 or bc_1) or ubiquinol cytochrome c oxidoreductase is a dimer with each monomer made up of 11 protein subunits encoded by mitochondrial and nuclear genes [16]. Complex III has three important subunits with known electron transport activity—binuclear Rieske Fe-S protein (2Fe-2S cluster), bis-heme cytochrome b, and cytochrome c_1 [18]. Cytochrome b contains two heme groups. Of the two heme groups, one is low potential heme (b_L) located near the outer surface of the inner mitochondrial membrane and the second high potential heme (b_H) at the center of the membrane about 20 Å from b_L [19]. The complex III has two separate ubiquinol and ubiquinone binding sites—Qo and Qi. Qo is located on the P (positive) side (outer surface) of the inner mitochondrial membrane and is ubiquinol oxidation site. Qi is located on the N (negative) side (close to the matrix) of the inner mitochondrial membrane and is the ubiquinone reduction site [20]. Complex III performs an important function of transfer of single electrons sequentially to cytochrome c and then to cytochrome IV since they can accept single electrons at a time unlike complex I and complex II by a cycle called Q cycle (**Figure 2a** and **b**). Q cycle begins with the binding of first molecule of ubiquinol to Qo site and ubiquinone at Qi site of complex III. The two electrons of ubiquinol follow two separate paths within complex III. One of the electrons from ubiquinol (yielding ubisemiquinone) is transferred to Rieske Iron-Sulfur protein to cytochrome c1 and finally to cytochrome c. The second electron from ubisemiquinone is transferred to cytochrome b and subsequently to ubiquinone bound at the Qi site of complex III converting ubiquinone to ubisemiquinone. Yet another ubiquinol binds at the Qo site with electron following the Rieske Iron-Sulfur, cytochrome c_1, cytochrome c pathway, and the second electron via cytochrome b reduces the ubisemiquinone at the Qi site to regenerate ubiquinol. Thus, one Q cycle involves oxidation of two ubiquinol molecules to ubiquinone with regeneration of one ubiquinol molecule and transfer of four protons from the intermembrane space from the matrix [21].

Superoxide can be generated at Qo and Qi sites at complex III by one electron reduction of oxygen to superoxide (**Figure 2a** and **b**) [21]. Ubisemiquinone that is formed repeatedly at Qo and Qi sites

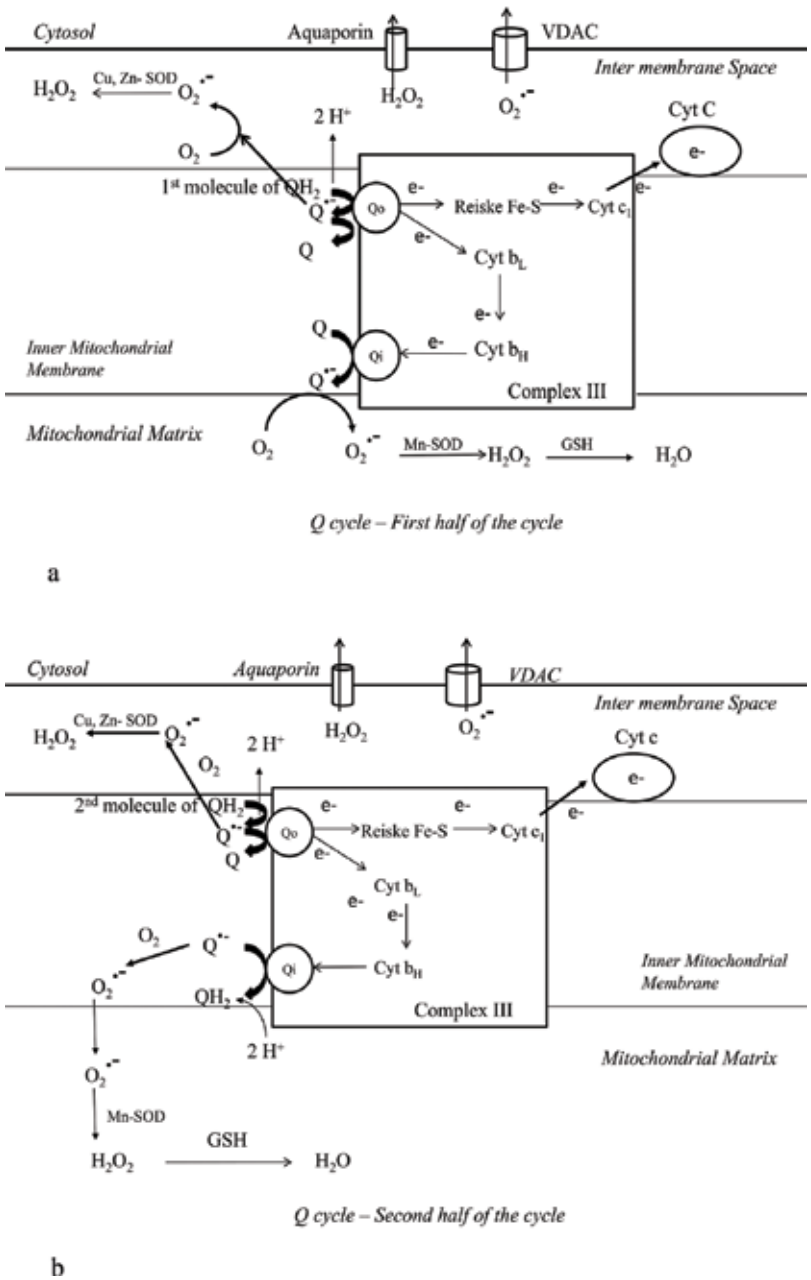

Figure 2. Schematic diagram depicting the Q-cycle at complex III of the mitochondrial electron transport chain and the generation of superoxide and H_2O_2 radicals at Qo and Qi sites of complex III.

of complex III is the site of ROS formation in hypoxia. The molecular oxygen that is lipophilic is dissolved in the hydrophobic environment within the membrane is highly electrophilic and can potentially capture electrons from ubisemiquinone forming superoxide radical [16]. Superoxide generated at Qo site is released into the intermembrane space and that generated at Qi site is

released to the mitochondrial matrix. Superoxide is an important source of H_2O_2 during hypoxia. Superoxide can be dismutated to H_2O_2 in the matrix by Cu, Zn-SOD and in the intermembrane space by Mn-SOD [18]. Hydrogen peroxide can travel to the cytosol via the membrane aquaporin channels [22]. Superoxide may also enter the cytosol through voltage-dependent anion channels (VDACs) [23]. The mechanism by which hypoxia increases ROS generation are poorly understood; however, several hypothesis have been proposed like O_2-dependent structural changes that prolong the lifetime of ubisemiquinone ("semiquinone lifetime" hypothesis), increase in the accessibility of O_2 to a site where single electrons can be captured ("oxygen access" hypothesis) or enhancement of the directional escape of superoxide to the intermembrane space versus matrix compartments ("vectoral transport" hypothesis) [16, 21].

The ROS produced can either participate in cell signaling or induce irreversible cellular damage and death [24]. There is substantial evidence to suggest the role of ROS produced at complex III of ETC in hypoxia signaling by stabilizing HIF-1α by preventing its hydroxylation by prolyl hydroxylases in low-oxygen microenvironment. This allows HIF-1α translocation to the nucleus and dimerization with HIF-1β initiating transcription of target genes (**Figure 3**) [17]. Prolyl hydroxylases (PHDs) belong to a family of mixed function oxidases involved in the hydroxylation of proline residues of HIF-1α that signals it for degradation. These enzymes require 2-oxoglutarate (α-ketoglutarate) and oxygen as substrates and non-heme iron as a cofactor [16]. The activities of PHDs have been extremely sensitive to inhibition by ROS although the mechanisms by which the ROS alter the activity of PHDs are unknown. However, the following hypotheses have been put forward: (1) possible oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) forbidding the mandatory binding of ferrous iron to prolyl hydroxylases; (2) recruiting ascorbate for free radical scavenging making it unavailable for

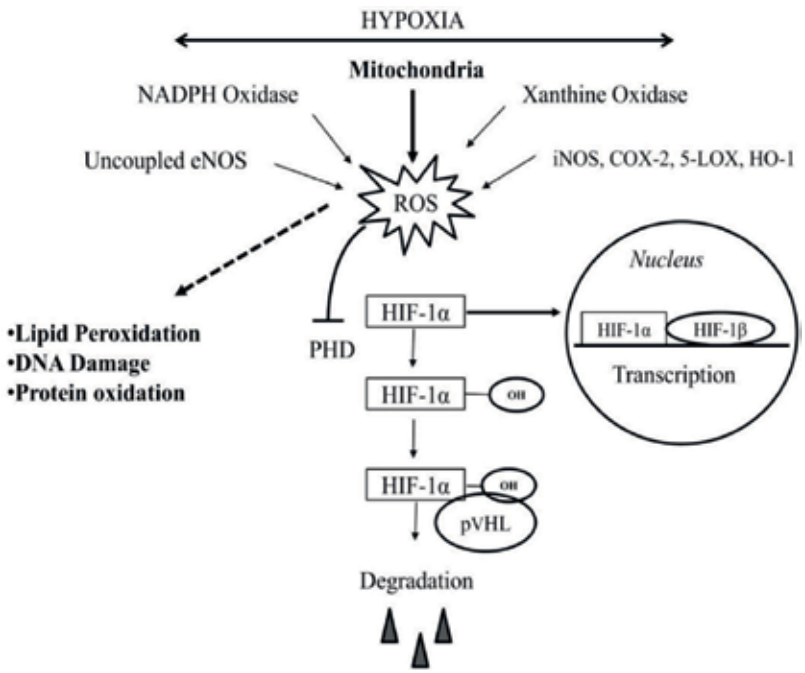

Figure 3. Schematic diagram depicting the various sources of ROD and the biological effects of ROS.

reducing ferric iron or probably by preventing direct binding of ascorbate to prolyl hydroxylase; (3) altering the concentrations of 2-oxoglutarate and succinate that might also have an prolyl hydroxylase activity [16, 25, 26]. Paddenberg et al. demonstrated a role of complex II of the electron transport chain in hypoxia-induced ROS generation particularly in the pulmonary vasculature. Complex II is the smallest of the protein complexes of the ETC located on the matrix side of the inner mitochondrial membrane. Under normoxia, this complex oxidizes succinate to fumarate meanwhile transferring two electrons to ubiquinone and reducing it to ubiquinol. During hypoxia, complex II switches its function from succinate dehydrogenase to fumarate reductase changing the direction of electron flow from ubiquinol to fumarate (fumarate accepting as electron acceptor), thereby generating ROS and accumulating succinate [27].

5. Conclusion

Hypoxia exposure induces generation of ROS (reactive oxygen species) and increases expression of p53, NF-kβ, AP-1, MAPK, and HIF-1α. The increase in expression of all these transcription factors leads to either cellular adaptation or cell death. The mechanisms by which mammalian cells adapt to acute and chronic alteration of oxygen tension are extremely important to understand the exact homeostasis regulation to counteract hypoxia-induced cell damage as therapeutic strategy.

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Section 2

Hypoxia Cell Signalling

Chapter 2

Mitochondrial KATP Channel Function under Hypoxia

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Additional information is available at the end of the chapter

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Abstract

Hypoxic states and conditions result in complex alterations of the energetics and metabolism at the level of the whole cell and mitochondria, including the modulation of metabolic pathways and activation of transcription factors and signaling events. Common feature of the alterations of mitochondrial functions under hypoxia is the activation of mitochondrial potassium channels. Most studied of mitochondrial potassium channels, ATP-sensitive K^+ channel (mKATP channel), is supposed to play important role in the adaptation to hypoxia. However, the main obstacles in the understanding of mKATP channel functions under hypoxic conditions are contradictory data on the direct bioenergetic effects of mKATP channels opening and the lack of knowledge on cell specificity of mKATP channel functioning and of cell signaling pathways triggered by mKATP channels opening. So, the aim of this review was to outline the present knowledge on mKATP channel functions under hypoxia and to discuss how alterations to mitochondrial energetics and metabolism caused by mKATP channels opening (primarily at the level of ROS production and ATP synthesis) could be involved in multiple adaptive responses of a living organism to oxygen deprivation conditions.

Keywords: hypoxia, mitochondria, mKATP channel, potassium transport, ROS production, ATP synthesis

1. Introduction

Hypoxia produces deep alterations in a living organism at systemic and cellular level. Cells respond to hypoxia by complex metabolic reprogramming and molecular mechanisms aimed to minimize detrimental consequences of the oxygen deprivation. Moderate hypoxia exposure (such as intermittent hypoxia) is supposed to possess a great therapeutic potential, while severe and prolonged hypoxia has pronounced pathophysiological consequences [1, 2]. Adaptive responses to hypoxia primarily involve metabolic and functional alterations in

mitochondria. Being the main consumers of cellular oxygen (up to ~90%) and highly sensitive to oxygen shortage [3–5], mitochondria are first to respond to oxygen deficiency. Hypoxia evokes complex network of the mechanisms aimed to adapt mitochondria, their morphology, functions and metabolism to oxygen deprivation. In agreement with the present knowledge, the first step in the adaptation to hypoxia is the expression, stabilization and activation of hypoxia-inducible factor HIF, which is the transcription factor that triggers metabolic reprogramming resulting in the shift from oxidative to glycolytic metabolism [6–8]. Multilevel mitochondrial response to oxygen shortage includes modulations at the transcription level [6, 8], morphological changes [2, 9, 10], alterations in the functioning of ETC at the level of respiratory complexes [11, 12], shift of ATP synthesis from oxidative to glycolytic pathway [7, 11, 13], alterations in the mechanisms of ROS production by the respiratory chain [3, 4, 14], triggering of signaling pathways specific for hypoxic conditions [6, 15, 16] and the modulation of ROS control by matrix antioxidants [17–19]. These mechanisms working separately or together could explain high effectiveness of the moderate exposures to hypoxia in the adaptation of a living organism to severe oxygen deprivation, such as ischemia and anoxia [1, 12].

One common feature of the metabolic alterations in mitochondria under hypoxia is the activation of mitochondrial potassium transport, which is thought to be a part of the adaptive responses of a living organism to oxygen deficiency [20–22]. Mitochondrial system of potassium transport is represented by several types of potassium channels, which are the pathways for potassium uptake (ATP-sensitive K^+ channel (KATP channel), large conductance Ca^{2+}-activated K^+ channel (BK_{Ca} channel), voltage-gated K^+ channels and others, reviewed in [23]), and K^+/H^+ exchanger, which is the pathway of potassium efflux (reviewed in [24]). In the literature, hypoxia was shown to increase the activity of both mKATP and BK_{Ca} channels [20, 25–27] and the overall potassium uptake in mitochondria [21, 28]. Potassium transport is an all-round modulator of mitochondrial functions: oxygen consumption, Ca^{2+} transport, ATP synthesis, ROS production and mitochondrial morphology [10, 24]. But which are the functions of potassium transport and what benefits could be gained by the activation of potassium channels under hypoxia?

Mitochondrial ATP-sensitive potassium channel (mKATP channel) is the most abundant of the K^+ channels present in the inner mitochondrial membrane, and the functional effects of ATP-sensitive potassium transport are best studied as compared to other types of K^+ transport in mitochondria [23, 24]. For this reason, primarily the functions of ATP-sensitive potassium transport under hypoxia and physiological relevance of mKATP channel functioning will be discussed below based on the published data and the results of the author's research.

2. The impact of hypoxia on mitochondrial functions and metabolism

2.1. The impact of hypoxia on mitochondrial morphology and the functions of the respiratory chain

2.1.1. The impact of hypoxia on mitochondrial morphology and subcellular distribution

"Mitochondrial response" to hypoxia, starting from the modulation of mitochondrial morphology and metabolism, is directed at the adaptation of the organelles to the conditions of

oxygen shortage. Morphological changes are highly dependent on the duration and severity of oxygen deprivation. Generally, it was reported that short-term and intermittent hypoxia resulted in the increase of the total number of mitochondria and the enrichment of their subsarcolemmal fraction [9, 10], while severe prolonged hypoxia, on the contrary, suppressed mitochondrial biogenesis and dramatically reduced the number of subsarcolemmal organelles [2, 11]. Apparently, "primary" response to oxygen deficiency is to improve oxygen supply to mitochondria, whereas response to severe prolonged hypoxia fits with the decrease of respiratory capacity and metabolic shift from oxidative mitochondrial to glycolytic metabolism [7, 8].

Thus, it was observed [10] that even short-term exposure to hypoxia (a 30-min hypobaric hypoxia) resulted in obvious changes in mitochondrial morphology and their subcellular distribution. As it was shown in cardiac and muscle tissues [9, 10], oxygen deficiency increases localization of mitochondria near the plasma membrane, in the close proximity to capillaries, and the enrichment of subsarcolemmal fraction of mitochondria, while not affecting interfibrillar one. Also, hypoxia exposure resulted in moderate swelling of cardiomyocyte mitochondria (by 25% from the initial diameter) and the formation of vesicular cristae [10]. Adaptive changes after exposure to 10–14% O_2 were also observed in cerebral cortex mitochondria [29]. More dense cristae package was found in the animals showing higher adaptive capacities to hypoxia [29]. It was observed too that short-term hypoxia exposure promoted mitochondrial division, thereby greatly increasing the number of mitochondria due to the formation of new microorganelles [10]. These changes were supposed to improve oxygen supply to mitochondria and the effectiveness of the oxygen consumption because of greatly increasing the total surface of mitochondria [10]. Interestingly, the effects similar to short-term hypoxia on mitochondrial morphology were produced by mKATP channel opener diazoxide. Thus, diazoxide administration *in vivo* enhanced division of mitochondria and increased the number of newly formed organelles while channel blocker 5-HD abolished this effect [10]. It is worth mentioning that higher adaptive capacities of the animals to hypoxia coincided with higher mKATP channel activity [28, 29], which implies the role of mKATP channel in the modulation of mitochondrial dynamics and agrees with supposed role of the channel in the adaptation to hypoxia.

In contrast, prolonged exposure to severe hypoxia has pathogenic consequences for the organism [1] and detrimental consequences for mitochondria and results in the loss of mitochondrial density, depletion of subsarcolemmal mitochondria, suppression of their biogenesis, decrease in the expression of the respiratory complexes, lowered pyruvate oxidation (complex I substrate), decreased respiratory capacity and de-energization [2, 11]. Thus, chronic exposure to hypobaric hypoxia (5000–6000 m) resulted in ~21% loss of total mitochondrial density and 73% loss of subsarcolemmal mitochondria accompanied by decreased expression of the complexes I and IV [2], which corresponds to the shift of metabolism from oxidative to glycolytic pathway. Thus, it seems reasonable to agree with Ref. [1] that therapeutic effect of hypoxia is a matter of dose and assumes that increase in oxygen-sensing properties of mitochondria is a first step in the adaptation of a living organism to hypoxia.

Hypoxia alters mitochondrial functions and metabolism primarily at the level of the respiratory chain. Thus, hypoxia affects sensitivity of mitochondria to oxygen and the functions of the respiratory complexes, the sources of electron supply to the respiratory chain, pathways

of ATP synthesis and the mechanisms of ROS formation and signaling. There we consider the changes in mitochondrial functions, which are supposed to be a part of non-pathophysiological adaptive responses of a living organism to moderate hypoxia exposures.

2.1.2. The functioning of the respiratory chain and ATP synthesis under hypoxia

Respiratory chain is the subject of complex modulation under oxygen deficiency. At the level of ETC, mitochondrial response to hypoxia is manifested by elevated ROS production and HIF stabilization [3–6], downregulation of the respiratory complexes [2] and ATP synthase, switch from the oxidative to glycolytic ATP production [7, 11–13] and triggering of the mechanisms aimed to suppress the respiration, e.g. by S-nitrosation of the respiratory complexes, which is supposed to save oxygen from excess consumption and prevent excess HIF activity [30]. Nitric oxide is supposed to be active player in hypoxia sensing by mitochondria [30, 31]. Thus, it was proposed that under low P_{O_2} electrons from the respiratory chain could reduce nitrite (NO_2^-) to $^{\bullet}NO$, which then reacts with oxygen producing superoxide ($^{\bullet}O_2^-$). The excess NO production in the presence of $^{\bullet}O_2^-$ results in S-nitrosation of mitochondrial proteins [31], particularly, complexes (I and IV) thereby suppressing respiration. The elevated production of hydrogen sulfide capable to directly donate electrons to the respiratory chain [32] opens the pathways of electron supply substituting for oxygen shortage. Also, the impact of hypoxia on the oxygen-sensing properties of mitochondria appears in the modified sensitivity of the electron transport chain to oxygen *via* the modified kinetic properties and increased oxygen affinity of cytochrome oxidase (complex IV) [9]. All these alterations are supposed to be directed at the adaptation of mitochondria to oxygen deprivation.

The primary step in sensing oxygen deficiency and complex reprogramming of mitochondrial functions under hypoxia is the activation of hypoxia-inducible factor HIF, a transcription factor that takes control over a multiplicity of genes [6]. HIF family counts three known at present members, HIF 1α, HIF 2α and HIF 3α. Most studied are the functions and the regulation of HIF 1, which is composed of constitutively expressed HIF 1β and three HIF α subunits (HIF 1α, HIF 2α, HIF 3α) that are highly sensitive to the changes in oxygen concentration. HIF α subunits are unstable under normoxic conditions (21% oxygen) but are stabilized under oxygen deficiency (10–14% oxygen) and assembled with HIF 1β forming functionally active heterodimers [6]. HIF life span is controlled by prolyl hydroxylases, which require oxygen for their activity and are inactivated when oxygen supply is insufficient. Oxygen-dependent hydroxylation of proline residues (402 and 564 in HIF 1α) by hydroxylase PHD2 and asparagine residue by factor inhibiting HIF (FIH) promotes binding of the von Hippel-Lindau tumor-suppressor protein (VHL), which in turn triggers pathway of HIF degradation by proteasome [6]. ROS formation by mitochondrial respiratory chain contributes to inactivation of prolyl hydroxylases and HIF stabilization, thus HIF stability is critically dependent on both oxygen concentration and ROS formation [4]. Silencing or pharmacological inhibition of prolyl hydroxylases enhanced HIF stability [4, 33], whereas ROS scavenging by antioxidants (N-acyl cysteine, and mitochondrial ROS scavenger mitotracker red) abolished HIF stabilization and HIF-dependent signaling even under oxygen shortage [4].

ROS-dependent stabilization and activation of HIF [3–5] triggers complex metabolic rearrangement resulting in a switch from oxidative to glycolytic metabolism, which is a hallmark of all

hypoxic states, including embryonic and tumor cells known to function in highly hypoxic environment. At transcriptional level, adaptive responses of a living organism to hypoxia involve upregulation of proteins and the enzymes along glycolytic pathway: glucose transporter (GLUT), hexokinase 2 (HK2) and lactate dehydrogenase (LDH). HIF-dependent upregulation of genes encoding glucose transporters [8] results in the enhanced uptake of glucose and the activation of glucose metabolism with eventual formation of the end product lactate.

As shown in literary data, under normoxic conditions about 25% of cellular ATP is supplied by glycolysis, which, for example, in dorsal root ganglion neurons constituted ~3.5 nmol/mg protein [13]. Hypoxia sharply changes relative contribution of the oxidative phosphorylation (OxPhos) and glycolysis to ATP production dramatically suppressing OxPhos pathway while simultaneously upregulating glycolysis. Thus, dependent on the conditions, hypoxia was capable of reducing ATP content by ~50% (which in the above example constituted from ~11 to as low as ~6 nmol/mg [13]), of which about ~5 nmol/mg (i.e. ~80%) was produced by glycolytic pathway. This pattern shows upregulation of glycolysis by ~1.5 times accompanied by nearly complete inhibition of the OxPhos.

ATP obtained by glycolysis, as well as ATP of mitochondrial origin, is consumed by several energy-consuming processes, such as maintenance of transmembrane ion gradients and membrane potential by the work of Na^+, K^+-ATPase, metabolic processes and protein synthesis. Literary data showed not only inhibition of ATP synthase caused by impaired mitochondrial bioenergetics but also its down-regulation along with down-regulation of the respiratory complexes.

As it was established in several studies (in embryonic cardiomyocytes [34], dorsal root ganglion neurons [13], malignant cell lines [35]), there was a reciprocal dependence between impaired mitochondrial bioenergetics, compromised ATP synthesis and upregulation of all steps of glycolytic pathway. Especially in tumors, which metabolic phenotype has much in common with that of normal tissues functioning under hypoxia, elevated expression of glycolytic proteins, starting from glucose transporter and ending with lactate dehydrogenase, and simultaneous downregulation of respiratory complexes and ATP synthase were observed in different cell lines [35].

Under oxygen deficiency, glycolysis was shown to be upregulated not only at transcriptional level but also at the level of metabolism. Thus, as it was shown in the early work of Hohachka et al. [7], insufficient production of ATP and lowering of cellular ATP content result in the elevation of cellular level of ADP that reaches the range of K_m (~100 μM) required for ADP-dependent kinases of glycolysis (phosphoglycerate kinase, pyruvate kinase), which is much higher than it is required for mitochondrial oxidative metabolism (~30 μM). Reduced level of ATP is reflected in lowered phosphocreatine/ATP ratio, which is one of the multiple hallmarks of oxygen deficiency [7, 11].

Another aspect of downregulation of the OxPhos is the conversion of ADP to AMP, the increase of the level of AMP and the activation of AMP-dependent protein kinase (AMPK), which was shown to afford several cytoprotective effects first established under the conditions of ischemia/reperfusion [36]. Thus, AMPK activation, which takes place when ATP demand exceeds the supply, i.e. under oxygen deprivation and compromised mitochondrial ATP production (ischemia, hypoxia [37]), was shown to protect tissues of oxidative stress, opening

of mitochondrial permeability transition pore (mPTP) and apoptosis induction. Also, it was shown to be indispensable for the activation of glycolysis as a part of adaptive responses to the lack of oxygen aimed to compensate for ATP deficiency [35, 36, 38].

At present, it still remains elusive, which is the "molecular link" between metabolic alterations and elevated expression of KATP channels observed under hypoxia in several works. Recently, it was shown [37] that AMPK activation under exposure to moderate hypoxia increased the level of the receptor SUR2A subunit of cardiac KATP channels, which was supposed to be a part of adaptive response to oxygen deprivation. Increased expression of SUR2A in cardiomyocytes was also observed after application of AMPK activator AICAR [37]. Thus, AMPK activation under hypoxia appears to be one of the mechanisms connecting metabolic rearrangements with KATP channels opening.

Thus, oxidative ATP synthesis under hypoxia gives way to glycolysis, and as it was shown in the literature, glycolysis becomes the prevailing source of ATP production, at dramatic diminution of ATP production by OxPhos. Upregulation of glycolytic metabolism primarily occurs because of ROS-dependent stabilization and activation of transcription factor HIF, triggering of HIF-dependent signaling and upregulation of glycolytic enzymes. Increased level of ADP and AMP because of compromised OxPhos and activation of AMP kinase [36] largely contribute to upregulation of glycolysis and glycolytic ATP production.

While metabolic alterations and redistribution of ATP production between OxPhos and glycolysis is one aspect of hypoxia's impact on mitochondrial and cellular functions, another as well important aspect of the functional rearrangements under hypoxia is triggering of redox signaling specific to the states of oxygen deficiency [5, 6, 14]. Elevated ROS production under hypoxia, resulting in HIF activation, is accompanied by the activation of redox signaling that in the literature was shown to be largely mediated by plasmalemmal and mitochondrial potassium channels and cytosolic Ca^{2+}.

2.2. The impact of hypoxia on mitochondrial ROS production and signaling

2.2.1. The sources of ROS under hypoxia

Even short-term exposure to hypoxia triggers complex network of cell-specific signaling pathways involving the induction of early genes [6, 15, 39] and activation of signaling pathways: PKC (phosphatydil-inositol-3-kinase (PI3K)/protein kinase B (Akt), mitogen-activated protein kinase (p38MAPK), AMP-activated protein kinase (AMPK) [15, 16, 40, 41]). Under hypoxia, signaling is known to be tightly coupled to mitochondrial ROS production. The major sources of ROS are NADPH oxidase (NOX), which activation was shown under the states of oxygen deficiency [16, 42, 43], and the respiratory chain [4, 14, 44]. Respiratory chain was shown to be a trigger of redox signaling in response to hypoxia [4, 5, 14]. Literary data show critical role of complexes I and III in HIF stabilization and redox signaling under hypoxia [4, 14, 43]. Mitochondrial complexes I, II and III are known to be the main sites of ROS formation in the respiratory chain [45]. Complex I releases ROS to the matrix space, while complex III releases ROS on both sides of mitochondrial membrane—to the matrix and the intermembrane space

[45]. Mitochondrial complex III is supposed to play a pivotal role in triggering ROS signaling [3, 4, 14]. Suppression of mitochondrial respiration by the respiratory inhibitors such as rotenone (complex I), myxothiazol and stigmatellin (complex III) [4]; genetic deletions within the complexes I [43] and III [4], and ROS scavenging by mitochondria targeting antioxidants (mitotracker red) have shown that under hypoxia ROS signaling and HIF stability were primarily dependent on the ROS production by the respiratory chain. As it was shown by many authors, under hypoxia, the primary site of ROS formation within the respiratory chain shifts to the complex III, and therein Q-cycle [3, 4] and Rieske protein (FeS cluster) [44] were shown to be the major sources of ROS responsible for HIF activation. It is worth notion that HIF stabilization by mitochondria-derived ROS exhibited site specificity. Thus, using approaches based on genetic ablation and pharmacological inhibition, it was shown that Q_o site facing cytosol and Rieske protein of complex III were critical for HIF stabilization under hypoxia, while ROS formation at Q_i site facing matrix space did not contribute to HIF stability [4]. Critical role of Q_o site for HIF stabilization allows an assumption that not so "bulk" ROS production or the lack of oxygen is most important for HIF stability and activation, as ROS signaling from outer boundary of mitochondrial membrane [4]. In line with this observation is the interplay between NOX- and mitochondria-derived ROS, which in the recent decades became a subject of keen interest [16, 42–44, 46, 47]. Voltage-gated K^+ channels of plasma membrane (Kv channels) and mKATP channels were shown to be important link mediating interplay between NOX and mitochondrial ROS formation, which triggers redox signaling specific to the states of oxygen deficiency [16, 42–44, 47–49].

Under hypoxia, the elevation of ROS production in the cell and mitochondria is accompanied by the increase in the level of cytosolic calcium, $[Ca^{2+}]_c$, which was shown to be ROS-dependent [44, 48, 49] and results either from Ca^{2+} entry *via* plasma membrane [49] explained by suppression of Kv channels by mitochondria-derived ROS and sarcolemmal depolarization [43, 46] or, alternatively, Ca^{2+} release from sarcoplasmic reticulum *via* ryanodine receptors [42, 44]. The role of ROS in the elevation of $[Ca^{2+}]_c$ was shown by its abolition by antioxidants (pyrrolidine dithiocarbamate, N-acetyl cysteine) as well as overexpression of glutathione peroxidase (GPx), catalase (CAT) and matrix Mn-SOD [48, 49], which showed its dependence on ROS formation by mitochondria. The elevation of ROS, activation of PKCε and the rise in $[Ca^{2+}]_c$, could contribute to the inhibition of Kv channels, extracellular Ca^{2+} influx [49] or Ca^{2+} release from intracellular stores [42, 44].

As it was shown in several studies, mKATP channels opening was capable to increase mitochondrial ROS production and trigger redox signaling mediated by PKCε [16, 47, 50, 51]. An interplay between NOX and mitochondrial ROS, dependent on mKATP channel opening; the rise in intracellular $[Ca^{2+}]_c$ and the activation of PKCε and other signaling pathways under hypoxia (Akt, MAPK, ERK [40, 41, 52, 53]) were shown in several works. Thus, hypoxia-induced NOX activation was shown to be dependent on mitochondrial ROS, and the suppression of ROS production by respiratory inhibitors (rotenone, myxothiazol) abolished NOX activation [42, 47]. As it was found, the role of mKATP channel in mediating NOX-mitochondria interplay was the direct activation of PKCε, resulting from the increase of mitochondrial ROS production following ATP-sensitive K^+ uptake [16, 50].

Alternatively, mKATP channel activation was supposed to be a part of a feedback loop mechanism, started by NOX activation [42, 47], ROS release and the increase in mKATP channel activity, which in turn triggered PKCε activation both in mitochondria and cytosol by increasing mitochondrial ROS production [16, 51]. The activation of mKATP channel shown under hypoxia by many authors as well could contribute to the increase in $[Ca^{2+}]_c$, because mKATP channel opening and uncoupling of the respiratory chain by potassium transport favors the elevation of $[Ca^{2+}]_c$, by reducing Ca^{2+} uptake in mitochondria [54–57]. Thus, an impact of mKATP channel activity on mitochondrial ROS formation and Ca^{2+} uptake becomes an important modulator of Ca^{2+}/ROS-dependent signaling under hypoxia.

2.2.2. The control of ROS production under hypoxia

Hypoxia evokes specific mechanisms to control ROS overproduction by the upregulation of antioxidant enzymes: SOD, catalase (CAT) and glutathione peroxidase (GPx). Exposure to different hypoxia regimens resulted in the increased expression and activity of SOD, CAT and GPx. Thus, chronic intermittent hypoxia was shown to upregulate the system of matrix antioxidants: SOD and (CAT), which exhibited elevated expression and activity after hypoxia exposure. Higher expression of SOD, CAT and GPx found in myocardium after the exposure to intermittent hypobaric hypoxia afforded preconditioning-like effect explained by the induction of antioxidant defense [19].The effect was similar to the pretreatment of the hearts with antioxidant mixture containing SOD and CAT, which helped to restore cardiac contractile function after ischemia/reperfusion [18]. Thus, while mitochondrial ROS generated by the respiratory chain are supposed to trigger the response to hypoxia shown by the increase in $[Ca^{2+}]_c$, HIF stabilization and triggering of redox signaling [3, 4, 42–44], elevated expression and activation of Mn-SOD, CAT and GPx are capable to abolish or attenuate this response [48, 49] and prevent excess lipid peroxidation and depletion of reduced glutathione [17].

Overexpression of the antioxidant enzymes in pulmonary artery smooth muscle cells showed selectivity towards the inhibition of hypoxic increase in ROS and $[Ca^{2+}]_c$ [48]. Common to other cell types, in smooth muscle cells hypoxia exposure increased both ROS production and $[Ca^{2+}]_c$. Overexpression of GPx and CAT, both cytosolic and mitochondrial, attenuated the response to hypoxia. Overexpression of cytosolic Cu, Zn-SOD had no effect on both ROS and $[Ca^{2+}]_c$, whereas overexpression of matrix Mn-SOD augmented $[Ca^{2+}]_c$ but had no effect on ROS signaling [48]. These data indicated H_2O_2 to be signaling molecule to trigger the response to hypoxia in smooth muscle cells. The absence of the effects of SOD on ROS signaling could be explained by increased H_2O_2 production and signaling explained by the increased SOD activity.

ROS-dependent stabilization and activation of HIF, downregulation of the OxPhos, lack in cellular ATP, activation of AMPK and other signaling pathways, elevation of ROS production and triggering of ROS-dependent signaling result in the opening and activation of mKATP channels, which is supposed to be a part of the adaptive response to hypoxia. As it will be shown below, multiple mKATP channel functions under hypoxia are aimed at controlling mitochondrial respiration, ATP synthesis and ROS production relevant to the conditions of oxygen deficiency.

3. Mitochondrial potassium transport under oxygen deficiency

Cells maintain high transmembrane gradients of sodium and potassium, which support cellular membrane potential built up by the work of Na^+, K^+-ATPase, plasmalemmal K^+ and Na^+ channels and transporters (Na^+/H^+, Na^+/Ca^{2+} and others), in order to maintain cellular functions and metabolism. Potassium is a prevalent cation of cytosol and mitochondrial matrix, where its concentration reaches 120–150 mM, and virtually there is no transmembrane gradient of this cation between the matrix and the cytosol [50]. Possibly, for this reason, K^+ transport for decades was not paid attention needed, till the discovery of mKATP channel (1991), and its importance for tissue protection first observed in experimental models of ischemia/reperfusion [58]. Shortly after it appeared that mKATP channel plays an equally important role in the adaptation of a living organism to oxygen deprivation [20], and later similar effects of the opening of large conductance calcium activated K^+ channel (BK_{Ca} channel) were observed [27]. These findings served as a powerful stimulus for extensive studies of the properties and functions of mitochondrial K^+ channels and cytoprotective mechanisms triggered by K^+ channels opening.

The system of mitochondrial potassium transport is represented by several types of potassium channels: ATP-sensitive K^+ channel (mKATP channel) large conductance Ca^{2+}-activated K^+ channel (BK_{Ca} channel), intermediate conductance Ca^{2+}-activated K^+ channel (IK_{Ca} channel), voltage-gated K^+ channel (Kv 1.3), twin pore potassium channel and other types of K^+ conductance (reviewed in more detail in [23]). Potassium uptake via K^+ channels is opposed by K^+/H^+ exchanger, which acting coordinately constitute potassium cycle [24].

As it was reported, different regimens of hypoxia exposure (such as intermittent hypobaric hypoxia [10, 21, 41], brief hypoxia exposure (hypoxic preconditioning) [25], chronic hypoxia [20, 27, 59]) resulted in the activation of potassium transport: mKATP channel [10, 20, 21, 41], BK_{Ca} channel [27] and K^+/H^+ exchange [21]. According to these data, mitochondrial K^+ channel opening and activation are ubiquitous consequence of oxygen shortage, indicating that K^+ channel opening is involved in the response of mitochondria to the lack of oxygen. So, in the light of the above metabolic and functional rearrangements caused by the oxygen deficiency, it is reasonable to ask which advantages are gained by the activation of mitochondrial potassium transport under hypoxia. Potassium uptake and potassium cycling are energy-dissipating processes affecting mitochondrial bioenergetics. So, with regard to the purpose of this review, most important is to consider how the modulation of mitochondrial functions by mKATP channels opening might affect oxygen-sensing properties of mitochondria and "mitochondrial response" to oxygen deficiency.

KATP channel is an octameric multiprotein complex ubiquitously present in plasma membranes and mitochondria. KATP channels comprise conducting subunit (Kir6.1 and Kir6.2) highly selective towards K^+ and receptor subunit SUR (SUR1A, SUR2A and SUR2B) differently distributed in tissues. The channel is specifically blocked by ATP in the presence of Mg^{2+}. Receptor subunit of the channel binds nucleotides and pharmacological modulators: sulfonylureas (glibenclamide, tolbutamide), which are channel blockers, and the openers (pinacidil, chromakalim, nicorandil, diazoxide). The properties and molecular composition of KATP channels are reviewed in detail in [60, 61].

In the literature, it was supposed that protection of tissues against the impairments caused by hypoxia afforded by mitochondrial K^+ transport is largely based on bioenergetic effects of K^+ transport and signaling triggered by K^+ channels opening [16, 41, 50, 53]. The impact of ATP-sensitive K^+ transport on the oxygen consumption, membrane potential, ATP synthesis, Ca^{2+} transport and ROS production is largely dependent on the abundance of the channel in mitochondrial membrane. Oxygen deficiency affects functional consequences of mKATP channels opening by modulating channels expression and activity [20, 53, 59]. As it was shown in cardiomyocytes, the activation of cardiac mKATP channels under hypoxia was mediated by the interactions of conducting subunit Kir6.2 with heat shock protein 90, HSP90 [25] and Kir6.1 with gap junction protein connexin 43 and PKCε [26]. Silencing or pharmacological inhibition of HSP90 and connexin 43 abolished protective effects afforded by mKATP channels opening [25, 26]. At present, there are scarce data on such interactions, which could contribute to cell specificity of molecular mechanisms regulating mKATP channels opening and its functional consequences under hypoxia. To assess how mKATP channels can be involved in mitochondrial response to oxygen deprivation, direct bioenergetic and functional effects of mKATP channels opening need to be considered.

3.1. Direct bioenergetic consequences of mKATP channels opening

In energized mitochondria, potential-dependent potassium transport directed to the matrix space takes place at the cost of proton-motive force ($\Delta\mu_H$), a free energy generated by the electron transport chain. As $\Delta\Psi_m$ is the main part of $\Delta\mu_H$, K^+ uptake, accompanied by the obligatory electroneutral water uptake [24], occurs at the cost of $\Delta\Psi_m$ and thus results in depolarization. Because of its dramatic effect on $\Delta\Psi_m$ and matrix swelling, K^+ uptake would be detrimental for mitochondria, if there was not the work of respiratory chain and K^+/H^+ exchange. Thus, the loss of $\Delta\Psi_m$ is opposed by the "compensatory" work of respiratory chain [62], which increases oxygen consumption proportional to the rate of K^+ transport in order to restore $\Delta\Psi_m$; on the other hand, matrix swelling is opposed by potassium extrusion *via* K^+/H^+-exchanger, which is accompanied by the matrix contraction [24]. Concurrent work of K^+ channels and K^+/H^+ exchanger constitutes mitochondrial K^+ cycle [24], of which potential-dependent component (K^+ uptake) dissipates $\Delta\mu_H$ and in this way uncouples mitochondria and affects potential-dependent mitochondrial functions: ATP synthesis, Ca^{2+} transport and ROS production.

The impact of mKATP channels opening on mitochondrial bioenergetics greatly depends on the channels' activity and their abundance in mitochondrial membrane, which is responsible for the effects of mKATP channels opening on mitochondrial energy state and decides for cell specificity of mKATP channel functions [63]. Thus, higher density of mKATP channel distribution in brain results in slight depolarization, which was observed in the literature and in our studies [63, 64], while lower amount of the channels in the heart and liver was of no effect on $\Delta\Psi_m$ even at full activation [24, 65, 66]. Elevated expression of mKATP channel and the channel activation that were observed under hypoxia [20, 21, 41, 59] increase the "weight" of ATP-sensitive K^+ transport in the regulation of mitochondrial functions and metabolism. This is still more visible in malignant cells functioning in hypoxic environment, in which overexpression of mKATP channel was shown [53].

Unlike protonophoric uncoupling that reduces transmembrane pH (ΔpH), uncoupling of the respiratory chain by mKATP channel opening is accompanied by the elevated ΔpH because of K^+ uptake into matrix occurring in exchange for protons. However, the activation of K^+/H^+-exchanger reduces this minor gain in ΔpH, and besides, simultaneous increase in the rate of oxygen consumption due to K^+ uptake dissipates $\Delta\mu_H$, largely at the cost of $\Delta\Psi_m$. Thus, the regulation of ROS production and other potential-dependent functions of mitochondria, dependent on cell type, are largely affected by the effects of ATP-sensitive K^+ transport on $\Delta\Psi_m$ and the rate of respiration.

3.1.1. The impact of mKATP channels opening on ROS production in mitochondria

Generally, it is supposed that cytoprotective effects of mKATP channels opening are primarily based on the modulation of Ca^{2+} transport and mitochondrial ROS production, which prevent Ca^{2+} overload [54, 56, 57] and trigger ROS-dependent signaling, thereby preventing the opening of cyclosporine-sensitive pore (mPTP) [50]. However, of all functional effects produced by mKATP channels opening (the modulation of mitochondrial morphology [9, 10], respiration [63, 65], Ca^{2+} transport [54–56], potassium cycle [24, 66], ATP synthesis [20, 67, 68] and ROS production [21, 50, 64]), the effects of ATP-sensitive K^+ transport on ROS production appear to be the most controversial. This diversity needs to consider the direct effects of mKATP channels opening on ROS production in mitochondria.

ROS production in mitochondria is regulated by a number of thermodynamic and kinetic factors [45]. The diverse, and even contrary, effects of mKATP channels opening on ROS production in mitochondria are difficult to evaluate because mitochondrial ROS production depends on a wide variety of conditions, which include mitochondrial energy state (quantitatively represented by $\Delta\mu_H$), redox potential of the main sites of ROS formation in the respiratory chain [69–71], the source of the electron supply to the respiratory chain, the rate of respiration [70] and, at last, the concentration of oxygen [3, 4], which is the end electron acceptor in the redox reactions in the respiratory chain.

Standard redox potential of one-electron oxygen reduction to superoxide constitutes −160 mV, and on this basis, the respiratory chain in highly energized mitochondria comprises multiple putative sites of ROS formation [45, 69]. At complex I, ROS formation largely occurs in the course of thermodynamically unfavorable reverse electron transport, which requires high $\Delta\mu_H$ and critically depends on both $\Delta\Psi_m$ and ΔpH [72, 73]. This mechanism of ROS formation is one best studied "classical" example of thermodynamically regulated ROS production in mitochondria. Unlike this, ROS production at complex III is dependent on both thermodynamic (such as the redox state of the ubiquinone pool) and kinetic factors [45, 69–71], such as the quantity and the life span of free radical intermediates of the redox reactions, which are regulated by the rate of respiration and the relations between the rates of ROS formation and the removal of these species. Q-cycle is supposed to be the main source of ROS in complex III [45], and ROS formation at this site exhibits a bell-shaped dependence on the redox state of Q-cycle [69]. Partially oxidized Q-cycle was shown to be most favorable for ROS production at complex III [74], which implies its dependence both on mitochondrial energy state and the rate of respiration.

The share of ATP-sensitive K^+ transport in the total K^+ transport in brain and liver mitochondria by our estimations, which agreed with literary data [24], was about ~30–35% [64, 66]. However, in spite of the well-defined characteristics of ATP-sensitive K^+ transport obtained in mitochondria of different cell types, the effects of mKATP channels opening on ROS production are difficult to quantify because of their dependence on several mutually dependent parameters. Overlay of the moderate alterations in mitochondrial functions caused by ATP-sensitive K^+ transport with closely interrelated thermodynamic and kinetic factors regulating ROS formation in mitochondria could explain apparently contradictory effects of mKATP channel opening on ROS production reported in the literature. Interestingly, both the elevation [16, 41] and suppression [21, 40] of ROS production were reported to improve cardiac and cardiomyocyte functions after the exposure to hypoxia in a way dependent on mKATP channel opening. Based on the published data, it is tempting to hypothesize that bidirectional regulation of ROS production by potassium transport observed in the literature could represent a flexible mechanism of the fast response to the elevation of ROS levels generally observed under hypoxia and that, dependent on conditions, could prevent ROS overproduction [57, 75] or trigger ROS-dependent signaling [16, 41, 50, 53], which makes this function of mKATP channel of especial importance under limited oxygen availability.

3.1.2. Direct effects of mKATP channel opening on F_0F_1 ATP synthase activity

In several works, including our own studies, an inhibition of both ATP synthesis and hydrolysis, ensuing from mKATP channels opening, was reported [67, 68, 76, 77]. Biochemical mechanism of this effect is not well understood, but, based on the published data, its physiological relevance can be considered.

As we have observed in our work on liver mitochondria, even full activation of mKATP channel by diazoxide moderately increased the rate of state 4 respiration and resulted in slight mitochondrial uncoupling not accompanied by depolarization [64]. However, these moderate changes in mitochondrial functions apparently suppressed phosphorylation, which could not be explained by the mild uncoupling effect. This was reflected in the decreased rates of state 3 respiration and phosphorylation, which were proved by measuring respective rates of proton transport after ADP addition [68]. It is worth mentioning that mKATP channel opening essentially reduced oxygen consumption in the course of phosphorylation and increased apparent P/O ratio [68]. These effects were coincident with concurrent activation of K^+ cycling, which was the cause of stimulation of state 4 respiration [66]. Based on the literature [78], we assumed that activation of K^+ cycling could be the plausible cause for inhibition of F_0F_1 ATP synthase functioning, not explained by respiratory uncoupling caused by ATP-sensitive K^+ transport.

Considering that ATP synthesis and hydrolysis are coupled to proton translocation across mitochondrial membrane, we supposed that concurrent K^+ cycling could disturb the molecular mechanism of F_0F_1 ATP synthase both at the stage of ATP synthesis and hydrolysis. Possibly, close mechanism of such molecular uncoupling called "decoupling" was observed in the literature under the action of K^+/H^+-ionophore gramicidin, which occurred without apparent changes in $\Delta\mu_H$ [78]. While the biochemical mechanism of such decoupling is not quite clear,

its physiological meaning appears to be more evident and needs to be considered. In agreement with the literature, we suppose that it is the regulation of cellular levels of ATP [67, 77], but what is still more important, the regulation of the oxygen consumption by mitochondria.

3.2. Functional consequences and molecular targets of mKATP channel opening under hypoxia

The functional effects of mKATP channels opening under hypoxia are similar to those observed in normoxic cells. Thus, under oxygen deprivation, mKATP channel activation reduced mitochondrial Ca^{2+} loading [57, 79], preserved ATP levels [67, 77] and increased cell survival [16, 40, 41, 80] by suppression of apoptosis *via* targeting glycogen synthase kinase 3β (GSK3β), an enzyme involved in triggering cell death by promotion of the opening of mitochondrial permeability transition pore, mPTP [81]. Suppression of cell death pathways resulted in stabilization of membrane potential [16, 59, 80] and the restoration of ATP synthesis [82]. Increased expression of both Kir6.2 [59] and SUR2A [37], similar to pharmacological mKATP channels opening, too was shown to improve the viability and the resistance of cardiomyocytes to hypoxia.

As one can see from the above examples, cell response to hypoxia was essentially dependent on the bioenergetic effects of mKATP channels opening. This allows assume that cytoprotection afforded by mKATP channels opening is largely based on a synergistic action of bioenergetic effects of mKATP channel functioning (primarily ROS production and ATP synthesis [20, 40, 41, 67, 79]), and the redox signaling critically dependent on ROS formation caused by mKATP channels opening [16, 50].

In the literature, it was obtained rather unambiguous evidence of the suppression of the OxPhos by mKATP channels opening [67, 76, 77]. However, it seems to be surprising that under hypoxia, similar to other metabolic stress conditions, cytoprotection was afforded by contrary effects of mKATP channels opening on free radical formation, and both the reduction [40, 80] and the elevation [16, 41, 50] of ROS production were shown to afford cytoprotective effects. To smooth this apparent contradiction, we recently proposed [83] that, dependent on the direct impact of ATP-sensitive K^+ transport on mitochondrial bioenergetics, mKATP channels opening could afford protection at least in two ways: either directly, by the direct reduction of ROS formation under certain conditions [40, 75, 80, 84] or indirectly, by the elevation of ROS production and triggering of ROS-dependent signaling shown to be cytoprotective under oxygen deprivation (ischemia and hypoxia) [16, 41, 50]. To shed more light on physiological role(s) of mKATP channels under hypoxia, functional consequences of mKATP channels opening on ROS production and ATP synthesis should be considered in more detail.

3.2.1. Triggering of ROS-dependent signaling and controlling of ROS production in mitochondria

With reference to hypoxia, it is generally supposed that mitochondria respond to oxygen deprivation by the generation of ROS and activation of ROS-dependent signaling pathways [3–5, 14]. mKATP channel was shown to be involved in ROS signaling triggered both upstream (by the activation of kinases PI3K/Akt, PKCε [16, 80]) and downstream (p38MAPK

[52], PKCε, Akt [16, 41]) of mKATP channels opening. This implies the ability of mKATP channel to sense and convey ROS signals, which agrees with the function of the channel as a "ROS sensor" proposed in the literature [75, 84]. The ability of mKATP channel to accept and convey ROS signals is well illustrated by the fast response to hypoxia exposure by NOX/ROS-dependent activation of PKCε *via* mKATP channel opening and feedback ROS/PKCε-dependent activation of NOX [16, 42, 47], PI3K/Akt and PKC activation upstream and feedback PKCε activation downstream of mKATP channel opening *via* increase in ROS formation [51] and ROS-dependent Akt and PKCε activation downstream of mKATP channel opening [41], which exerted anti-apoptotic effect by the inhibition of GSK3β and mPTP opening. The ability of ATP-sensitive K^+ transport to trigger cytoprotective signaling based on the modulation of ROS production has adverse effects in tumors functioning under limited oxygen supply and known to exhibit high mKATP channel activity. Thus, radioresistance of malignant glioma cells overexpressing mKATP channel was shown to be dependent on mKATP channel opening, increasing mitochondrial ROS emission and triggering of MAPK/ERK signaling, which also resulted in suppression of mPTP opening and prevention of tumor cell death [53].

As shown in the above examples, a hypothesis of mKATP channels acting as ROS sensors [75, 84] could be useful in the appraisal of physiological functions of mKATP channel under hypoxia. It is well known that mKATP channel can be activated by ROS [85], and elevated channel activity in response to excess ROS formation could serve to regulate mitochondrial metabolism and prevent ROS overproduction [57, 79]. This enables us to consider mKATP channel as the trigger of both ROS-dependent signaling and "ROS sensor" involved in the regulation of mitochondrial ROS production *via* modulation of mitochondrial bioenergetics. Oxidative modification of mKATP channel can represent a feedback mechanism for the regulation of mKATP channel activity. Being at one time a subject of an oxidative modification and a regulator of ROS formation, mKATP channel could be an effective tool in controlling of mitochondrial ROS production under hypoxia.

The impact of mKATP channel opening on mitochondrial energy state, dependent on the channel activity, could serve as a regulatory mechanism directed either on triggering of redox signaling or prevention of ROS overproduction. Apparently, controversial data on the regulation of ROS production by mKATP channel opening possibly reflect one integrated mechanism regulating fast response of mitochondria to the changes of ROS levels in the mitochondrial environment.

3.2.2. ATP-sensitive K^+ transport in the regulation of oxidative phosphorylation

Physiological role of mKATP channel functioning under hypoxia is not limited to the regulation of ROS production and antiapoptotic effects. In our recent work [68], we proposed that F_0F_1 ATP synthase can be one of the principal targets of mKATP channels opening. The modulation of ATP synthesis by ATP-sensitive K^+ transport can play particularly important role under hypoxia, which is the regulation of cellular ATP and controlling of oxygen consumption:

Generally, it is supposed that suppression of ATP hydrolysis by mKATP channels opening is a plausible explanation for the preservation of cellular ATP of excess depletion observed

after application of pharmacological mKATP channel openers under pathophysiological conditions [67, 77]. This assumption was supported by the data showing that inhibition of hydrolytic activity of F_0F_1 ATP synthase by mKATP channel openers helped to preserve cellular ATP levels under ischemic conditions [67]. Possibly, under hypoxia, suppression of ATP hydrolysis would be helpful in saving ATP available from the glycolytic pathway. Besides, the dramatic fall of the total level of ATP under hypoxia and suppression of ATP synthesis by ATP-sensitive K^+ transport should keep mKATP channel in functionally active state in order to maintain other physiological functions of the channel. However, we suppose that inhibition of ATP synthesis could be of particular significance under oxygen deprivation.

Under hypoxia, controlling of cellular oxygen level becomes important for cell survival [3–5, 46]. Mitochondria consume most part (up to 90%) of cellular oxygen. With reference to hypoxia, it needs to be considered that ATP synthesis, which continually occurs in a living cell, is a highly oxygen-consuming process. Thus, it is reasonable to suppose that controlling of oxygen consumption by controlling the rate of ATP synthesis and the reduction of oxygen expenses for oxidative phosphorylation is one vitally important function of mKATP channel under hypoxia. This is in line with other mechanisms suppressing mitochondrial respiration and OxPhos reported in the literature: the activation of AMPK and glycolysis, S-nitrosation of the respiratory complexes and downregulation of F_0F_1 ATP synthase. Possibly, this function of ATP-sensitive K^+ transport (and K^+ transport on the whole) to reduce oxygen consumption and save oxygen for oxygen-dependent processes by suppression of the oxidative ATP synthesis could move into first place under hypoxia. Concomitant suppression of ATP hydrolysis should prevent excess ATP consumption, which was confirmed by the data showing a preservation of cellular ATP ensuing from the mKATP channel opening.

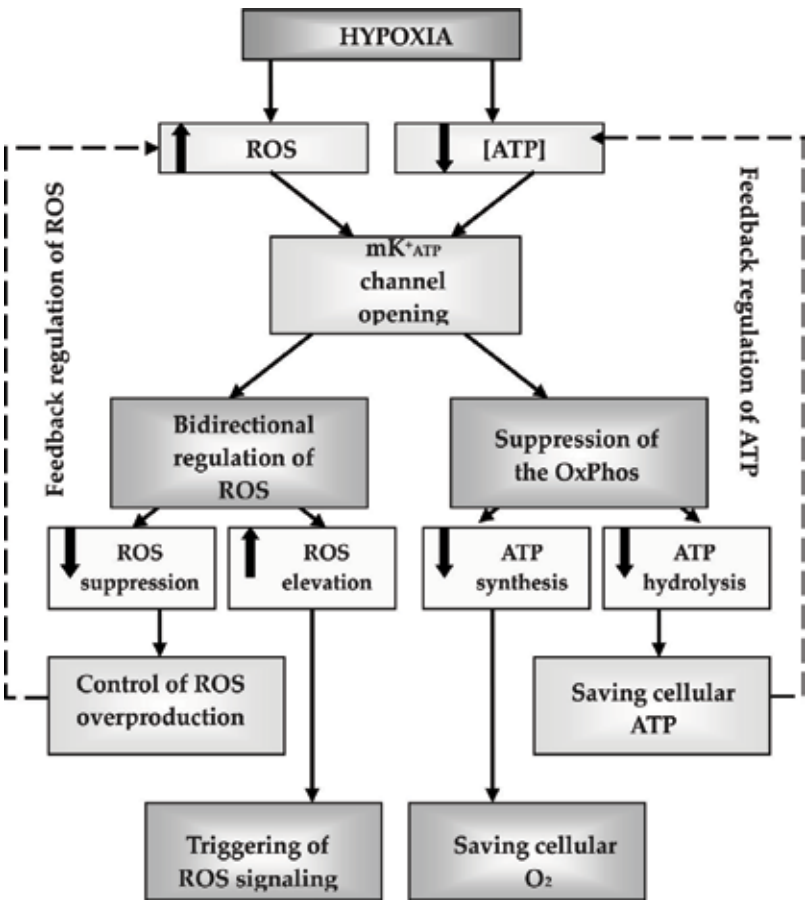
4. Conclusions: physiological relevance of mKATP channel functions under hypoxia

Mitochondria respond to hypoxia by triggering ROS signaling, HIFs activation, controlling of oxygen consumption, ROS production and the level of cellular ATP. Potassium channels of mitochondria and plasma membrane were shown to be both oxygen sensors and ROS sensors and thus are first to respond to the changes of ROS and oxygen levels in the cell. Several published data discussed in this review allow us suppose that activation of potassium transport in mitochondria and controlling the above processes *via* mKATP channel opening could be one of the key events in the adaptive responses of the organelles to hypoxia.

As it was supposed by many authors, mitochondria, being the main oxygen consumers, deprive the rest of the cell of oxygen. Under these conditions, ATP synthesis *via* OxPhos becomes too oxygen expensive function of mitochondria. So, phosphorylation should be down-regulated in order to rescue the whole cell from severe oxygen deficiency. Thus, under hypoxia, several mechanisms are brought into action in order to reduce oxygen consumption by mitochondria, i.e. by downregulation and nitrosylation of respiratory complexes, by

producing H_2S as electron donor to the respiratory chain, by downregulation of the OxPhos and by the activation of mitochondrial ATP-sensitive K^+ transport to reduce ATP synthesis and oxygen expenses for one of the most oxygen-consuming mitochondrial functions. Thus, hypoxia upregulates glycolysis in order to save oxygen and preserve cellular ATP needed for energy-consuming processes, such as maintenance of membrane potentials, metabolism, protein synthesis and other cell functions. Inhibition of ATP hydrolysis by potassium transport helps to save ATP obtained by glycolytic pathway.

The activation of mitochondrial potassium transport is a ubiquitous phenomenon under the limited oxygen availability. The above brief survey of the literature enables us to propose the following important functions of mKATP channels relevant to hypoxia: (1) ability to accept and convey ROS signals, triggering of ROS signaling specific for hypoxia; (2) controlling of mitochondrial ROS production and preventing overproduction; (3) controlling the level of cellular oxygen by oxygen-saving control of OxPhos and ATP production and (4) saving cellular ATP (obtained from both oxidation and glycolysis) by suppression of ATP hydrolysis. Multiple mKATP channel functions under hypoxia discussed in this work can be summarized in the following scheme.



Scheme. mKATP channels functions under hypoxia.

Being important for the understanding of physiological role of mKATP channel, these aspects of mKATP channel functions, largely based on bioenergetic effects of ATP-sensitive K^+ transport, cannot yet help in appraisal of the specificity of mKATP channel, as compared to other potassium channels present in mitochondria. Possibly, novel concepts of physiological role(s) of mKATP channels based on the molecular and cellular mechanisms regulating mKATP channel functioning are required to extend our understanding of physiological relevance and the mechanisms regulating mKATP channel functions under hypoxia.

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Chapter 3

Hypoxia Signaling in Cardiovascular Diseases

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Additional information is available at the end of the chapter

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Abstract

Cardiovascular diseases such as stroke, coronary artery disease, and thrombosis remain a global health burden. Understanding the mechanism of these diseases paves the way for development of prophylactics/therapeutics. It is well known at cellular levels; the pathophysiology of most of the cardiovascular disease involves a complicated yet coordinated signaling networks triggered in response to either cellular or tissue levels of hypoxic milieu. Information related to types of hypoxia and signaling mechanism associated to such complications if complied and presented in a comprehensive manner shall prove relevant in proposing common therapeutic targets for wide array of cardiovascular complications. The relative functional roles of hypoxia-triggered signaling pathways are also an area of current research. Based upon these facts, this chapter discusses the types of hypoxia and role of hypoxia-mediated signaling pathways in various types of commonly occurring cardiovascular disorders.

Keywords: hypoxia, signaling, cardiovascular disorders, thrombosis, therapeutics

1. Introduction

Oxygen concentration below the tissue specific physiological levels is termed as 'Hypoxia'. Depending upon the cause of oxygen scarcity, hypoxia can be classified into Hypoxic hypoxia (occurs due to deficiency in oxygen exchange in lungs or arises due to reduced partial pressure of oxygen in air), Anemic hypoxia (arises when the transport of oxygen is affected), stagnant hypoxia (due to delayed blood renewal, or insufficient blood flow) or histotoxic hypoxia (body is not able to use the available oxygen) [1]. Among its various types, stagnant hypoxia and hypoxic hypoxia are most common types associated with pathophysiology of a variety of cardiovascular disorders (CVDs) such as hypoxic milieu developing in veins due to reduced blood flow promotes thrombus formation [2, 3], whereas, environmental hypoxia at

high altitude exposures also promotes a prothrombotic tendency [4, 5]. Physiological alterations are ultimately orchestrated as a myriad of changes at both cellular and molecular levels. These changes involving the activation of transcription factors (Hypoxia-inducible Factors-1, NF-kB), their downstream signaling pathways, generation of reactive oxygen species and many other molecular adaptive responses in cells, also contribute toward the development of diseased phenotype. A better understanding of these signaling pathways would lead to the identification of putative targets for development of therapeutics and prophylactics to reduce the burden of CVDs. The current chapter discusses the hypoxia-associated pathophysiological changes toward disease progression and major transcription factors playing a role in hypoxic conditions, the signaling and molecular events involved in commonly occurring CVDs. Expanding the understanding of the hypoxia-associated molecular-signaling pathways and cross-talk between them will provide new avenues of therapeutic opportunity of the disease.

2. Master regulators of hypoxia responsive factors

2.1. Transcription factors

2.1.1. Nuclear factor (NF)-kβ

Nuclear factor (NF)-kβ is a eukaryotic transcription factor that mediates inflammatory processes through Rel family of proteins and was originally described as a nuclear factor required for immunoglobulin k light-chain transcription in B cells [6]. Normally, in most of the cells, NF-kβ lies in its inactive state by binding to the inhibitor IkB and is retained in cytoplasm. Upon sensing an inflammatory stimulus, IkB undergoes ubiquitin-mediated degradation and NF- kβ translocation takes place to the nucleus [7]. Inside the nucleus, NF-kB regulates the transcription of a number of genes. NF-kβ plays a central role in inflammatory processes by orchestrating the expression of numerous factors (cytokines, adhesion molecules and enzymes) [8, 9].

Activation of NF-kβ also occurs in cardiovascular tissue with a concomitant increase in expression of *i*NOS (inducible nitric oxide synthase) protein. Increased NF-kβ activity in circulating neutrophils and raised plasma levels of NF-kβ, controlled gene products, soluble E-selectin and soluble vascular cell-adhesion molecule-1(VCAM-1) are a response to hypoxia in patients of obstructive sleep apnea syndrome (OSAS) [10].

2.2. Hypoxia-inducible factor-1 (HIF-1)

HIF-1 is a heterodimeric transcription factor consisting of a constitutively expressed-β subunit and an α-subunit containing an oxygen dependent degradation (ODD) domain [11]. Under normoxic conditions, hydroxylation of ODD occurs in an oxygen dependent manner rendering α-subunit vulnerable to proteasomal degradation [12]. Therefore, HIF-1 is suppressed in normoxia, whereas under hypoxic conditions, HIF-1 is stable and active, capable to bind to the regulatory regions of its target genes and inducing their expression. HIF-1 is

the major regulator of oxygen homeostasis, for adaptation to hypoxia involving increasing tissue reperfusion, and oxygenation, thereby, overcoming initial hypoxic insult [13]. Even under normoxic conditions also HIF-1 regulates the shift to increased glycolysis and anaerobic metabolism at low oxygen tensions [13]. HIF-1 regulates a number of target genes (such VEGF-1, EPO). Furthermore, mammalian HIF-1α has three isoforms viz. HIF-1α, 2α, and 3α. HIF-1α accumulation is the key regulatory subunit for assembly of HIF under low O_2 conditions. Regulation of HIF-1α occurs post-translationally in response to low O_2 levels [14, 15].

2.3. Interaction between NF-kβ and HIF-1 pathways

Although, NF-kβ and HIF-1 has independent roles in gene regulation, their cross-talk plays equally important role in pathophysiology of a number of diseases. Structurally, there lies an active NF-kβ binding site, in the proximal promoter site of HIF-1 gene and NF-kB, regulates the basal levels of HIF-1 gene expression [16]. Even under hypoxic conditions, HIF-1 transcription is upregulated through NF-kβ dependent mechanism [17]. Reports are there to show that hypoxia-induced transcription of NF-kβ depends upon the presence of HIF-1 and HIF-1 also directly, regulates neutrophil survival in hypoxia via NF-kβ modulation [18]. Higher expressions of HIF-1 are related to increased NF-kβ activity and an enhanced inflammatory response [18, 19]. Some of the common gene products are shared by HIF-1α such as eNOS, a potent vasodilator whose bioavailability is increased by coordinated action of HIF-1 and NF-kB. In OSAS even crosstalk of NF-kβ and HIF-1 also play a central role [20].

3. Hypoxia in various CVDs

3.1. Hypoxia in obstructive sleep apnea syndrome

OSAS have been recognized as a major health problem affecting developed countries. The disorder is characterized by obstruction of upper airways during sleep resulting in sleep fragmentation and excessive day time sleepiness. OSAS has shown to have a causal relationship with CVDs [21–23]. Although pathogenesis of CVDs in OSAS is multifactorial, the proposed mechanism by which OSAS predisposes to CVD includes sympathetic excitation, vascular endothelial dysfunction, metabolic dysregulation as well as oxidative stress and inflammation induced by cyclical intermittent hypoxia [24]. Evidences are there to show that inflammatory pathways mediate the pathobiology of cardiovascular complications in OSAS. The pattern of intermittent hypoxia in patients of OSAS can be either repetitive cycles of hypoxia/reoxygenation or it can be with prolonged periods of sustained hypoxia allowing for development of an adaptive response, associated with increased tissue perfusion and oxygenation whereas shorter intermittent hypoxic exposures may also activate inflammatory pathways [25, 26]. Intermittent hypoxia directly promotes the production of cytokines and inflammatory cells in OSAS patients. The inflammatory response in OSAS is regulated by NF-kβ and HIF-1α. A rise in NF-kβ activity and its downstream product TNF-α has been observed in OSAS. Levels of TNF-α has also been found to be higher in serum samples of OSAS patients as compared to age and sex matched controls. OSAS patients also show elevated monocyte

NF-kβactivity [25]. In OSAS, patients with several nocturnal hypoxemia, HIF-1, can be viewed as a pro-inflammatory contributor to hypoxic response by promoting inflammatory cell survival [27, 28].

3.2. Venous thrombosis: role of valvular stasis-associated hypoxia

Venous Thrombosis involves the formation of a thrombus inside deep veins usually in legs. Such thrombus can break off and travels in circulation and may lodge at pulmonary vasculature leading to Pulmonary Embolism, which may cause death. As per current understanding the luminal thrombus in veins develops in the presence of increased stasis and hypoxia resulting from the outgrowth of a progressively occlusive thrombus extending from valve to lumen [2, 29]. Evidences for the role of stasis (reduced blood flow), include clinical scenario like long term immobilization due to hospitalization. Pressure of stasis in venous valves is supported by the observation that contrast media used in venography lingers in the veins for up to 60 min after the procedure in the elderly with a clear gradient of increasing stasis with age [30]. Further, pO_2 measurements in sinuses of dogs by Hamer et al. have established that prolonged stasis leads to severe hypoxia at venous valvular sinus. A steeply declining pO_2 gradient from 5 to 1 kPa was observed after 2 h of stasis [31]. However, the anatomical location of the severe hypoxia and thrombus initiation were same site [29]. Changes in blood flow pattern are attributed to generation of hypoxia. Role of HIF-1α is venous thrombus is contradictory and interplayed. An earlier study revealed that HIF-1α stimulates, vein recanalization and thrombus resolution [32] however, study by Gupta et al. suggest that HIF-1α plays a role in thrombus development [33].

3.3. Pathways associated with ischemia-associated thrombosis

Reduced blood flow in veins (also called as stasis), is associated with reduced intravascular O_2 tension and thrombus progression. However, only reduced levels of O_2 have not been found sufficient to trigger fibrin clot formation. Although interplay of hypoxia with different cell types, majorly mononuclear phagocytes and polymorphonuclear leucocytes, can contribute, the association between hypoxia and hypoxemia has remained strong, despite extensive mechanistic explanations [3, 34]. In thrombotic episodes, hypoxemia is found severe in proximity to venous valve cusps and nascent thrombi develop on apparently intact endothelial surface at the parietal aspect of valve cusps during hypoxemia [3]. In addition, *in vivo* exposures to intermittent hypoxia/reoxygenation are also associated with thrombus formation. Under such settings, hypoxia/hypoxemia is sufficient to cause venous thrombosis. Stasis leads to ischemia that is associated with a myriad of changes in vascular microenvironment, increased vascular permeability [3].

In a mice model of hypoxia-induced thrombosis, administration of blocking Ab to tissue factor (TF) suppressed fibrin deposition [35]. This observation was also supported by the evidence generated when TF expression was analyzed in hypoxic murine lungs. Hypoxic exposure produced ~20 fold rise in TF transcripts in hypoxic lungs in comparison to normoxic ones [36]. In such cases, early growth receptor-1 (egr-1) has been identified as the primary driving motif for hypoxia-induced TF transcriptional upregulation. The biological importance of

egr-1 has also been validated in *in vivo* model system where egr-1 null animals when exposed to hypoxia showed a minimal rise in TF mRNA levels with no change in antigen levels in comparison to normoxia exposed controls [37]. Earlier evidences indicate that oxygen deprivation promotes egr-1 synthesis due to binding of ternary complex factor to serum response element (SRE) sites in egr-1 promoter region [38]. Egr-1 also plays a central role in monocyte expression of TF under hypoxia. Expression of egr-1 initiated mechanism in pathologic changes associated with hypoxia point toward novel strategies to prevent these events, that is, target egr-1 rather than directly targeting coagulation mechanism. These series of events producing exposure of TF in hypoxemic vasculature especially in mononuclear phagocytes and smooth muscle cells provide a new biologic context to consider mechanisms underlying and possible interventions to prevent hypoxia-induced thrombosis.

3.4. Hypoxia signaling in inflammation and tissue regeneration, with role in chronic obstructive pulmonary disease (COPD) (studies with zebrafish models)

Most of the *in vitro* studies have been complemented by *in vivo* model systems to obtain a more physiologically relevant setting to understand the inter-relationship of hypoxia and disease. Rodents are most widely used. Mice and rats are highly amenable to manipulation and are small enough to fit into hypoxic chambers for longer periods of time. With advent of technology and in present era, Zebra fish are also used as a new whole-organism model of disease to understand the complex physiology involved [39]. One of the primary reasons is that zebrafish have optically transparent larvae; an opportunity to visualize disease processes *in vivo* using fluorescence microscopy. In addition, high-throughput drug screening can be done easily by addition of small molecule compounds to the embryo in water [40]. An injury to blood vessel is often associated with tissue hypoxia when blood flow is restricted in localized milieu. Even, inflammation can occur and recruitment of immune cells at the injury site occurs and clearance of damaged cells takes place to prevent infection [41]. Innate immune cells (leucocytes) are the first one to respond to injury and sense change in local oxygen levels. This inflammatory response is highly regulated by HIF, signaling, contributing to the regulation of immune activity and lifespan of leucocytes, as timely resolution of inflammation is also necessary otherwise failure to timely resolution may result in inflammatory diseases such as COPD [42, 43]. Once inflammation is resolved, tissues thus regenerate and homeostasis is restored. In zebrafish models, cardiomyocytes regeneration is dependent upon HIF-1α signaling by virtue of which cardiomyocytes can survive and regenerate with an injury of 20% of heart tissue thereby identifying HIF-1α as a potential therapeutic target [44, 45].

3.5. Hypoxia in heart and cardiac dysfunction: role of reactive oxygen species (ROS)

Myocardial gene expression highly depends upon the levels of O_2. O_2 levels change either during isolated hypoxia or ischemia-associated hypoxia, as a result gene expression patterns are altered. In experiments, conducted with myocardial infarction-induced mice, HIF-1α stability was found to reduce infarct size and decrease the number of apoptotic cells [46]. The possible explanation is an upregulation of cardiotrophin-1 (member of IL-6 family) by HIF-1α in hypoxic environment. Further, impaired cardiac muscle contractility due to reduced calcium

ion uptake by cardiomyocytes along with certain amount of dilation in muscles was the additional factor involved [47–49]. This elevated HIf-1α levels may lead to better cardiomyocyte survival under hypoxia.

In addition to the regulatory role of transcription factors, formation of ROS is another major event occurring under oxygen regulation conditions. ROS participates as a benevolent molecule in cell signaling processes and can induce irreversible cellular damage. Formation of ROS in heart or other tissues may occur by several mechanisms either by xanthine oxidase (XO), NAD(P)H, oxidases, and cytochrome P450, or by auto-oxidation of catecholamines and by uncoupling of NO synthase (NOS) [50–52]. Presence of unpaired e- on NO facilitates its reaction with O_2- to form peroxynitrites ($ONOO^-$), an oxidant. Further, Formation of ROS is also induced by cytokine stimulus, growth factors such as angiotensin II (ATII), PDGF and TNF-α [50, 53]. As an adaptive response, production of ROS is counterbalanced by several enzymatic (such as superoxide dismutase (SOD), Catalase, Thioredoxin) and non-enzymatic mechanisms (intracellular oxidants such as vitamins E, C, β-carotene, ubiquinone lipoic acid and glutathione) [54–56]. Deletion of Thioredoxin reductase leads to cardiac abnormalities and even cardiac death, secondary to severe dilated cardiomyopathy [57]. Activation of ROS occurs in response to various stressors and in failing heart as well (**Table 1**).

3.6. Hypoxia-mediated inflammation in atherosclerosis

Inflammation and hypoxia are integral parts in development of atherosclerosis. Data from recent reports suggest that HIF-1α is involved in the pathogenesis of atherosclerosis. Smooth muscle cells extracted from coronary arteries showed that HIF-1α increased activity was related to increased VEGF expression required for proliferation of smooth muscle cells (SMC) [70]. Moreover, hypoxia also produces HIF-1α dependent increase in macrophage migration inhibitory factor (MIF)-required for escalation of migration increased proliferation of vascular SMCs during progression of atherosclerosis [71]. In developing atherosclerosis chronic inflammation and various types of cells (SMC, EC monocytes/macrophages, and T lymphocytes) are involved in plaque formation [72]. Even, the oxygen supply from the luminal blood strongly affects the cells of blood vessel wall [73]. In developed atherosclerosis, tissue hypoxia occurs at the plaque lesion and HIF-1α expression occurs at the macrophage rich center of plaque [72, 74].

HIF-1α also upregulates the expression of low density lipoprotein receptor related protein-1 (LRP1) associated with cholesterol independent progression of atherosclerosis [75]. In fact, bone marrow transplantation of muscle specific HIF-1α deficient mice reduced the plaque burden in aorta of Ldlr−/− mice. Furthermore, expression of inflammatory genes (M1 macrophage accumulation) was also suppressed in HIF-1α deficient mice [76]. It is also known that tissue hypoxia in plaque lesion is not a consequence of increased plaque burden but a consequence of HIF-1α signaling-mediated M1 macrophage activation [72, 77].

3.7. Congenital heart diseases (CHD): role of spatially differential hypoxia

CHDs are the major inborn abnormality with major role of environmental factors. The role of non-physiological hypoxia during early pregnancy also induces CHD. Reports are there to show that cells in the mouse heart tube are hypoxic while cardiac progenitor cells (CPCs) in the secondary heart field are normoxic. This spatial difference in the oxygenation of developing

Disease	ROS related mechanism	References
Heart failure function (In ischemic syndrome, heart failure is a sequelae of myocardial ischemia and necrosis is a major cause of death worldwide)	ROS contributes to the formation of oxidized LDL, the key molecular player in progression of atherosclerosis. Even the activation of MMPs by ROS contributes to plaque rupture initiating coronary thrombosis and occlusion	[58–60]
Myocardial infarction	ROS plays a role in necrosis and reperfusion, injury. Overexpression of SOD (an antioxidant molecule), reduces the infarct size. Evidences that ROS play an important role in myocardial infarction (MI). Around 20% of patients suffering from MI, often develop heart failure which is also determined by the healing and remodeling patterns of ventricles. The latter highly depends upon ROS. Even inhibition of XO with allopurinol diminishes ROS production in myocardium and attenuates maladaptive LV remodeling, leading to post MI cardiac function.	[61–65]
Cardiac hypertrophy (cardiac hypertrophy often serves as a maladaptive precursor to heart failure)	ROS activates either directly or indirectly many extracellular factors as well as downstream signaling pathways that mediate hypertrophic growth response to these factors. Molecules such as PKC, MAPKs, p38, JNK, ERK1/2, Akt, Tyrosine kinase, NF-kB. For instance, AgII-induced hypertrophy is mediated by induction of extracellular signal, whereas direct activation involves ROS-mediated activation of PKC via oxidation of cysteine residues.	[66–69]

Table 1. Different diseases associated to ROS signaling.

heart serves as a signal to regulate the expansion of CPC and cardiac morphogenesis. The response is also mediated by HIF-1α, where HIF-1α forms a complex with notch effector HES family bHLH transcription factor 1 (HES 1) and protein deacetylase sirtuin1 (SIRT1) at the ISL1 gene (islet gene) where ISL1 repression occurs in hypoxic heart tube or as a response to ectopic hypoxic response and prevents CHDs. Thus this is an example where spatial difference in physiological hypoxia maintains the homeostasis for CPCs and provides mechanistic explanation for non- congenital CHDs [78].

3.8. Pulmonary arterial hypertension (PAH)

PAH is clinically manifested as elevated BP in pulmonary artery with resulting right ventricular heart failure [79, 80]. Hypoxia is known to elicit pulmonary vasoconstriction and arterial remodeling [81, 82]. Hypoxic exposures are the commonly used murine models of PAH. Recently, pulmonary endothelial specific HIF-2α deficient mice showed tolerance to hypoxia-induced PAH as compared to HIF-1α deficient or control mice [83]. As a molecular mechanism HIF-2α regulates NO production in pulmonary vasculature via induction of arginase-1, thereby indicating that HIF-2α-Arginase-1 axes may be used as a therapeutic target to improve NO availability in PAH [83].

3.9. High-altitude hypoxia and thrombosis

An imbalance between tissue demand and actual oxygen supply also develops due to environmental hypoxia or reduced oxygen content in ambient air [4]. Such episodes are commonly

found on exposure to high altitude, mountain climbing or while traveling through air travel, (commercial flights). In commercial flights, when cabin pressure reduces and becomes equivalent to an altitude of 1.5–2.5 km hypoxia is often followed by reoxygenation in majority of such cases, serves as an exacerbating factor for thrombus development in veins [84, 85]. In an earlier study, such observations have been recorded with simulated mouse models where hypoxia-reoxygenation is known to promote thrombosis in mouse model of DVT thereby validating incidence of DVT under H/R conditions. The mechanistic explanation given is that hypoxia promotes the secretion of Weibel-Palade bodies, thereby initiating thrombosis in stenosis model [5]. In addition, a recent report also elucidated the possible early factors for hypoxia-induced venous thrombosis; however, in these cases, animals were exposed to hypoxia/normoxia post-thrombus induction (by ligation method) and the study has reported the role of novel regulators NLRP3-Caspase-1-IL-1β signaling axis under the transcriptional regulation of HIF-1. Using the pre-clinical rat model for hypoxia-induced thrombosis, the investigators have clearly demonstrated that under hypoxic environments (as found at high altitude), NLRP3-Caspase-1-IL-1α signaling axis could serve as therapeutic target to prevent thrombogenesis under hypoxic settings. Nonetheless, the translational potential of these pre-clinical observations were also made evident in patients of altitude induce venous thrombosis obtained from army soldiers posted at regions of High altitude [33]. In another parallel study, aimed to investigate the role of hypoxia-induced platelet hyper-reactivity, platelet specific novel regulator protein 'calpain' was found to be involved in promoting prothrombotic tendency on ascension to high altitude [86]. A genome wide expression analysis of genes in patients of high altitude-induced venous thrombosis revealed that the progression of venous thrombus formation is attributed to the differential expression of hypoxia responsive genes in response to environmental hypoxia [87].

4. HIF-1–independent responses

These responses become functional to promote ATP conservation by limiting energy consuming processes such as ribosome biogenesis ion channel activity. Such types of responses include mTORC1 & UPR pathways-mediated regulation of mRNA translation [88]. Responses include inhibition of protein synthesis by affecting the assembly of active eukaryotic initiation factor (eIF) 4F & eIF2-GTP-met-tRNA ternary complex. mTOR is a highly conserved serine/threonine kinase which integrates environmental stimuli to regulate metabolism, translation & structural organization in cell in response to growth factors and O_2 availability [89, 90].

mTOR occurs in two distinct complexes. mTORC1 (comprising of raptor and GBL/mLST8) and mTORC2 (raptor and GBL/mLST8). mTORC1 plays a role in ribosome biogenesis, mRNA translation, and nutrient import. mTORC2 regulates Akt catalysis and actin organization. Hypoxia regulates the translational activity via involvement of mTORC1 and C2-mediated action. The activity of mTORC1 is regulated by different types of kinases (upstream such as PI3K/Akt/MAPK) by phosphorylation of tuberous sclerosis complex (TSC) [90].

4.1. Biological manifestation of mTORC1 pathways in cardiovascular cells

Cells of cardiovascular system respond to hypoxic environments by exhibiting increased growth and program of vascular remodeling operating in tissues and cells (SMC, EC, fibroblasts), involving signaling pathways (mTORC1 mediated and its downstream targets) [91, 92].

In the cell types of pulmonary artery, adventitial fibroblasts proliferation occurs under reduced O_2 conditions [93]. Signaling pathways include MAPK, PKC. In fact, hypoxic exposure leads to mTORC1 activation as in aortic SMC [92]. Along with mTORC1 activation, $P70^{S6K}$ activity and 4E-BP1 phosphorylation increase, thus affecting the rate of protein synthesis changing with hypoxic gradient [94].

Ischemia is characterized by exposure of cells to O_2 followed by O_2 availability that produce interesting effects on mTORC1 pathway. In experimental models of ischemia/reperfusion cells showed failure in response with mTORC1 inhibition. Additionally, reperfusion also resulted in increased mTORC1 signaling with increased $P70^{S6K}$ and 4E-BP1 phosphorylation. mTORC1 signaling during ischemia imparts/contributes to withstand the associated stresses and help in recovery following ischemic insult [95].

5. Conclusion

Activation of hypoxia-induced signaling mechanisms form an integral component in development of widely known CVDs (**Figure 1**). These mechanisms are activated as an adaptive response toward hypoxia, and involve a coordinated action of Transcription

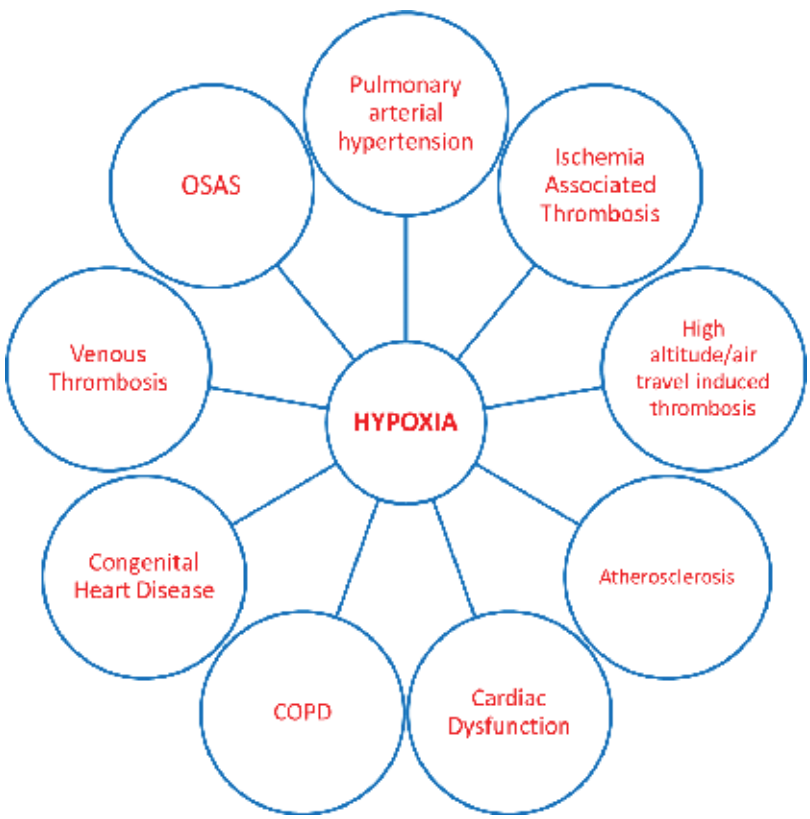

Figure 1. Commonly known CVDs with pathophysiology as a function of hypoxia signaling mechanisms.

factors (HIF-1, NF-kβ), reactive oxygen species and downstream effector molecules, which can serve as therapeutic targets to control the development of the related disease.

Conflict of interest

The authors declare no conflict of interest.

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Section 3

Hypoxia Biochemistry

Chapter 4

Glycolysis Fermentative By-Products and Secondary Metabolites Involved in Plant Adaptation under Hypoxia during Pre- and Postharvest

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Additional information is available at the end of the chapter

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Abstract

Floods inducing hypoxia (reduction of available O_2) in the plants are current major constrains for agricultural production. Oxygen deficiency in the plant cells induces the secondary response of anatomical and physiological modifications. Hypoxia triggers glycolysis fermentative pathway and other alternative pathways, when the plant lacks energy. During cultivation, some submerged plants can adapt themselves to survive by modifying some parenchyma cells in the roots to be aerenchyma cells to detain available oxygen for oxidative phosphorylation. Furthermore, carbon sources in the cells will be accumulated in N store that recovers back to a C source at the end of hypoxia. In postharvest, long period in modified atmosphere storage could activate hypoxia in the plant parts that produce off-flavor perception. However, in some fruits at a particular maturity, ethanol, a hypoxic product, can be modified into ethyl ester compounds as the detoxification.

Keywords: oxygen deficiency, fermentative by-products, adaptation, aerenchyma, off-flavor

1. Introduction

Recently, climate changes on Earth have frequently been fluctuated due to unbalances of natural resources used. The consumption of fossil fuel and industrial activities produce a lot of gas pollutions endangering the atmospheric conditions. Cosmic radiation from outside directly passes through the Earth surface and cannot be reflected out to the space, resulting in increasing temperature of the troposphere. Subsequently, ice packs from mountain tops or icebergs from the North and South poles are gradually melting, causing higher sea mean

level, in particular near the equator areas. Furthermore, when heavy rains come, mass of water may sometimes not be completely absorbed by the soil and slowly runoff into the sea. Lands close to the sea are risk to encounter flash flooding. As a result, flooding is frequently invaded over some agricultural areas where the crops are forced to be damaged and then perhaps dead depending on the level of submerging and the duration of flooding. Although floods are crucial disasters to agricultural crops across the world, the evidence occurs repetitiously in low lands of the tropics such as South America, Africa, and South Asia.

Floods are typically classified into two types including water logging and flooding. Water logging conducts low levels of water flooded and slowly runoff only over the plant root system in which an anaerobic condition is alternately taken place. On the other hand, water flooding, showing excessive water, could be subcategorized to partial and complete submergences. For the partial submergence, some plant parts are immersed in stand-still water, whereas the whole plant is being under the water level for the complete submergence. The respiration of plant parts under water is forced to be switched to an anaerobic pathway due to slower O_2 diffusion from the air. Thus, hypoxia (low O_2 concentration) is mostly generated in partial submerged plants, when metabolisms of upper water parts are under normoxia, but those of submerged parts are switched to an anaerobic condition. Flooded soil leads the decrease of O_2 concentration surrounding the plant roots. The severity of cell damage is relied on the responses of the plants. Water depth and turbidity are important factors defining this scenario.

Although water is important for agricultural cultivation in particular for industrial propose, excessive water supply may cause changes in the anatomy and physiology of cultivated plants. Flooding is caused by heavy raining under poor draining of soil, which could result in the losses of field crop production. The degrees of losses are due to types of plants, stages of development, and duration of water flooding. Horticultural crops need time of recovery when the levels of flood tolerance are different. For example, durian, pummelo, and jackfruit trees are very sensitive to flooding. In contrast, some evergreen trees containing big canopy such as mango will not be damaged by flash flooding as the root system receives some metabolites from the normoxic leaves for surviving. As a result, it is crucial to understand the response and adaptation of plants to hypoxic conditions that would be beneficial for proper management of agricultural supply chains.

1.1. Evidences involved in hypoxia

Water logging and partial submerging could cause hypoxia in agricultural crops during the growth and development. Referred to a whole plant under water logging, the shoots are in normoxia, when the roots turn to be under an anoxic condition. Although hypoxia could be a major abiotic stress, inhibiting the growth and development in many higher plants, hypoxic tolerant plants can generate some metabolites and modify cell structure for recovery to survive. The level of damage from flooding is apparently relied on soil structure. Soil with high porous in the structure contains high O_2 concentration. Nevertheless, when rain falls, soil is then saturated with water which is the trigger mechanism generating the plant response or adaptation. Most triggers include the by-product substances surrounding the plant roots such as soil redox, pH, and decreasing O_2 level. Soil potential (Eh) is used as a key indicator for chemical changes throughout flooding. The Eh is generally reduced when soil is flooded. Under anaerobic conditions, Eh comprises approximately 350 mv which leads to a high

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competitive demand for O_2. However, the changes in soil Eh result that Fe^{++}, Mn^{++}, and other cations will be dissolved out and changed into ferrous ions. Furthermore, in contrast to Eh, soil pH has a trend to be increased when flooded. The increasing pH can be indicated from dissolving carbonate and bicarbonate at the initial stages of flooding. Soil pH affects turn-over of soil organic matters and nitrification [1].

Floods induce the decrease of available O_2 in the plant parts submerged in water. Gas diffusion in the air is 10,000 times faster than in water when O_2 diffuses at the rate of 0.201 cm^{-2} s^{-1} in air, compared to 2.1 × 10^{-5} cm^{-2} s^{-1} in water [2]. The O_2 available level will affect plant cellular metabolisms in three different levels (**Table 1**, [3]):

1. Normoxia comprises aerobic respiration and the ATP production is mainly derived from oxidative phosphorylation.
2. Hypoxia generates when the available O_2 reduces until reaching the limiting factor for oxidative phosphorylation.
3. Anoxia starts when the ATPs are generated only from fermentative glycolysis. This indicates that there is no longer O_2 available. An anaerobic condition in plant during flooding enhances the plants to produce fermentative by-products that accumulate in the roots. In this situation, available energy has dramatic consequence on cellular processes, resulting the generation of unbalances between water and minerals. The plants will be then suffered to other stresses especially disease infection contaminated from flooding.

Apart from plant cultivation, postharvest treatments can either induce hypoxia in stored fresh produce. Fresh fruits and vegetables comprise high rates of respiration after harvest. Thus, some storage conditions such as controlled or modified atmosphere (CA/MA) for long period can generate hypoxia in some parts of the fresh produce. Oxygen cannot diffuse through all tissues causing partial normoxia, hypoxia, and anoxia in the fruit that are responsible for different metabolisms and energy supplies in the fruit tissues.

1.2. Metabolic adaptation plants under hypoxia

Under hypoxia with O_2 deficiency, an inter-conversion of free amino acids is sharply increased. Among the amino acids, alanine is increasingly predominant [4–6]. The production of alanine is come from the inter-conversion of free amino acids derived from proteolysis. Alanine production is related to anaerobic assimilation of NH_4^+ which, in this case, indicates the detoxified of NH_4^+ in the cells [7].

	Normoxia	Hypoxia	Anoxia
Metobolism	Aerobic	Increasing anaerobic	Anaerobic
NAD^+ regenration	Oxidative phosphorylation	Alcoholic and lactic fermentation pathways	Alcoholic and lactic fermentation pathways
ATP production	36-38 mol ATP·mol^{-1} glucose	Dependent on species	~4 mol ATP·mol^{-1} glucose
ATP/ADP content	Normal	Low ATP	Low ATP and High ADP
O_2 content	8-8.5 mg O_2·L^{-1}	1.5-6.0 mg O_2·L^{-1}	0 mg O_2·L^{-1}

Noted: 8.5 mg O_2·L^{-1} = 21% O_2 = 270 μM O_2 =100% O_2 saturation

Table 1. Different levels of available O_2 deficiency of plant cells (modified from [3]).

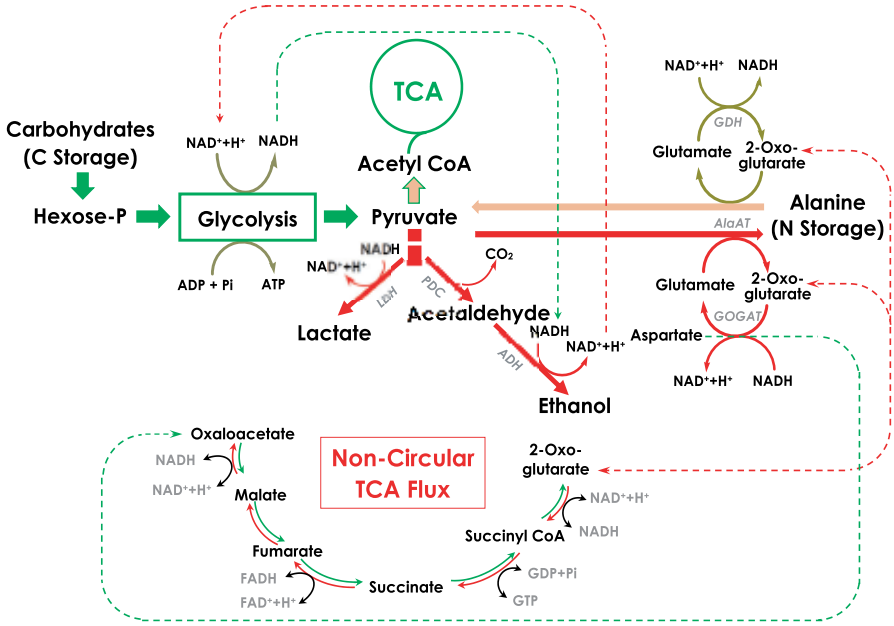

Figure 1. Schematic pathway of carbohydrate and nitrogen metabolisms in plant cells under hypoxic conditions (modified from [1, 8, 9]). LDH: lactate dehydrogenase; PDC: pyruvate decarboxylase; ADH: alcohol dehydrogenase; GDH: glutamate dehydrogenase; AlaAT: alanine aminotransferase; GOGAT: glutamine oxo-glutarate aminotransferase.

The adaptation of plants to survive under O_2 lacking atmosphere includes various evidences in the anatomical, physical, and biochemical changes. Adjustment of N metabolisms in the plants conducts two key enzymes including alanine aminotransferase (AlaAT) and glutamate dehydrogenase (GDH) [8]. Under O_2 lacking conditions, pyruvate derived from glycolysis is alternatively modified to be alanine by AlaAT in coupling with GDH to NAD^+ production. All carbon sources will not be lost through ethanolic fermentative pathway of anaerobic respiration, but some are temporarily accumulated in the form of available N sources instead. When being at the post-stage of hypoxia, alanine is mobilized back to be used as a carbon source by the AlaAT/GDH route. Pyruvate is re-produced again by the reverse reaction of AlaAT in simultaneous with deaminating of GDH to produce NADH and 2-oxoglutarate. Finally, the re-produced pyruvate can go through TCA cycle in mitochondria for oxidative phosphorylation process (**Figure 1**).

2. Plant growth and flowering induced by hypoxia

Hypoxia could affect plant metabolisms throughout the growth and development. This present chapter exemplifies the interesting responses of plant parts to hypoxic conditions that would occur during plant cultivation through the postharvest period.

2.1. Case study: elongation of rice stem during flash flooding

Many rice (*Oryza sativa* L.) varieties can be adapted well with low oxygen conditions to flooding. The crucial adaptations include rapid stem elongation and growth of adventitious roots, and

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metabolic changes. Rice plants will resume aerobic metabolisms and photosynthesis by raising their shoots and leaves above the water surface. Young rice stem at the vegetative growth stage can be elongating when encounters flooding, but after flowering, the mature plant, however, lose the ability. Low O_2 and high CO_2 of hypoxia during flooding promote the ethylene biosynthesis and then enhance the growth-promoting effect of ethylene [10]. *Sub1* family genes (*Sub-1A*, *Sub-1B*, and *Sub-1C*), transcriptional factors involved in ethylene response domains increase in submergence-tolerant rice cultivars. *Sub-1A* interferes with the normal ethylene-response pathway leading to faster extension growth [11]. Thus, rapid internodal growth of rice under flash flooding results in an increase of ethylene mediates. Endogenous ethylene then alternately induces a reduction of abscisic acid (ABA) concentrations and an increase of gibberellic acid (GA) production in the internodes that promote the stem elongation.

Furthermore, the fast-elongating shoot dramatically retrieves non-structural carbohydrates (NSCs) from other developed parts to avoid complete submergence [12]. New developing shoot and leaves of the submerged rice are supplied by NSCs from the developed leaves under flooding, increased in the carbohydrate consumption for cell division and elongation.

2.2. Case study: flowering induction of wax apple by water logging

Success of fruit production during off-season can be done in commercial orchards of wax apple induced by abiotic stresses. The induction of flowering is related to the management of the root system. In general, farmers either prune the root system or apply a short-term flood that is a famous procedure in Taiwan. Water logging induces hypoxic soil environment when the roots respond to the stimulus. In anatomical study, wax apple roots, being changed, acquire a special type of protective tissues called "**polyderm**" that consists of suberized and non-suberized alternating layers. The change in the cell wall by accumulating lignins and suberins, secondary cell wall components, is a developmental program when the surrounding environments are changed. In addition, in the root system, some parenchyma cells dramatically adapted and transformed to be "**aerenchyma**" cells in the cortical areas under water logging due to hypoxia [13]. Modified to be an aerenchyma cell, several parenchyma cells are fused together and the cell then produces and accumulates lignins and suberins in the secondary cell wall, resulting detaining available O_2 in the cell. Consequently, the development of air space allows the diffusion of O_2 from aerial portions of plants into the roots. An increase of lignification and modification of anatomical structure of aerenchyma are recently reported in carrot tap root grown under hydroponic cultivation [14].

Under water logging stress, wax apple tree, a flood tolerant plant, undergoes the different balances of C and N metabolisms in the shoots and roots. The oxidative response in hypoxic root could be via H_2O_2 signaling [15]. In the leaves, starch content increases and the total N decreases 14 days after flooding (DAF), whereas the total soluble sugars increase in the roots. After flooding, a C accumulation in the leaves and an increase of sugars in the roots are responsible for a reduction of the growth and metabolisms in the roots in which reduces the sink demand of carbohydrates. Furthermore, the roots reveal a reduction of soluble protein content 7 DAF, directly related to an increase of free amino acid content. Glutamate dehydrogenase activity in the leaves reduces 7 DAF, but it is higher in the roots that is responsible for high N compound accumulation (**Figure 1**). As a result of high C:N ratio in the leaves/stems, formation of floral buds is activated after a short period of water logging to the wax apple trees [16].

3. Fruit development and ripening under hypoxia

During fruit maturation on the tree, many kinds of fruits in particular climacteric fruits acquire high biological changes including high respiration and ethylene production rates. The physiological changes during environmental changes could affect the quality of the fruit.

3.1. Case study: mangosteen translucent flesh induced by rain fall during on-tree fruit maturation

Fruit flesh is typically developed from the ovary wall of fertilized flowers, but the edible parts of some tropical fruit are developed from other else. For example, durian flesh so anatomically called "aril" is developed from the seed funiculus, while mangosteen aril is developed from integument of the seed coat. Mangosteen fruit contains 4–5 aril segments developed from apomictic seeds. Each big fruit segment typically contains a complete seed [17]. Mangosteen takes 11–12 weeks after anthesis for fruit development. Rain falls above 20 mm/day for 2–3 consecutive days during mangosteen fruit maturation on the tree induces translucent flesh for 30–60% which is specifically progressed only in ripe fruit. Translucent flesh is an internal disorder which the white opaque flesh turns to translucency and the texture changes from soft to crispy firm. Translucent flesh is usually found in the big segment containing a complete seed which behaves high vitality. The pericarp (peel) of fruit containing translucent flesh absorbs high water matter from rain, when water content in the arils is non-significantly different between translucent and normal fruit. Lignins highly accumulate in the cell wall of translucent aril. During ripening of fruit, solubility of pectin increases due to high de-methylation and de-esterification by pectin methylesterase (PME) and polygalacturonse (PG), respectively. Healthy aril behaving white flesh contains an increase of water soluble pectin (WSP), whereas the EDTA-SP and Na_2CO_3-SP are reduced, resulting in rapid reduction of the firmness. On the other hand, translucent aril contains a mild increase of WSP, but the Na_2CO_3-SP significantly increases in parallel of high lignin accumulation in the aril cell wall, especially in the segment containing a complete seed. Thus, the aril firmness of translucent flesh is higher than healthy aril, exhibiting stiffness flesh [18].

The actual cause of translucent flesh in mangosteen fruit during raining is due to water absorption into intercellular space of the pericarp by capillary force. The capillary water functions as a barrier of air movement and circulation in the pericarp resulting generating a hypoxic condition of O_2 deficiency in the fruit. The evident induces higher respiration of the aril, but low energy and high free radicals are released. Thus, this suggests that the high respiration of fruit could be caused partial anaerobic respiration. A high accumulation of CO_2 is found in the intercellular space that could suppress succinate dehydrogenase activity [19, 20], causing non-circular TCA flux (**Figure 1**). The level of reactive oxygen species (ROS) in aril increases during O_2 deficiency [18]. However, adaptability of mangosteen fruit under hypoxia is detected by inducing lignification in the aril flesh, which is related to the phenylpropanoid pathway, including phenylalanine amonialyase (PAL), cinnamyl alcohol dehydrogenase (CAD), and peroxidase (POD) activities. For a hypoxic tolerant mechanism, the ROS production in particular O_2^- that damages cellular membrane and macromolecules is detoxified by modifying

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O_2^- to H_2O_2 by superoxide dismutase (SOD). The H_2O_2 is then served as a co-substrate for lignification by POD. In mangosteen fruit ripening on-tree during rain fall, the aril pectin structure is re-esterified, responsible for more covalent crossed-link network and forming building boxes for cell-to-cell adhesion. Consequently, the cell wall structure generates stiff texture of insoluble jelly-like translucent pectin in the middle lamella (**Figure 2**, [18]).

To prevent the internal disorder of translucent flesh in mangosteen fruit, cultivation management would be applied. Firstly, protection of fruit during on-tree maturation from rain fall would be a great deal, but it is difficult for the practice in out fields. Thus, fruits are usually harvested at early stages of maturation to avoid the risk from rain fall that is a typical procedure of Thai farmers for commercial practice. The harvested fruit will turn to full ripening quickly at room temperature. The other recommendation for the preharvest treatment is to spray some waxes covering the fruit at onset of maturation. The thin covering wax will protect the pericarp from the force of capillary water from runoff during rain fall.

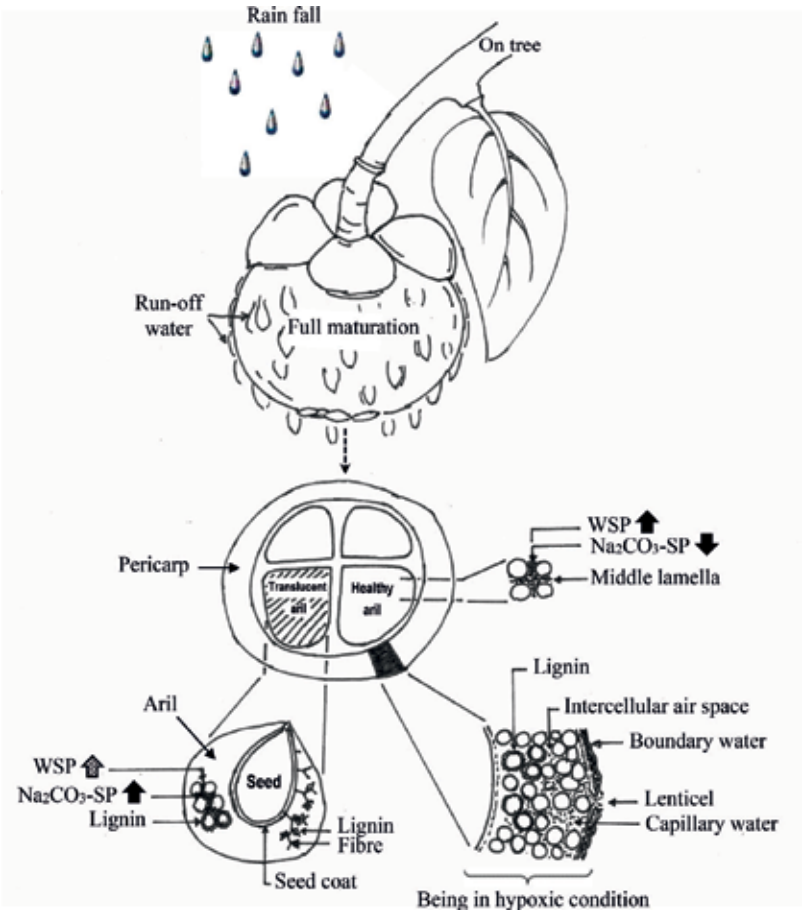

Figure 2. The proposed mechanisms of generation of translucent aril in mangosteen fruit during fruit maturation on tree. WSP: water soluble pectin; Na_2CO_3-SP: sodium carbonate soluble pectin.

Figure 3. Internal disorder of ripe tissues surrounding the seed of "Num Dokmai" (Left) and "Oak Rong" mango fruit (Right) during on-tree ripening.

3.2. Case study: internal tissue disorder of mango during on-tree fruit ripening

The off-flavor of ripe tissue around seed of mango is an internal disorder of some mango fruit cultivars including "Num Dokmai" and "Oak Rong" of Thailand during on-tree ripening (**Figure 3**). The ripe yellow tissue turns to jelly-like translucent tissues with a bid fizzy taste, often generating during on-tree ripening. The disorder would be induced from hypoxia in the fruit upon the physiological changes. Mango among pear and apple produces thick cuticle covering the fruit [21] and the cuticle is even thicker during fruit maturation [21, 22]. Furthermore, as a climacteric fruit, mango shows a climacteric respiration and a peak of ethylene during fruit ripening. Some mango ripening-related genes including alcohol dehydrogenase (*MiADH1*) are sharply expressed at onset of the process [23]. As a result, fruit ripening on the tree could undergo the metabolisms under a hypoxic condition of low O_2 in tissue near the seed. The cuticle thickness of mature mango is a good barrier for O_2 to diffuse into the fruit. The deep tissue could be in a hypoxic condition and start to accumulate acetaldehyde and ethanol compounds, resulting mild off-flavor. Moreover, some modifications in the cell wall of tissue adjacent to the seed are expected to be similarly related to the changes in translucent flesh of mangosteen. In commercial practice, mango fruit are harvested at around 80–90% of maturity and an artificial ripening by applying some ethylene-related compounds is used to the fruit for accelerating the complete ripening to prevent the internal disorder.

4. Hypoxia affecting fruit quality during storage

After harvest, fresh produce lost their quality attributes quickly. Postharvest techniques have been developed and applied to preserve the fruit quality and extend the storage life. However, some evidences of storage treatments could induce hypoxia that is responsible for the quality changes.

4.1. Case study: MA storage of fresh produce

Controlled and modified atmosphere (CA/MA) conditions have been used for extending storage life of agricultural commodities for many decades. Under low O_2 and high CO_2 for long storage, the biological metabolisms of horticultural crops are stimulated by hypoxia to retard

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the ripening and ethylene response. Hypoxic conditions enhance the synthetic of anoxic proteins as well as the cell ability to survive to the subsequent of anoxia [24, 25]. Hypoxia can reduce the respiration and the ATP biosynthesis by reducing the flow of glycolysis and TCA cycle [8, 9]. This condition retards ripening due to the disruption of ethylene biosynthesis by inhibition of ACC-oxidase. The ripening of stored fruits would be delayed. As a result, the quality of horticultural crops especially climacteric fruits can be preserved, and the storage life is extended. Furthermore, for long period under CA/MA storage, some tissues of the fresh produce may be involved in anoxia that induces an enhancement of the activity of alcohol dehydrogenase (ADH) [14, 24]. This increases the accumulations of acetaldehyde and ethanol in the cells that lead to the perception of off-odor.

4.2. Case study: flavor changes in ripe mango fruits under artificial hypoxia

Our current study (unpublished data) revealed that mango fruit at different maturities showed different response to hypoxic conditions. In this case, the maturities of mango affected different responses to hypoxia. Mature green and ripe "Num Dokmai" mangoes were incubated under artificial hypoxic conditions of continuous air flowed through water and through 10% ethanol solution at 25°C. Under air flowed through 10% ethanol, mature mango fruit behaved severe off-flavor after 3 days of storage, whereas ripe mango obviously released additional volatiles in particular ethyl esters on day 7, compared to the control under normal air flowed (**Figure 4**; **Table 2**).

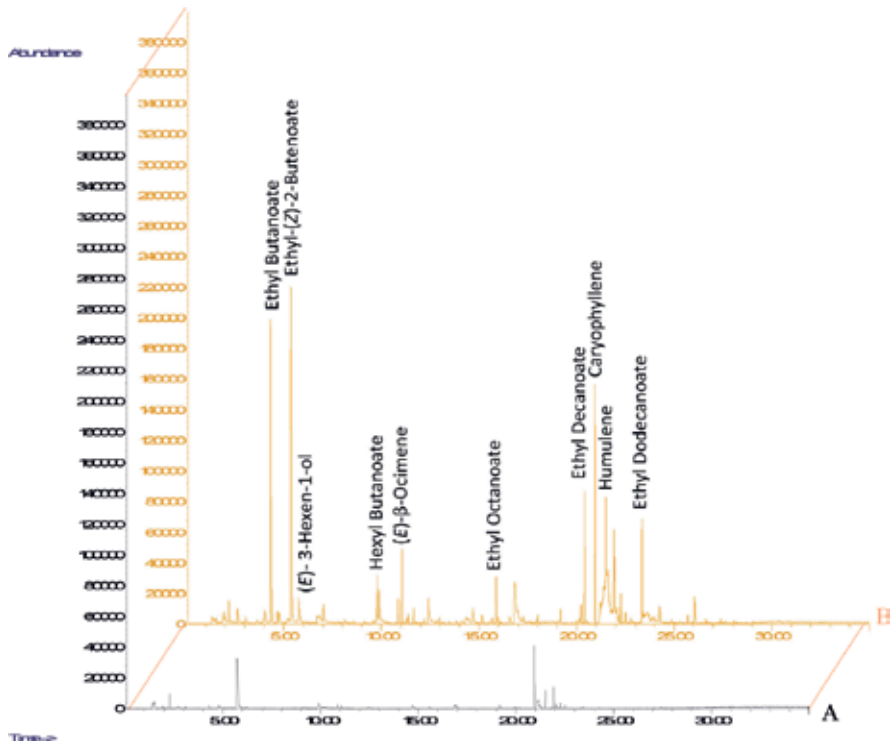

Figure 4. GC-MS volatile profile patterns of ripe flesh of "Num Dokmai" mango fruit incubated with continuous air flowed through water (A) and continuous air flowed through 10% ethanol (B) for 7 days at 25°C.

RT (min)	Normal air	10% Ethanol solution
4.329	-	Ethyl Butanoate
5.369	-	Ethyl-2-Butenoate
5.740	(*E*)- 3-Hexen-1-ol	(*E*)- 3-Hexen-1-ol
9.804	-	Hexyl Butanoate
11.067	-	(*E*)-β-Ocimene
15.879	-	Ethyl Octanoate
20.423	-	Ethyl Decanoate
20.949	Caryophyllene	Caryophyllene
21.503	Humulene	Humulene
23.360	-	Ethyl Dodecanoate

Table 2. Lists of key volatiles released from ripe "Num Dokmai" mango fruit incubated with continuous air flowed through water and continuous air flowed through 10% ethanol for 7 days at 25°C, accorded to **Figure 4**.

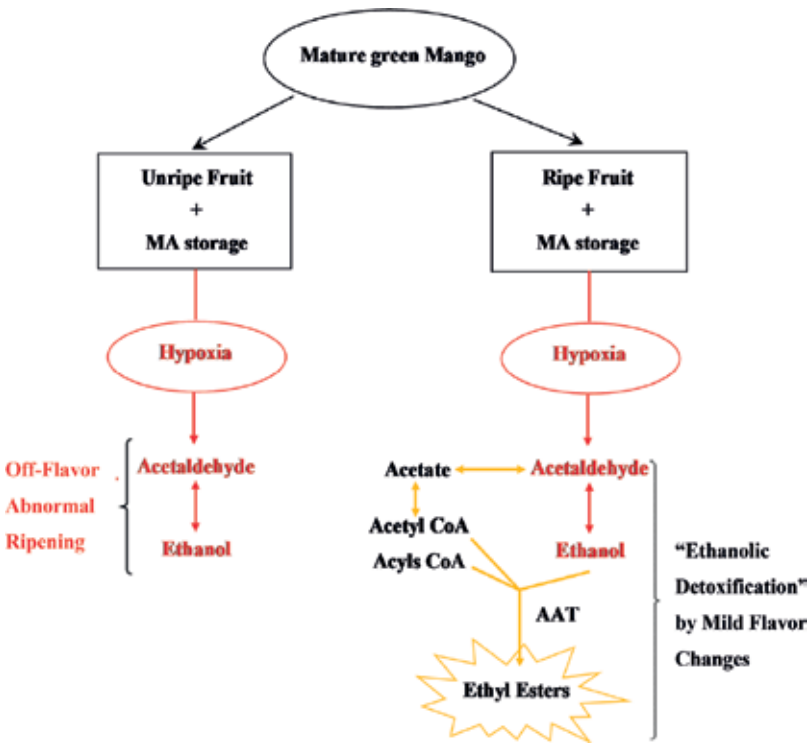

Figure 5. Putative schematic pathway of ripe and unripe mango fruit stored for long period under hypoxic condition.

There is no report about alcohol acyltransferase (AAT), an enzyme producing ester compounds, isolated from mango. Ripe "Num Dokmai" belonging to the Indo-Chinese mango exhibits yellow peel and pulp and high turpentine flavor, whereas many ripe mangoes in the Indian type mango comprising intense peel color, strong aroma and fragrance, and high nutrition value [26] such as "Tommy Atkins" produces a variety of ester volatiles [27]. Alcohol acyltransferase (AAT) has been reported in many fruit as a fruit ripening specific enzyme and is the rate-limiting step for ester biosynthesis regulated by ethylene [28, 29]. It implies that mature green "Num Dokmai" mango fruit contains no AAT activity. When incubated under artificial hypoxia of saturated ethanol vapor, the mango tissues absorbed high amounts of ethanol and released strong off-flavor in a short period. On the other hand, there could be some AAT activities expressed in the ripe fruit, even though typically there is a trace of esters in ripe "Num Dokmai" mango. This is suggested by the conversion of ethanol absorbed in the tissue to be a series of ethyl ester compounds at the late storage as shown in **Figure 4** and **Table 2**. It is in consistent with the report of Jin et al. [30] that ethanol vapor improved aroma profiles especially ester compounds during sweet melon storage. Consequently, some ripe fruits at particular maturity stages can adapt themselves and detoxify the glycolysis fermentative metabolite under hypoxic storage condition by converting ethanol accumulated in the cell to ester compounds by AAT (**Figure 5**).

5. Conclusions

Hypoxia in plants is generated whenever the plants are under conditions of available O_2 reduced such as under water logging/flooding, physiological changes during fruit maturation, and MA/CA storage of plant parts at postharvest. Oxygen deficiency in the cells induces the secondary response of anatomical and physiological modifications. Normoxia, hypoxia, and anoxia can be simultaneously generated in different tissues of the same plant part. Hypoxia triggers glycolysis fermentative pathway and other alternative pathways. Hypoxic tolerant plants are depended on types of plants, maturities, and degrees of hypoxia. Plant defensive mechanisms under hypoxia are signaled mainly by increasing endogenous H_2O_2 and/or ethylene, which are responsible for cascade controls of further endogenous hormones. The responses include an increase in cell wall lignification, different changes in cell wall component, and the production of hypoxic by-products such as fermentative mediate, N-store, and ethyl ester compounds.

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Section 4

Hypoxia, Ischemia and Hypoxic Preconditioning

Chapter 5

Perinatal and Neonatal Hypoxia Ischaemia: The Unique Challenges of Treating the Infant Brain

Lancelot Jamie Millar

Additional information is available at the end of the chapter

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Abstract

Hypoxic ischaemic injury can damage the brain at any age. However, the infant brain displays a unique profile of sensitivity and resistance compared to adult ischaemic stroke patients. Both pathology and response to treatment are uniquely affected by the molecular landscape of the neonatal brain. With new revelations in the biology of brain injury in perinates and neonates being discovered, as global mortality and morbidity increases research funding into infant brain injury, it is important to raise awareness of the unparalleled challenge of treating these young patients. This chapter will review currently known differences between the infant and adult brain response to hypoxia, and address existing treatments alongside proposed treatments not yet evaluated by clinical trial.

Keywords: perinatal, neonatal, hypoxia, ischaemia, ischemia, hypothermia, development

1. Introduction

The clinical definition of neonatal hypoxic ischaemic (HI) injury is "asphyxia of the umbilical blood supply to the human foetus occurring at 36 gestational weeks or later" [1–4]. Neonatal HI has also been referred to as hypoxic-ischaemic encephalopathy (HIE), where the neonatal period is interchangeably referred to as "term" [2, 4]. If the injury occurs prior to 36 gestational weeks, the condition is described as perinatal hypoxia ischaemia.

Neonatal hypoxia ischaemia is diagnosed based on a range of factors which correlate with clinical outcome [1, 5, 6]. These include: 5-min Apgar score of less than 5 [7, 9]; need for delivery room intubation or cardiopulmonary resuscitations [8]; umbilical cord arterial pH below 7.00 [9]; and absence of normal neurological signs, such as the infant sucking reflex

[7, 10]. These are only a selection of risk factors assessed postnatally, and there is an enormous range of clinical outcomes amongst infant patients diagnosed with HI [11, 12].

Globally, hypoxia ischaemia is the single most common cause of death and disability in human neonates [13–15], making further research into pathophysiology and treatment an international priority. Persistent disability is common is surviving infants. Clinical outcomes can range from death to normal neurological profile at 2 years follow-up [16]. Meta-analysis studies have documented that 5–10% of patients developed a persistent motor disability, with up to 50% of patients displaying cognitive or sensory disorders in childhood or adolescence [17–20]. Between 0.7 and 1.2 million infants are born with evidence of hypoxic ischaemic brain injury every year, accounting for 23% of global infant mortality [21]. Survival rates have increased since the 1990s [22], perhaps in part due to improvements in intensive care technology, yet the prevalence of morbidity associated with infant HI remains undiminished [23, 24]. These sobering statistics should draw greater attention to the study of hypoxia in the developing brain, and the need for protective therapies to administer in these vulnerable infants.

2. The unique molecular landscape of the infant brain

Hypoxia exacts damage on the neonatal brain in a unique profile incomparable to the effect of ischaemic stroke on the adult brain. The fundamental anatomy and chemistry of the immature brain creates sites of increased sensitivity and resistance, many of which basic science is only beginning to understand [16, 25]. This chapter will examine several key areas where the physiology of the neonatal brain, and its susceptibility to hypoxic brain damage, requires special consideration. The section will cover: the effect of structural immaturity on the development of hypoxic ischaemic brain injury; alterations to the balance of cell death cascades; and the surprising sex differences in severity of neonatal injury.

2.1. Hypoxia ischaemia and the structurally immature brain

The basic anatomy of the foetal brain is far from the oxygen-rich vasculated tissue familiar from the adult brain, as summarised in **Figure 1**. Outlining the full range of age-dependent processes is beyond the scope of this chapter, but one excellent review [25] expands substantially on the information presented here.

The cerebral microvasculature is known to exhibit a significant risk of rupture, especially in premature neonates [26, 27]. Fluctuations in cerebral blood flow have been correlated with increased rates of intracerebral haemorrhage in infant patients [26, 28], an effect enhanced by altered CO_2 partial pressure in the blood [29] and haematocrit levels [30]. One influential model [31] of the neonatal blood brain barrier (BBB) describes the cerebral vasculature as undergoing a state of flux, remodelling vessels from basal-ganglia dense to a predominantly cortex enriched state. This immature, incomplete vascular structure has not formed permanent vessels by the time of birth. Research in animal models support this assessment, suggesting that neonatal blood vessels are surrounded by fewer astrocyte end-feet [32], demonstrating that the regulatory basement membrane which surrounds mature blood vessels is still forming in the neonatal brain.

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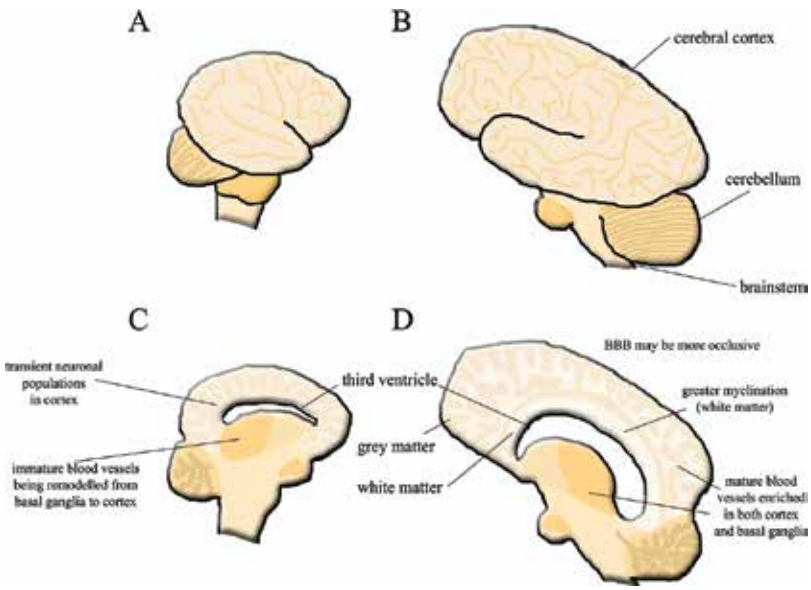

Figure 1. Schematic summarising gross anatomical differences between the adult and neonatal human brain. Side profile of whole neonatal (A) and adult (B) human brains. Sagittal cross-section of neonatal (C) and adult (D) human brains, revealing structure visible by magnetic resonance imaging (MRI). Several key differences are highlighted between the structure of the brain around birth and in the mature adult. BBB = blood brain barrier.

The infant cerebrovasculature is often described as operating on a "pressure passive" autoregulatory system [29]. Impaired vascular autoregulation has been reported as a risk factor for poor clinical outcome in cases of perinatal and neonatal hypoxia ischaemia, or other infant brain injuries [33, 34]. Low vascular tolerance of fluctuations in arterial CO_2 partial pressure and mean arterial blood pressure have been associated with severity of brain lesion in human patients [35]. Nitric oxide synthetase (NOS) inhibitors were shown to be effective at increasing tolerance of hypertension in neonatal pigs by increasing the upper cerebral blood flow limit for vascular autoregulation [36], an effect not replicated in juvenile animals. Currently, the mechanisms behind this "pressure passive "vascular regulation seen in the neonate remain unknown [25], yet the success of NOS in preserving the cerebral vasculature of neonatal pigs suggests that different molecules drive vascular autoregulation in the developing brain compared to the adult.

Another key differentiating factor between the adult and infant brain is the blood brain barrier. The BBB is composed of capillary endothelial cells, astrocytes, pericytes, and the basement membrane, forming a structure that regulates the transport of molecules between the blood and the extracellular matrix of the brain. The accepted view in the literature for some time has been that the immature BBB is less occlusive than that of the adult, enhancing brain damage when the infant brain is subjected to hypoxia ischaemia [16, 25].

Some researchers have reported increased 'leakage' through the BBB in the immature brain. In postnatal day 7 (P7) rat pups subjected to unilateral common carotid artery occlusion followed by exposure to hypoxia, BBB permeability to immunoglobulin G (IgG) was increased compared to P14 rats undergoing the same procedure [37]. When blood brain barrier transfer coefficient was measured in perinatal and neonatal sheep, a greater vulnerability to hyperosmolarity was

detected compared to postnatal sheep [38]. Conversely, matrix metalloproteinase 9 (MMP9) knock-out mice, which display reduced BBB permeability to IgG, were protected against neonatal HI, displaying reduced brain lesion size [39]. Pharmacophores which reduce BBB leakage are also protective [40, 41].

However, assumptions concerning the vulnerability of the BBB are now coming under revision [25]. Some experiments suggest that the increased BBB permeability in young rodents is a secondary consequence of brain inflammation [42, 43], which suggests that reducing inflammation in the hypoxic ischaemic brain may preserve BBB function. It is now known that tight junctions, the molecular structures within the BBB responsible for its occlusive properties, are present from the day embryonic blood vessels invade the foetal brain [16, 25, 44]. These foetal BBB units have been demonstrated to possess occlusive properties, excluding water molecules in the developing opossum brain [44, 45], and in piglets subjected to hypoxia ischaemia [46].

This brief overview highlights the importance of immature brain anatomy to the creating a unique set of factors influencing the outcome of hypoxic ischaemic brain injury in the infant brain. More basic research is needed to clarify the structure and functional capacities of the cerebral microvasculature in the perinatal and neonatal brain. This information will be essential prior to development of future therapies for oral or intravenous administration.

2.2. Cell death in the neonatal brain: excitotoxicity, oxidative stress, and inflammation

There are several other areas of divergence between the infant and adult brain in addition to vascular architecture. The immature brain also responds differently to major molecular cell death pathways. Hypoxia ischaemia mediates brain damage through three overlapping molecular cell death cascades: excitotoxicity, oxidative stress, and brain inflammation [16, 25], summarised in **Figure 2**. The following section will outline the unique vulnerability of the developing brain to each of these processes.

Excitotoxicity is a major cause of cell death in hypoxic ischaemic brain injury. During excitotoxicity, over-activation of physiological glutamate neurotransmission leads to excessive influx of positive ions through postsynaptic receptors, leading to cell death [16, 25, 47]. The *N*-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamate receptor stimulated during excitotoxicity, is expressed at a substantially higher level in the developing brain compared to the adult. In P6 rats, the NMDA receptor is expressed at 150–200% of adult levels [48]. The combination of NMDA receptor subunits also differs in the perinatal period. The subunits expressed in foetal rat favour prolonged calcium influx for a given excitation [49], increasing the sensitivity of the immature brain to excitotoxicity. Intracerebral NMDA injection in rats produces more extensive cell death in the neonate than in the adult [50]. Increased glutamate concentrations have also been documented in the cerebrospinal fluid (CSF) of human infants who have suffered severe HI injury [51].

Many factors contribute to the sensitivity of neurons to excitotoxic cell death, which is not solely mediated by NMDA receptors. Much of this unique molecular landscape remains to be understood. For example, it is known that neonatal brain is more prone to seizure activity than the adult brain [52], with severe seizures potentially resulting in permanent brain damage by

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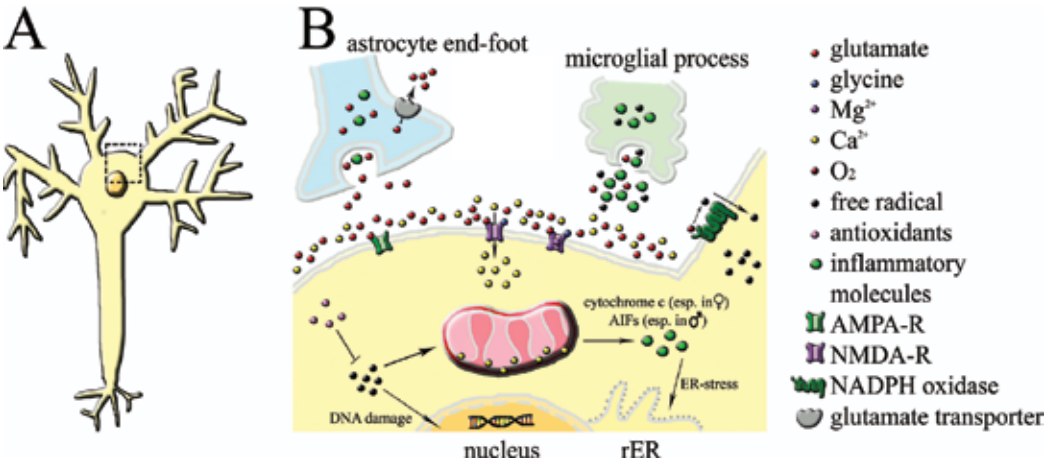

Figure 2. Schematic showing major molecular cascades contributing to the neuropathology of neonatal hypoxia ischaemia. (A) Schematic of an immature cortical neuron. (B) Sub-cellular molecular interactions in neonatal hypoxia ischaemia. The inset corresponds to the area of the neurons in panel A outlined by the dotted line. Molecules involved in excitotoxicity, oxidative stress, and inflammation closely interact. rER = rough endoplasmic reticulum, AIF = apoptosis-inducing factors, free radicals in H_2O_2, O_2^-, NO, inflammatory molecules include interleukins (IL1β, IL6), tumour necrosis factor alpha (TNFα), cytokines.

excitotoxic mechanisms. However, the mechanisms behind this sensitivity remain debated [52, 53]. Perinatal exposure to hypoxia is known to elicit seizures in rodent models [54]. Yet it is not clear if this is caused by the unique receptor complement of the developing brain, transcriptional responses to hyperexcitability, long-term remodelling responses to inflammation, or the paradoxical excitatory activity of the neurotransmitter γ-amino butyric acid (GABA) in the developing brain [53].

The oxidative stress molecular cell death cascade is integrally linked to that of excitotoxicity. Oxidative stress is the term for high levels of free radical production generated during oxygen metabolism under pathological conditions [55]. Hyperexcitability causes energy depletion, mitochondrial dysfunction, and calcium ion accumulation in the cytoplasm, which in turn lead to generation of free radicals, the damaging particles responsible for oxidative stress, which then trigger increased excitotoxicity [16, 55]. Free radicals are atoms or molecules containing an unpaired valence electron which makes these molecules highly chemically reactive and capable of stripping electrons from other molecules in the brain, particularly in the mitochondria [55].

In the adult brain, there exist several protective mechanisms which reduce the damage caused by oxidative stress, such as stores of antioxidants and nucleic acid or protein repair enzymes, which are not yet fully developed in the infant brain [56]. Expression of the enzyme nitric oxide synthetase (NOS), which inhibits mitochondrial respiration and generates free radicals based on NO, is up to 250% higher in the early postnatal rodent brain than in the adult [57]. Free radical scavenging cascades, which render these highly reactive molecules harmless, are present in the neonatal brain but less effective than in the adult brain [55, 58]. Immature oligodendrocytes were far less effective at degrading the free radical H_2O_2 *in vitro*, where scavenger

enzyme catalase was expressed at constant levels throughout development, but glutathione peroxidase was expressed at less than half of adult levels in oligodendrocytes from the neonatal brain [58]. There is also evidence that the scavenging cascades are less organised in the developing brain, with some rodent studies documenting decreased expression of key enzymes following exposure to hypoxia ischaemia [59].

In the first minutes after birth, the low oxygen environment of the foetus abruptly experiences an increase in O_2 partial pressure, which creates a pro-oxidant condition highly susceptible to oxidative stress prior to the development of healthy protective mechanisms [60]. Another potential reason for the vulnerability of the infant brain is the high polyunsaturated fat content, particularly in the white matter, making this region vulnerable to lipid peroxidation [61, 62]. Rodent studies have found that neonatal neurons may contain as little as a quarter of the full complement of mitochondria expressed in adult cells, with those neonatal mitochondria exhibiting altered calcium metabolism and internal matrix density as assessed by electron microscopy [63]. The complex molecular response to free radical generation is still only beginning to be understood in the infant brain.

The final factor known to contribute to neonatal hypoxic ischaemic brain injury is intracerebral inflammation [16, 64]. In humans, intrauterine infection is strongly associated with preterm birth and brain injury [64, 65]. In one long-term study, over 1000 premature infants diagnosed with early- or late-onset sepsis at birth were assessed for neurodevelopmental outcome at the age of five [66]. There was a strong correlation between sepsis at birth and diagnosis of cerebral palsy at age five, however, there was no correlation between sepsis and milder cognitive impairments. Although the infections were successfully treated in these patients, it is clear that there are persistent effects of infection-induced inflammation.

Molecular biology experiments in animal models have directly linked brain inflammation to neuronal cell death. Intracerebral inflammation triggered by injection of bacterial cell wall component lipopolysaccharide (LPS) caused neurodegeneration in young mice via activation of Toll-like receptor 4 [67]. One study investigating the effect of administering a single dose of LPS prior to hypoxia ischaemia in neonatal rats found lesion size increased by more than 100% compared to littermate animals that underwent hypoxia ischaemia alone [68]. This sensitivity to hypoxia in the presence of brain inflammation has been termed the "double-hit hypothesis" [69]. Interestingly, the injury-exacerbating effect of LPS on hypoxic ischaemic brain lesions may be specific to the infant brain. In one investigation, low-dose pre-treatment with intrauterine LPS increased injury severity in neonatal hypoxic ischaemic mouse, whereas the same pre-treatment was protective in adult animals [70, 71].

Despite the potential for inflammation to cause injury in animal models of infant hypoxia ischaemia, not all elements of the brain's inflammatory response are necessarily detrimental. The resident macrophages of the central nervous system, known as microglia, are activated within hours of the hypoxic ischaemic insult [72]. Microglia are known to produce a range of cytokines, excitotoxic neurotransmitter glutamate, and molecules known to induce oxidative stress such as nitric oxide and free radicals [16]. Additionally, chloroquine and minocycline, drugs which inhibit microglia and monocytes, decreased lesion size in a mouse model of neonatal hypoxia ischaemia [73]. However, microglia are complex secretory powerhouses with

multiple active states, and there is growing support for a balanced understanding of these neuronal support cells as capable of causing both damaging and beneficial effects in neonatal hypoxia ischaemia [74, 75]. For example, when microglia were depleted in the brains of neonatal mice, lesion volume increased, along with the concentration of various cytokines and reactive oxygen species in the neonatal brain [76]. This suggests a neuroprotective function for microglia, at least under specific conditions.

2.3. Sexual dimorphism in the response to neonatal hypoxia ischaemia

One finding clearly illustrates how much remains to be understood about the neuropathology of neonatal hypoxia ischaemia. This is the recent discovery of sexual dimorphism in the developmental outcome of HI in human patients [77, 78]. Male babies are at higher risk of cerebral palsy than females [79]. Not only are motor deficits significantly more severe in male infants [77], but structural magnetic resonance imaging (MRI) has demonstrated a qualitatively different pattern of injury in males and females. One study reported that white-matter injury patterns predominated in male babies, whereas females were more likely to demonstrate a grey-matter injury pattern [80]. These relatively recent discoveries led to reanalysis of a clinical trial of prostaglandin inhibitor indomethacin as a preventative treatment in infants at high-risk of intraventricular haemorrhage [77, 81]. When cognitive and motor development were assessed in a mixed-sex group at age 3, there was no difference between treated and untreated groups. However, when boys and girls were analysed separately, the anti-inflammatory drug improved functionality in boys given indomethacin compared to boy who did not receive treatment. New contributing factors for hypoxic ischaemia injury continue to be uncovered, and this surprising revelation reinforces the argument that our current models should remain under revision.

The physiological basis of this sexual dimorphism remains poorly understood [16, 77, 78]. Neuronal culture models have identified sex-specific differences in cell death cascades induced by hypoxia *in vitro*. One of the first studies to suggest a molecular basis for this sexual dimorphism cultured XX (female) and XY (male) neurons separately and triggered neuronal cell death by administering nitric oxide (NO) and glutamate [82]. The mitochondria of male and female neurons released different molecules, and the male neurons were less able to maintain antioxidant expression. This finding has been expanded upon considerably since. The putative treatment 2-iminobiotin appears to have different effects on neurons from male and female rats [83]. In males, there was no significant effect, whereas female rats showed decreased activation of the cytochrome C cascpase-3 pathway, and its downstream cell death markers. Another investigation found that female neonatal rats expressed greater levels of cleaved caspase-3, the activated form of an important cell death promoting molecule, than male brains, although there was no difference between the sexes in nitrotyrosine or autophagy [84].

Sexual dimorphism in neonatal hypoxia ischaemia is receiving increasing attention. This is an expanding area of research, with recent *in vivo* studies uncovering unexpected results. There is now robust evidence that the increased vulnerability seen in male human patients extends to rodents [77, 84]. When equivalent procedures were used to generate hypoxia ischaemia in rats over a range of developmental stages, the only significant difference in lesion outcome

between the sexes was detected at a perinatal age, with no difference in older rat pups or fully-developed adults [84]. Animal models of neonatal HI support a fundamental difference in mitochondrial respiratory function in the developing male and female brain [85, 86], with female mitochondria posited as more resilient. Mitochondrial function may not fully explain the difference between the sexes, as evidence is now emerging that drugs targeting neurotransmitter receptors may only be effective in one sex, males [87]. Sex hormone therapy, such as progesterone treatment, is protective against HI injury in male rats but had no effect in females [78, 88]. Despite suggestions that the adult brain's response to ischaemic stroke may also be sex-dependent [77], the evidence currently suggests that this difference is largely a neonatal phenomenon.

3. Treatments of neonatal hypoxia ischaemia

Despite the high rates of disability in human survivors of neonatal hypoxia ischaemia [4, 16], only one treatment is currently licenced in the UK: hypothermia. This therapy reduces the infant's body or head to approximately 33°C [16, 89]. Hypothermia was first demonstrated to improve survival in cases of cardiac arrest [90], and has since been applied as a neuroprotective treatment in acute neonatal hypoxia ischaemia patients [89, 91]. One meta-analysis of over 1200 infants found that hypothermia reduced death and neurological handicaps at 18 months follow-up across all severities of neonatal hypoxia ischaemia [89].

However, hypothermia alone is not sufficient to prevent all brain injury or neurological symptoms [4, 16, 89]. Since the discovery of the neuroprotective effect of hypothermia, little progress has been made towards additive therapies. Few potential treatments have reached clinical trials. The development of novel treatments to supplement hypothermia is imperative. In this section, current research into novel treatments for neonatal hypoxia ischaemia will be reviewed and approaches for therapy development will be evaluated.

3.1. Review of therapies under development for infant hypoxia ischaemia

Two additional interventions have been deemed safe to trial in neonates. Resuscitation at room temperature [92] and xenon gas administration [93] have been investigated in a clinical environment alongside hypothermia. The limited success reported in these studies is now under speculation. Recent randomised clinical trials have demonstrated that although xenon gas is a safe treatment, there is little or no therapeutic effect of combined hypothermia and xenon gas in moderate and severe cases of neonatal HI at 18 months follow-up [94]. A parallel experiment in rats found that xenon made no difference to lesion size or neuronal cell numbers in cases of severe hypoxia ischaemia [95].

Pharmacological agents have also been investigated in human neonatal patients, resulting in limited success. Barbiturate anticonvulsants had no effect on long-term neurological development when given to hypoxic ischaemic neonates [96]. A more promising result from recent clinical studies suggests that high-dose erythropoietin (EPo) treatment in term neonates reduces disability [97]. However, even proponents of this potential treatment advise caution

in interpreting early results. The therapy does not completely prevent neurological symptoms. There is hope for erythropoietin as a future additive treatment, yet the field should be concerned that this is currently the only pharmacological molecule being pursued in clinical trials for neonatal hypoxia ischaemia.

Many more small molecules are being investigated in animal models of neonatal hypoxia ischaemia, where the translational value of the research, and the safety of the treatment in vulnerable newborns, remain uncertain. For example, free radical scavenger N-acetylcysteine and systemic hypothermia reduced infarct volume after focal hypoxic ischaemic injury in rats [98, 99]. Another free radical scavenger, allopurinol, reduced cerebral oedema and neuropathological damage [99, 100].

One example of the difficulty involved in selecting targets for new therapies is the lack of clinical translation of the extensive work on NMDA receptor-mediated excitotoxicity in the neonatal hypoxic ischaemic brain. Drugs that block NMDA receptors are protective against HI injury in neonatal rodent models [101]. Despite the efficacy of NMDA receptor antagonists in reducing infarct volumes in rats, this work has not been pursued in humans as intact NMDA-mediated classical neuronal plasticity is essential for normal brain development [16, 102, 103]. Effective NMDA antagonists could cause more damage to the circuitry of the neonatal brain than is justified by their anti-excitotoxicity function, undermining the medical philosophy enshrined in the Hippocratic oath: to do no harm.

There are several novel treatments being developed by dedicated scientists, although it is extremely difficult to predict which of these will be deemed safe enough to allow clinical trials in the developing brain. Perhaps the translation from bench to bedside for putative treatments could be improved through a different approach to treatment selection and funding. Some essential factors demanding consideration at the earliest point in treatment development are outlined below.

3.2. Proposed approaches to therapy development for infant hypoxia ischaemia

New approaches are required to identify potential treatments for neonatal hypoxia ischaemia which will be better suited to advance into clinical trials. Three essential properties must be satisfied in a new therapy. These have been suggested in a previous publication I authored [16], and are summarised in **Figure 3**. First, all potential treatments should be safe for vulnerable neonates and not interfere with essential developmental milestones. Second, treatments should be specific, to avoid extreme adverse effects in vulnerable infants. And third, an ideal treatment would target molecules common to the excitotoxicity, oxidative stress, and inflammation pathways. Targeting common mediators would allow a single therapy to be efficacious against multiple mechanisms of brain damage, instead of merely eliciting a reshuffle to favour a different method of cell death [62, 102]. These three qualities can be summarised as safety, specificity, and breadth.

The suitability of any future small molecules for use in human neonates will depend greatly on the severity of any adverse effects on brain development. A wide range of molecules contribute to healthy brain development in the neonatal period [16, 103, 104], a time of widespread

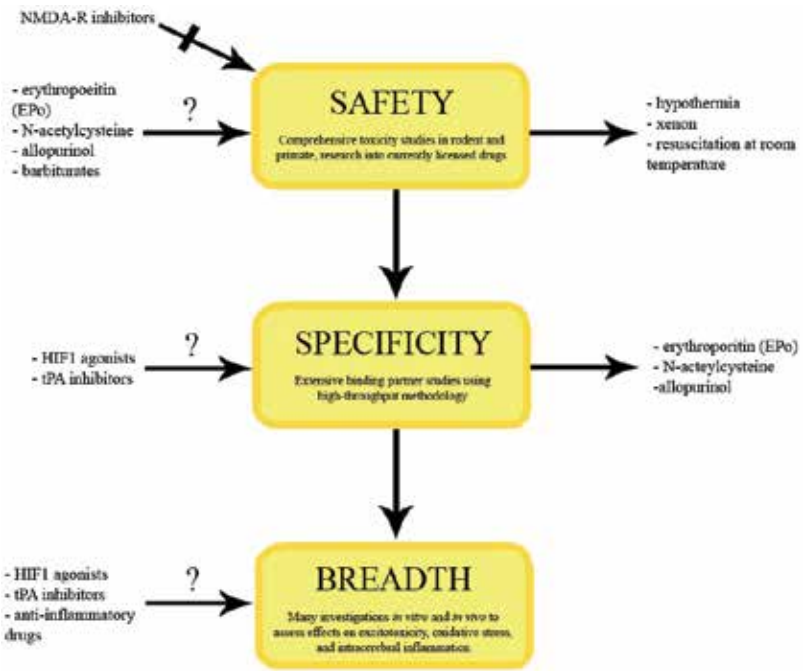

Figure 3. Schematic depiction of proposed filters for putative therapies for infant hypoxia ischaemia. All putative treatments should satisfy safety prior to investigation in human infants. Specificity may also decrease off-target effects, and breadth may increase treatment efficacy. Several current treatments are listed by which criteria these satisfy. HIF1 = hypoxia inducible factor 1, tPA = tissue plasminogen activator, NMDA-R = *N*-methyl-D-aspartate receptor, EPo = erythropoietin.

remodelling and plasticity within the brain. Selecting molecular targets known to be expressed in the neonatal brain could reduce the chances of general toxicity, but does not preclude the possibility that endogenous proteins may have a narrow therapeutic range, with slight increases or decreases interfering with development. Future studies of potential treatments should examine developmental plasticity processes, to ensure safety in the neonatal brain.

Specificity is also a desired characteristic of any potential pharmacological therapy for neonates, as faithfulness of a single molecular target minimises the likelihood of off-target side-effects. The cardiovascular and respiratory systems of neonates are vulnerable in premature birth and following hypoxic ischaemia injury [5, 8], so brain-specific neuroprotective treatments are desirable. Molecular specificity is essential in addition to organ specificity. Extensive characterisation of the binding partners of not only the pharmacological molecule, but also its biological molecular target, is time-consuming but essential work if a therapy is to be estimated as safe enough for trial in human neonates. As high-throughput screening methods are increasingly refined for use throughout the pharmaceutical industry [105, 107], capturing specificity is becoming a realistic research goal.

Breadth of action is essential for the efficacy of a therapy designed for neonatal hypoxia ischaemia, in which a wide range of neuronal death pathways are active simultaneously in

the injured brain. These cascades, which include excitotoxicity, oxidative stress, and inflammation, are not entirely independent of one another. It may be possible to identify a "master regulator". This hypothetical single molecule would dampen multiple brain damage pathways, inhibiting a key activator (or activators) of each respective process, and perhaps trigger other neuroprotective cascades. But how probable is it that a "master regulator" will be discovered? Is it possible that one has already been documented and simply remains to be exploited?

The concept of a "master regulator" for any complex disease appears enticing, but is its promise only linguistic trickery? Identification of candidate proteins will not be a simple process, likely requiring many experiments spanning multiple methods. One possible starting point is microarray data collected following neonatal hypoxia ischaemia in rodents [106, 107]. Microarrays are highly sensitive to the time of tissue collection post-injury, and do not detect changes in functional protein content mediated by translational modification or secretion. For example, no published microarrays detected changes in tissue plasminogen activator (tPA) or hypoxia inducible factor 1 (HIF1) transcription, although these proteins play a substantial role in injury pathogenesis [108, 109]. Generating a neonatal hypoxia ischaemia 'secretome' [110] could help identify the earliest changes in protein activity directly following neonatal brain injury.

Some proteins are already known to span multiple cell death cascades [16, 62, 102]. These are clearly the most accessible candidates for "master regulator" properties. NMDA receptors, major mediators of neuronal death by excitotoxicity, can be directly or indirectly activated by free radicals, combining two lethal molecular cascades often treated as separate in the literature [16, 25, 47]. Inflammatory pathways also mediate excitotoxicity and oxidative stress. In rodents, pre-treatment with IL-1ß, IL-6, IL-9, or TNF-α enhances brain damage caused by NMDA agonists [16, 64, 73]. It is these overlaps between cascades at which a "master regulator" could act. One candidate is HIF1 [109]. This transcription factor is known to regulate a minimum of 60 genes, including the putative therapeutic molecule erythropoietin, several growth factors, and mitochondrial proteins. Another possible "master regulator", tissue plasminogen activator, is currently one of the best-documented possibilities [16, 108]. tPA has established roles crossing boundaries between excitotoxicity, oxidative stress, and brain inflammation. The relative dearth of candidates proposed here perhaps reflects our incomplete knowledge of the molecular mechanisms underpinning neonatal hypoxic ischaemic brain damage. There is no clear single "master regulator" protein documented in the literature of this complex neurodevelopmental disorder. However, this does not restrain future experimenters from seizing on those few currently supported candidates for further development.

4. Conclusions

As the most common single cause of infant mortality and morbidity globally, neonatal and perinatal hypoxia ischaemia deserve wide recognition and funding within the research community. These conditions require careful examination in animal models closely matched to the

level of brain development at birth in humans, as there is a plethora of differences between the adult and infant brain which create infant-specific challenges for understanding neuropathology and developing new therapies. Infant-specific obstacles also exist, as any treatment should not interfere with normal brain development, or the vulnerable infant cardiovascular system. Despite these constraints, it should be possible to develop novel therapeutics closely guided by the criteria of safety, specificity, and breadth. Current research has suggested some promising candidate neuroprotective treatments, such as erythropoietin and tissue plasminogen activator, and these could yet inform future approaches to therapeutic development.

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Conflict of interest

The author declares that the review article was written in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 6

Hypoxic Preconditioning: The Multiplicity of Central Neurotransmitter Mechanisms and Method of Predicting Its Efficiency

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Abstract

In rats, a single moderate hypobaric hypoxia (HBH) increased the resistance to severe hypoxia (SHBH). The HBH efficiency and neurotransmitter mechanisms of its preconditioning action were investigated by biochemical and pharmacological methods. It will be substantiated in the chapter: (1) HBH preconditioning has its own mechanisms that do not depend on an innate resistance to SHBH and prior hypoxic experience of rats; (2) the same preconditioning effect can be achieved by diverse neuronal pathways and synaptic plasticity means; (3) cholinergic and, presumably, serotoninergic, GABAergic and/or glutamatergic systems of the caudal brainstem, cortex and some other brain structures are involved in HBH realisation; (4) the rate of sensorimotor gating estimated in the model of acoustic startle pre-pulse inhibition (PPI) predicts the efficiency of hypoxic preconditioning and (5) the cholinergic system, including α7 nicotinic receptors, is involved in the mechanisms of HBH-PPI-dependent preconditioning effects.

Keywords: hypoxic preconditioning, resistance to severe hypoxia, apnoea, adaptation to hypoxia, mechanisms of hypoxic preconditioning, brainstem, cortex, central neurotransmitter systems, pre-pulse inhibition in acoustic sensorimotor startle reaction, cholinergic system, nicotinic receptors

1. Introduction

1.1. Protective action of moderate hypoxia

It is well known that pathological factors (many poisons, pathogenic viruses) in low doses can initiate protective mechanisms and increase resistance to the corresponding pathologies. Similarly, it is possible to increase resistance to severe hypoxia or ischaemia by exposing the organism or organ to adaptation in conditions of moderate hypoxia [1].

Short-term hypoxic adaptation is a single 1- or 3-h moderate hypoxic continuous or intermittent exposure in which hypoxia alternates with normoxia or hyperoxia [2–5]. Such hypoxic adaptation is characterized by the mobilization of available cellular reserves and its effect may be manifested within 24 hours [2, 5–7]. In experimental practice, Murry et al. were the first to describe the protective effect of the short ischaemic exposure against ischaemic stroke, suggested a preconditioning term for this phenomenon [8]. Later, the same authors identified the reperfusion (re-oxygenation) as the second most important adaptive component [9].

Hypoxic factor is the main in ischaemic preconditioning. Therapeutic potential of the hypoxic and ischaemic preconditioning are closely related. Protection from hypoxic damage is also very relevant because the hypoxic component is involved in pathogenesis of many diseases. *In vivo,* protective action of the hypoxic or ischaemic preconditioning was identified in the diseases of brain [3, 10–15], heart [8, 16–18], liver [19–21] and kidneys [22, 23]. Direct hypoxic preconditioning or drugs that mimic the hypoxic protective response could reveal promising therapeutic targets. Today, understanding of the hypoxic or ischaemic preconditioning mechanisms is a high priority [15].

The brain is central in the problem of hypoxic adaptation not only as the most sensitive organ to hypoxia but also as the coordinator of the functions of all body organs and systems. In the nervous tissue, the functional specificity and individual sensitivity to hypoxia of separate neuronal populations and the corresponding brain structures are of fundamental importance. However, in search of key targets of hypoxic preconditioning, the neuronal mechanisms remain the least studied. It is the aspect of hypoxic preconditioning that is in the centre of our attention. On the other hand, the neuronal autonomous mechanisms of respiration and blood circulation in the norm and under severe hypoxic conditions are intensively studied. These data serve as a serious help in the analysis of neuronal mechanisms of hypoxic preconditioning.

Another important problem that we have tried to solve is the methodology for studying the mechanisms of hypoxic preconditioning.

2. Systemic reaction of autonomic systems to hypoxia

The primary and immediate response to hypoxia is always recorded in the autonomic respiratory and cardiovascular systems. Central representation, the neurons of both systems, is

located in the medulla oblongata and pons Varolii (caudal brainstem) and spinal cord. Autonomic systems are functionally closely interrelated by the "respiratory centre", groups of respiratory neurons, which support respiratory rhythm [24–26].

It has been shown that the "hypoxic" response involves activation of the autonomic sympathoexcitatory reticulospinal pathways, primarily from the peripheral or central chemoreceptors and the stimulation of respiration, heart activity and blood circulation aimed at restoring the blood level of O_2 and CO_2 exchange and pH [27–32].

The sequence and development of hypoxia-induced events are carefully researched [30, 31]. In general, this systemic response is a result of the wide cooperation of different functional groups of neurons of the central autonomic respiratory and cardiovascular systems: sensory, primary and secondary chemo and baroreceptors of the nucleus tractus solitary (NTS) and ventrolateral medulla (VLM); relay neurons; reticulospinal neurons (area C1 of the rostral VLM); efferent neurons of the preganglious parasympathetic nuclei of cranial nerves and spinal motoneurons.

Another important adaptive express response to hypoxia is non-sympathetic activation of the cerebral blood flow that is based on the redistribution of blood flow towards the brain [31, 33–36]. Two centres have been detected: the "parasympathetic cerebrovasodilator centre" or in another way "dorsal facial area" (DFA) and the "medullary cerebral vasodilator area" (MCVA). Both centres are located in the rostrolateral part of medulla oblongata (and DFA also partially covers the pons Varolii), and their innervation, including hypoxia, initiates elevation of the cerebral blood flow by parasympathetic [33, 37, 38] or relay cerebripetal pathways [34, 39]. Also, the connection of MCVA with the sympathetic vasomotor mechanisms is shown: in the sympathoexcitatory zone C1, the presence of the O_2-sensitive neurons of which activation excites the cerebripetal pathway; dependence of the cerebrovascular MCVA efficiency of safety of the reticulospinal pathway and existence of collaterals from the sympathoexcitatory neurons in the cerebripetal direction [34, 39].

All of these systemic reactions are revealed in cats [40] and respiratory reactions from those in rats [36, 41, 42] with high, intermediate and low resistance to very severe hypoxia (3% O_2). Differences between these groups were mainly in expression and duration of the responses before apnoea. Under moderate hypoxia, all the compensatory reactions, including cerebral blood flow elevation, are maintained during the whole session of hypoxic training in rats [36, 43, 44], and all of them are the physiological basis of hypoxic preconditioning [44–46].

It should also be noted that the structures of the forebrain are the most unstable to ischaemic/hypoxic injuries [47], and the most interesting have been shown to be the cortex and hippocampus, as these are the higher brain structures responsible for cognitive functions. Both the cortex and hippocampus interact with the cardiorespiratory systems, participating in the regulation of voluntary respiration and hypothetically adaptive reactions of the respiratory and cardiovascular systems [25, 26, 48, 49].

But we assumed that under moderate hypoxia conditions (10–12% O_2 for rat), the key role in the preconditioning belongs to the autonomic systems.

3. Autonomous regulation of respiration: overview of neuronal populations responsible for generation of apnoea

In our experiments on rats, the preconditioning effect of moderate hypoxia was evaluated under conditions of severe hypoxia by the time (T) until agonal inspiration (apnoea).

Thus, among the key neurotransmitters of caudal brainstem are of interest, involved in the generation of apnoea. The most studied in this respect are inhibitory neuromediators or neuromodulators of the opioid, serotoninergic, GABAergic, glycinergic and adenosinergic systems. And we will certainly touch upon the cholinergic system as an object of our research, which, according to our data, occupies not the last place in the preconditioning mechanisms.

In the overview, special attention was paid to the synaptic transmission; since in our studies, we evaluated the response of the synaptic pool of the caudal brainstem and some other brain structures.

3.1. Opioid system

Opioids cause apnoea selectively through μ-receptors. The action of the majority of opioid analgesics is associated with the stimulation of μ-receptor type. However, μ-receptor agonists cause side effects, among them respiratory depression. It was shown in cats and rats that μ-receptor agonists morphine and/or fentamine depressed respiration, initiated central apnoea or apneusis breathing [50–54]. μ-Receptors are widely distributed in the brain, but their mechanisms of action and targets are still poorly understood. According to some data, Bötzinger Complex (BötC) and especially pre-Bötzinger Complex (preBötC) are responsible for opioid-initiated destruction of respiration [51, 52]. According to other data, such opioid-sensitive sites are numerous in the brainstem [53]. An endogenous ligand of μ-receptors is β-endorphin. However, endorphinergic fibres or terminals in the caudal brainstem have not been described to date. Hormonal mode of distribution of endorphins through the blood is well known. In consideration of the chemical stability of the ligands of the opiate receptors, it is assumed that they penetrate into the respiratory centre through the cerebrospinal fluid [25].

3.2. Serotoninergic system

I.v. administration of serotonin (5-hydroxytryptamine, 5-HT) or 5-HT3 receptor agonist phenylbiguanide provoked "von Bezold-Jarisch" or C-fibres reflex (bradycardia, drop in blood pressure, apnoea) passing, the authors believe, through 5-HT3 receptors in the nucleus tractus solitary NTS [55]. Really, bradycardia from the triad of Bezold-Jarisch reflex was potentiated by the i.c. administration of phenylbiguanide and was dose-dependently weakened by the i.c. administration of receptor antagonist granisetron [55]. Granisetron microinjected into NTS significantly attenuated both bradycardia and hypotension [55].

Under normoxic conditions (cat), both i.v. administration and microinjection into preBötC of 5-HT1A receptor agonist 8-OH-DPAT produced apnoea and arrested respiratory neuronal activity [32]. Previously, it has been found that stimulation of the nucleus raphe obscurus provoked

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that apnoea and 5-HT1A receptors, which are abundantly expressed in the respiratory neurons of ventral respiratory group, were involved in this mechanism [56].

Under hypoxic conditions (cat), using microdialysis and registration of the phrenic nerve and respiratory neurons of the ventral respiratory group activity, it was shown that elevation of 5-HT levels in the extracellular space of the ventral respiratory group clearly coincided with the beginning of hypoxic depression and apnoea (5–10% O_2) [32]. The authors revealed that such high correlation with hypoxic depression was selective for 5-HT in this respiratory region because it was absent with the levels of other investigated mediators or modulators (GABA, glutamate and adenosine). In the same study, microinjection of 8-OH-DPAT into preBötC on the apneustic patterns background, initiated by prolonged moderate hypoxia (cat, 15% O_2), resulted in normal respiratory parameters. In contrast to the 8-OH-DPAT effects, blockade of 5-HT1A receptors during hypoxia by antagonist NAN-190 resulted in dramatic enhancement of apneustic inspiratory activity patterns.

The molecular signalling pathway was later traced through these receptors on the glycinergic respiratory neurons of preBötC and, possibly, neighbouring regions of the ventral respiratory group [51]. The activation of 5-HTR1A potentiated glycinergic currents in all postsynaptic neurons receiving glycinergic inputs through glycine alpha3 receptors (GlyRalpha3) that not only excitatory (glutamatergic) but also inhibitory (glycinergic) neurons and enhanced their inhibition. It is proved that the 5-HTR1A-GlyRalpha3 signalling pathway can restore the respiratory circuitry and disturbed by hypoxia or some other factors (opioid intoxication) [26, 51].

In the rat, the long deep apnoea occurs when stimulation of special neurons within cluster of the serotoninergic neurons in the medullary raphe nuclei (the raphe pallidus, magnus and obscurus) [57]. The point of these neurons is called the "midline apneic site" (MAS) and suggested their 5-HTergic nature. By the morphoimmunological methods, the same researchers proved the relationship of MAS with many higher brain regions and some areas of the medulla oblongata, including the ventral respiratory group [58].

It was also revealed the action of 5-HT on the cerebral circulation. Intravenously (i.v.) injections of 5-HT (cat, rat) may have different regional actions on the brain blood vessels of various categories, but its decisive value was the development of cerebrovascular constriction, a decrease in the rate of cerebral blood flow and a drop in blood pressure [59], which was enhanced by ischaemic exposure [60]. All cerebrovascular reactions appeared similar or were more pronounced at i.c.v. injections (cat) or the application of 5-HT on the brain (rat) indicating the central nature of its actions [59]. It has been proven the central action of 5-HT in the DFA on the cerebral circulation. Injected into DFA, 5-HT or alaproclate, a 5-HT reuptake inhibitor, synaptically inhibited the glutamatergic activation of the parasympathetic preganglionic cholinergic motoneurons and thus reduced the rate of blood flow in the common carotid arteries [38]. Also, by i.v. administration of 5-HT, a significant drop in the rate of the cerebral circulation was revealed in the cortical parietal area as well as in the frequency and depth of breathing [61]. The drop in the rate of the cerebral circulation coincided with the accumulation of CO_2 in the blood. It should be noted that some serotoninergic neurons in the **dorsal** raphe nucleus (in the midbrain), connected with MAS [58], have CO_2/pH chemoreception and deep hypercapnia (9% CO_2) produced an increase in their firing rate [62].

These data suggest that the reduction of the serotoninergic influences on, presumably, any site of the autonomous cardiorespiratory regulation, except the ventral respiratory group facilitates breathing and/or conduces to a delay of generation of apnoea.

3.3. GABAergic system

GABA side by side with glutamate is the most widespread mediator in the central nervous system and is involved virtually in all nervous processes. Central GABAergic effects on the cardiorespiratory functions are not unidirectional. GABA is a principal inhibitory neurotransmitter of the sympathoexcitatory and baroreflex sympathoinhibitory glutamatergic pathways from peripheral chemo- and baroreceptors through the second-order sensitive neurons of NTS [63, 64]. The GABAergic neurons of caudal VLM are interneurons in the baroreceptor reflex arc and directly inhibit the sympathoexcitatory C1 zone neurons of rostral VLM [64].

In the NTS (rat), the agonist of $GABA_B$ receptor baclofen attenuated the cardiorespiratory reflexes of C-fibres, provoked by phenylbiguanide, bradycardia and decrease in frequency of breathing with no effect on hypotension and apnoea when microinjected into any point of dorsomedial NTS in dose of 60 pmol [65]. When it was injected into the inhibitory zone of the dorsal respiratory group of NTS only (0.5–0.6 mm caudal to the obex [66]), baclofen removed C-fibre-provoked apnoea and the antagonist of $GABA_B$ receptor CGP 35348 (2.8 nmol) newly restored it. Similar effect was obtained by the systemic administration of high doses of the $GABA_B$ receptor agonists hydroxybutyrate and phenibut (6.9 mmol/kg, i.v., and 2.3 mmol/kg, I.P., respectively, rat) [67, 68]. Both agonists attenuated the decrease in the frequency of breathing and abolished or shortened the duration of apnoea of C-fibres reflex, which are provoked by 5-HT. Hydroxybutyrate (i.v.) and phenibut (I.P.) caused a complete loss of sensitivity of the respiratory system to vagotomy are supposed by central blocking transmission of afferent impulses from the baroreceptors of lungs and airways to the second-order barosensitive neurons in NTS [54, 67].

Under the normoxic conditions, baclofen also prolonged the inspiration and increased the heart rates injected into NTS in the same dose of 60 pmol in rats [65]. In the high doses in cats, in hundreds nmoles for baclofen and micromoles for GABA and sodium hydroxybutyrate, the decrease in respiratory frequency and apneusis breathing arose in the majority of intact animals when GABA or the $GABA_B$ receptor agonist was microinjected into the dorsal respiratory group region (ventrolateral NTS) [69]. The same respiratory reactions were obtained after the i. v. administration of hydroxybutyrate in cats and rats [25, 69] and after the I.P. administration of phenibut in rats [54].

Under hypoxic conditions, similar to our HBH (10% O_2, 45–50 minutes, rat), participation of GABA in respiratory mechanisms of hypoxia was investigated [43]. Moderate hypoxia initiated a primary pronounced ventilatory increase (minute respiratory volume) followed by a gradually decline to a second-stable level above pre-hypoxic level and a sustained increase in respiratory frequency during the hypoxic exposure. Under these conditions, GABA had a depressant effect on the ventilation. By *in vivo* microdialysis, the elevation of GABA concentration in NTS coincided with the ventilatory decline. By microinjections into sensitive non-apnoeic

region of NTS of agonists and antagonists of $GABA_A$ and $GABA_B$ receptors, both the agonists muscimol (150 pmol) and baclofen (400 pmol) were injected 10 minutes before the hypoxic exposure significantly attenuated the early increase of ventilation, and on the contrary, the antagonists of $GABA_A$ receptor bicuculline and of $GABA_B$ receptors saclofen (400 pmol) and CGP-35348 (2.5 nmol) in the 40-minute hypoxic exposure abolished the late ventilatory decline and reduced the GABA elevation [43]. The authors have shown that for GABA activation in NTS, peripheral chemoreceptor stimulation is essential because it is the normoxia or under denervation of the carotid body, GABA level in the NTS did not change and the effects of GABA antagonists did not appear.

Another study also showed that endogenous GABA in the NTS inhibits the carotid chemoreflex (rat) [70]. Microinjection of the selective GABA uptake inhibitor nipecotic acid into the commissural sub-nucleus of NTS attenuated the increases in respiration and elevation in arterial blood pressure elicited by carotid chemoreceptor stimulation. These effects were completely antagonised by the $GABA_A$ antagonist bicuculline (20 pmol) but not by the $GABA_B$ antagonist saclofen (400 pmol), injected into the same site.

I.v. administration of $GABA_A$ receptor antagonist picrotoxin enhanced ventilation through an increase in respiratory frequency and minute tidal volume (rat) [42]. A severe hypoxia (3% O_2, rat) in the first few minutes, like in the moderate hypoxia, initiated the same dynamics in the increase of minute tidal volume and respiratory frequency [40–42], and picrotoxin (i.v.) significantly potentiated the activation of both respiratory functions (cat and rat) [42]. Also, the authors observed that these effects of picrotoxin were most pronounced in the high resistance rats compared with low and intermediate resistance rats. Moreover, it was found that under these hypoxic conditions, picrotoxin greatly extended the time before apnoea in all resistance rat groups [42].

Note that these data indicate that systemic administration of the GABA receptor agonists and antagonists reflects the central action of these drugs within NTS.

In the ventral respiratory group of VLM, GABAergic neurons have other effects and can have a protective action on respiration under hypoxic conditions. By *in vivo* microdialysis, the level of GABA (and glutamate) in the respiratory region of VLM increased transiently during early periods of severe hypoxia (5–10% O_2, cat), coinciding with augmented phrenic nerve activity and fell below the control levels during central apnoea [32]. The authors suggest that GABA may be important for regulation of level of enhanced respiratory network activity at the onset of hypoxia. In addition, in BötC and preBötC of VLM, the pacemaker nature of some GABAergic respiratory types of neurons is assumed [26, 71]. Microinjections of GABA into BötC facilitated respiration (increased the tidal volume) and into preBötC significantly inhibited respiration (reduced the tidal volume) [72]. The same lack of uniformity was observed under the action on GABA receptors of B and A subtypes at BötC/preBötC.

Participation of $GABA_B$ receptors in preBötC and BötC respirator functions was revealed using agonist baclofen. Under the normoxic conditions, the influences through $GABA_B$ were directed towards the respiratory depression when baclofen was administered into both BötC and preBötC [73] or BötC only and, on the contrary, towards the weak respiratory stimulation

when it was microinjected into preBötC [72]. These two studies were performed on rabbits (first) and rats (second). However, we believe that the main difference was in doses. It seems that baclofen is more selective at 15–25 times smaller dose (2 pmol) [72] and therefore caused the opposite effects and influenced the breathing of $GABA_B$ receptors in BötC, which was more expressed than in preBötC. At the same time, it should be noted that $GABA_B$ receptor stimulation by baclofen at BötC suppressed breathing in both doses.

The blockade of $GABA_A$ receptors within these respiratory complexes by the antagonist bicuculline or gabazine disturbed respiration until apnoea [71, 73, 74]. Blocking $GABA_A$ receptors by picrotoxin also disturbed respiratory rhythm and provoked apneusis breathing when it was injected into the fourth ventricle [42]. At the same time, microinjections of bicuculline into preBötC recovered the respiration against the background of apnoea caused by the blockade of $GABA_A$ receptors in BötC [73].

Thus, the natural reduction of GABAergic synapses would help the delay of generation of apnoea by cutting back the impacts of GABA in the sympathoexcitatory C1 zone neurons of rostral VLM, through both $GABA_A$ and $GABA_B$ receptors in the sympathoexcitatory conductive sensory pathways and dorsal respiratory group within NTS and through at least $GABA_B$ receptors in BötC and possibly $GABA_A$ receptors in preBötC within VLM.

3.4. Glycinergic system

Glycinergic neurons of the medulla oblongata were identified in the ventral respiratory group. In BötC and especially preBötC, glycinergic neurons are more than half of all respiratory neurons [74]. It was shown *in vivo* that many of them generate the respiratory activity, that is, pacemakers [71]. Both in preBötC and in BötC, the blockade of glycine receptor by the antagonist strychnine resulted in the suppression of respiratory activity until apnoea [26, 51, 73] or disturbance of the respiratory cycle, the modification of activity of the post-inspiratory neurons and, as a consequence, the transfer of the normal three-phase cycle into the pathological biphasic [26, 75]. These data indicate that the inhibitory effects via glycine receptors are required for normal respiratory function.

By other data [76], glycine is also required for normal respiratory function in the dorsal respiratory group. In the intermediate sub-nucleus of NTS, the secondary barosensitive neurons receive glutamatergic afferent inputs from the pulmonary rapidly adapting stretch receptors and have inhibitory influences on respiration. The authors found that these secondary neurons receive the phasically acting inputs from glycinergic neurons, which inhibit their activity in the inspiratory phase.

In another networks of the medulla oblongata, participation of glycine was identified as a sympathoexcitatory or sympathoinhibitory modulator. Sympathoinhibitory influence on glutamate pathway from caudal to rostral VLM was mediated by glycine in a manner independent of $GABA_A$ and $GABA_B$ receptors [77]. Microinjections of glycine into NTS decreased arterial pressure and heart rate [78], inhibited the pressor but not bradycardic responses produced by L-glutamate microinjection in the same site [79] and, at the same time, inhibited the depressor and bradycardic responses to L-glutamate [78].

Thus, on the certain sites of the glutamatergic pathways of VLM and NTS, attenuation of the glycinergic influences may contribute to the delay of apnoea generation.

3.5. Adenosinergic system

Adenosine is present in the CNS at pharmacologically active concentrations [80–82], and it is now recognised as a neuromodulator. The extracellular concentration of adenosine in the brain increases dramatically during hypoxia or ischaemia [32, 80, 83]. Adenosine has a depressor effect on the neuronal activity through A1 and A3 receptor types and antagonistic effect through A2 receptors [83, 84]. Autoradiographic and immunohistochemical studies illustrate the presence of A1 and A2 (mainly A2a) binding sites/receptors in NTS, VLM and other brainstem regions that are important in cardiorespiratory control [81, 85].

The presence of the enzyme 5′-nucleotidase, which converts AMP to adenosine in the fractions of synaptosomes (cortex and hippocampus, rat) [86] and adenosine in the fraction of the synaptic vesicles (rat brain) [87], indicates the existence of adenosinergic pre-synapses in the brain. In addition, a high-affinity transport system for adenosine and adenosine deaminase, an enzyme of adenosine cleavage in the synaptic cleft, was revealed in the crude synaptosomal fraction of NTS (rat brain) [88, 89].

In the caudal brainstem, adenosine and as a rule, the following selective agonists and antagonists of A1 and A2a adenosine receptors used to study the role of adenosine in the regulation of cardiorespiratory functions: the agonist N6-cyclopentyladenosine (CPA) and antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) of A1 receptors; the agonist 2-[p-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoade (CGS 21680) and antagonists 9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo(1,5-c)quinazoline-5-imine (CGS 15943A) and 4-(2-[7-amino-2-[2-furyl][3,2,4]triazolol[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM 241385) of A2a receptors. Also, the non-selective adenosine receptor antagonist 8-phenyltheophylline (8-PT) and A1/A2 receptor antagonist 8-(p-sulphophenyl)theophylline (8-SPT) with higher affinity for A1 receptors [85] were applied.

In the chemo- and baroreceptive sites of the NTS, opposite effects on cardiovascular parameters were revealed under the action of these two types of receptors in the intact rat brain. Microinjection into NTS of A1 receptor agonist CPA stimulated and of A2a receptor agonist CGS21680 decreased the blood pressure, and both agonists provoked bradycardia [82, 85, 90–92]. It was found that the opposite effects of A1 and A2a receptor agonists on blood pressure are due to the action on the glutamatergic afferent nerve fibres from arterial and pulmonary baroreceptors. Accordingly, the stimulation of A1 receptors inhibited glutamate release, and the stimulation of A2a receptors activated it [91, 92]. The effects of A2a receptors considerably dominated over A1 receptors in NTS [82, 90, 91], and the decrease of blood pressure in all of these studies was removed by the antagonists of A2a receptor CGS15943A or ZM241385 and of A1 receptors agonist DPCPX, while bradycardia was selectively antagonised by CGS15943A but not by DPCPX. The same agonist of A1 receptors CPA had the hypotensive action followed by microinjections into NTS at doses far exceeding those used in the cited studies and was antagonised by i.v. infusions of A1 receptor antagonist DPCPX [93].

From the above data, under certain conditions of the disturbance of cardiorespiratory functions, the NTS A2a receptors in concert with A1 receptors are involved in the activation of the sympathetic functions. At the same time, the decrease of synaptic influences on the A2a receptors and possibly on the A1 receptors of NTS could contribute to the delay of apnoea generation.

Stimulation of 5HT3 receptors by agonist phenylbiguanide (i.v. injection) initiated "cardiopulmonary chemoreflex" (hypotension, bradycardia and apnoea) [82, 94], aka reflex of C-fibres or von Bezold-Jarisch in other sources. This reflex was attenuated/blockaded or conversely potentiated by microinjections into NTS of the A2a receptor agonist CGS21680 or antagonist ZM241385, respectively [82]. The same reflex, when developed under a severe haemorrhage model, was inhibited by endogenous adenosine; this inhibition was removed by microinjections into NTS of the antagonist 8-SPT [82]. The authors suggested that A2a receptors were responsible for the activation of inhibitory influence on the "cardiopulmonary chemoreflex" pathway and later proved a participation of $GABA_A$ and much weaker $GABA_B$ receptors in this mechanism within NTS [94].

The role of A1 receptors in the cardiorespiratory functions was revealed in the VLM, where this receptor type had the highest density [85]. In the rostral VLM (rat), microinjections of adenosine into the pressor zone C1 augmented the sympathoexcitatory reflex of increase in blood pressure evoked by electrical stimulation of the "hypothalamic defence area" and, on the contrary, the microinjections of 8-SPT into C1 or both peripheral and central injections of the selective A1 receptor antagonist DPCPX this reflex reduced [80]. Vice versa, microinjections of adenosine into the ventral respiratory group (cat), acting on pre- and postsynaptic A1 receptors, led to the depression of spontaneous and stimulus-evoked synaptic activity of the respiratory neurons and to the fall of mean respiratory drive potentials. The depressive effects of adenosine were abolished after the i.v. administration of the antagonist DPCPX [95].

Under severe hypoxia (5–10% O_2, cat), i.v. DPCPX administration retained the same direction of action on respiration. This A1 antagonist showed marked protective properties, namely preventing the early hypoxic depression of stimulus-evoked activity of the respiratory neurons in the ventral respiratory group, significantly delayed the onset of apnoea and reduced the recovery time [95]. In the same hypoxic conditions in the ventral respiratory group using microdialysis, an intensified increase of a level of the endogenous extracellular adenosine was identified, and this reaction was developed in the background of the apnoea after it began [32]. The authors noted that the hypoxic release of adenosine was occurred surprisingly late than hypoxic depression of respiration and cannot be responsible for the onset of the hypoxic depression of respiratory neurons, and that it is noteworthy that increases in adenosine levels outlasted hypoxic periods, which were not associated with pronounced depression of phrenic nerve activity.

However, endogenous adenosine may be involved in the mechanism of a secondary suppression of the hypoxic activation of the cerebral blood flow. As mentioned above, the fall in the rate of cerebral blood flow under the von Bezold-Jarisch reflex conditions also occurred after the apnoea beginning in the background of its end and coincided with the accumulation of CO_2 in the blood [61]. Under severe hypoxia (8% O_2, rat), i.v. administration of the non-selective antagonist 8-PT, penetrating through the blood-brain barrier unlike not penetrating 8-SPT, potentiated hypoxic alkalosis and hypocapnoea that arise from the initial hyperventilatory

response, extended the increase in tidal volume and heart rate, reduced the decrease in arterial pressure, stopped the progressive increase of the carotid vascular conductance and, at the same time, showed a pronounced tendency for cerebral blood flow to be better maintained during hypoxia [31, 96]. The authors proved that all effects of 8-PT were central and were a consequence of the influence of the antagonist on the secondary fall in ventilation.

Taken together, under hypoxia, endogenous adenosine of the ventral respiratory group can contribute to the fall in the ventilation and, as a consequence, initiate hypercapnia. Accordingly, attenuation of these effects of adenosine can contribute to maintaining the cerebral blood flow.

3.6. Glutamatergic system

It should also be added that apnoea can be also caused by microinjection of the main excitatory neurotransmitter glutamate into certain sites of the medulla. Therefore, the neurons of the raphe nuclei were stimulated by glutamate for apnoea in MAS [57]. Also, apnoea occurred when glutamate was microinjected into the inhibitory site of the respiratory neurons of the dorsal group of NTS (behind the obex) [66]. Apparently, it is the action of the described above glutamate afferents triggering baroreflex [64, 78]. In NTS, these afferents switched on bulbar interneurons, mainly glutamatergic, which, as shown, transfer drive signals to the parasympathetic motor nucleus ambiguous and stimulate bronchoconstrictor reflex [97] and also through the caudal VLM, having an inhibitory action on the sympathoexcitatory reflex [64].

3.7. Cholinergic system

Starting from Loeschcke studies [27, 98], the central effects of ACh and its analogues on the respiration and blood circulation are intensively investigated. As for many other neurotransmitters, the cholinergic participation is detected in the majority of functional sites of cardiorespiratory networks as well as the ambiguity of the cholinergic effects depending on drug dose, application site and reception [39, 99].

Caudal brainstem structures include several cholinergic sources: (1) projections from the reticular formation of the midbrain tegmentum [100–103]; (2) afferents of the nodose ganglion sensory neurons from the lung mechanoreceptors to the NTS [97, 104, 105] and (3) the neurons of pons Varolii and medulla oblongata, including reticular areas, NTS and efferent parasympathetic preganglionic neurons of the motor cranial nerves nuclei [99, 102, 103, 105–107].

Concerning the connections and functional effects of the cholinergic system of the caudal brainstem in response to hypoxia, we recently published an overview similar to the above [108, 109].

4. Preconditioning effects on resistance to severe hypoxia and synaptic pool of caudal brainstem, cortex and hippocampus

The sub-chapter briefly describes our experimental approaches on the study of neuronal mechanisms of hypoxic preconditioning. During the planning of experiments, we were guided

by the data that the short-term adaptation, especially after continuous hypobaric hypoxia, had the pronounced and rapid preconditioning effect in the first minute of re-oxygenation [2, 6, 7].

The experiments were carried out according to two protocols.

4.1. General experimental conditions and procedures

Animals. The male outbred albino rats aged 2–2.5 months (200–250 g) at the beginning of the studies. All animal care and experimental procedures were conducted in accordance with the official regulations of the European Communities Council Directive on the use of laboratory animals of November 24, 1986 (86/609/EEC).

Hypoxic models. Hypoxic preconditioning, the continuous hypobaric hypoxia (HBH): an altitude of 5000 m (11% O_2), 60 minutes. Test for resistance to hypoxia, severe hypobaric hypoxia (SHBH): the critical altitude of 11,500 m (4.5% O_2). In the latter case, resistance to hypoxia was recorded with respect to time (T) until agonal inspiration (apnoea) in combination with a loss of voluntary control of body tone. Apnoea was a defining attribute.

Re-oxygenation after HBH. Four minutes.

Brain structures for biochemical investigations. The caudal brainstem, cortex and hippocampus.

Preparative methods for biochemical investigations. From each brain structure, the sub-fractions of synaptic membrane and synaptoplasma were isolated from the fractions of "light" and "heavy" synaptosomes by routine preparative methods using discontinuous sucrose gradients.

The sub-synaptic level of fractionation made it possible to study the largest functionally different pre-synaptic compartments, and it was very informative. Moreover, synaptic membrane sub-fractions were considerably cleaned from glial, mitochondrial and free (not docked) synaptic vesicle contaminations.

Analytical methods. In the sub-synaptic fractions, the choline acetyltransferase (ChAT, functional marker of cholinergic neurons) activity by radiometric method [110] and protein content by spectrophotometric method [111] were assayed.

For details of the experimental procedures, see [112–114].

4.2. Experimental protocol number 1

It is important to bear in mind that animals (and humans) are very different in their resistance to severe hypoxia, and this implied different mechanisms. Because of this, since the publication of Purshottam and Ghosh [115], animals were divided into resistance to severe hypoxia, using pre-testing them under the same hypoxic conditions [116–118]. Later, the pre-testing under severe hypoxia was applied to rats for the investigation of mechanisms of hypoxic preconditioning [2, 7] and in our experiments [108, 109, 113, 119].

So, in our experiments (**Figure 1**) in each sample, most of the rats were pre-tested under SHBH and divided into groups of low, high and intermediate resistance to hypoxia with T1 < 3.5 minutes, T1 > 7 minutes and between them, respectively. For the following 4–5 weeks, all pre-testing rats

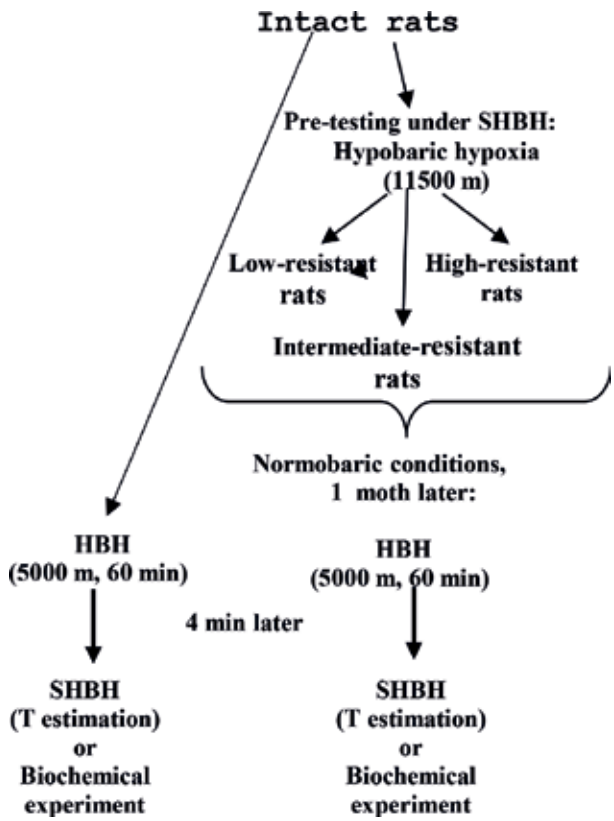

Figure 1. Scheme of experimental protocol number 1.

were kept under standard vivarium conditions after which the rats in each pre-tested group and the rats in not pre-tested group (intact group) were sub-divided into experimental (HBH) and control groups, and the rats of all experimental groups were subjected to a single HBH session. Four minutes after the end of HBH, the rats from each experimental group, which were subjected to SHBH and T2 (or T1 in intact group), were estimated or taken in the biochemical experiment. The control groups underwent all the procedures after HBH simultaneously with the corresponding experimental groups.

4.3. Experimental protocol number 2

In these experiments, the pre-testing under SHBH was excluded. Instead, all rats were pre-tested in the model of acoustic sensorimotor startle reaction, and the magnitude of pre-pulse inhibition (PPI) was estimated [120]. Two to four days after pre-testing, the experimental rats were subjected to a single HBH session, and 4 minutes after the end of HBH were subjected to SHBH (as in Scheme number 1). The control rats underwent all the same procedures except HBH.

Using this experimental scheme, pharmacological experiments were also carried out (**Figure 2**). The cholinergic nicotinic mechanisms of HBH preconditioning were investigated using the selective agonists of nicotinic receptors (nAChRs) $\alpha 4\beta 2$ type metanicotine RJR 2304 (RJR) and $\alpha 7$ type

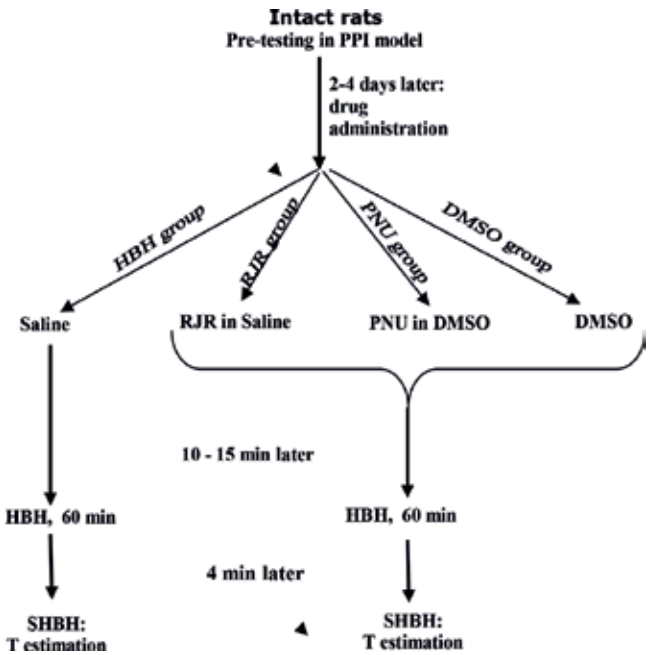

Figure 2. Scheme of experimental protocol number 2.

PNU-282,987 (PNU, Tocris Bioscience, Bristol, UK for both agonists) and a bipolar aprotic solvent for PNU dimethyl sulfoxide (DMSO, LLC "Tula Pharmaceutical Factory", Tula, RF).

Rats in the RJR group received a single I.P. injection of RJR (26 nmol/kg, n = 8) in the physiological saline. Rats in the PNU group received a single I.P. injection of PNU (26 nmol/kg, n = 23, or 260 nmol/kg, n = 12) in 3% DMSO. Rats in the DMSO group received a single I.P. injection of 3% DMSO (n = 16). Rats in the HBH group received a single I.P. injection of the saline (n = 23). Both drugs, DMSO and saline, were injected 10–15 minutes before HBH session.

5. Experiments on the protocol number 1

5.1. The effect of HBH on the resistance of rats to SHBH

HBH markedly increased the mean values of the resistance of rats to SHBH in all the investigated groups (**Figure 3**). After HBH session, all rat groups showed a similar range of values for resistance to SHBH. In fact, the T values of these groups formed the same variational series (**Figure 4**) [113].

In biochemical experiments, the reaction on HBH of synaptic pool of caudal brainstem and cortex (no reaction was shown in the hippocampus) in the low- and high-resistant rats and intact rats showed that the same preconditioning hypoxic effect can be achieved by various neuronal pathways and plastic synaptic tools. For a detailed analysis, see [108, 109, 112]. Briefly, it was revealed in the following.

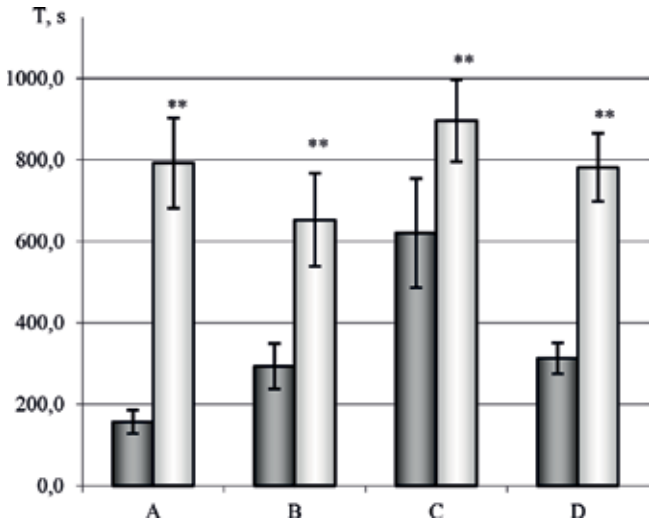

Figure 3. Preconditioning effects of HBH on the resistance to SHBH of the low-resistant (A), intermediate-resistant (B), high-resistant (C) and intact (D) rats. *T* values, a time before apnoea, are expressed as means ± SE. For each group of bars: grey bars, T2 values in the control pre-tested rat groups (A, B, C; $n = 12, 9, 14$, respectively) and T1 value in the control intact rat group (D; $n = 18$); light bars, T2 (A, B, C) or T1 (D) values in the corresponding HBH groups ($n = 11, 8, 10$ and 19 in A, B, C and D, respectively). $^{**}p < 0.025$ compared to the respective control, Fisher's exact test.

5.1.1. In the low-resistant rats

In the low-resistant rats, in the caudal brainstem (**Figure 5a**), the inhibition of water-soluble ChAT activity in the pre-synapses of heavy synaptosomal fraction corresponded to the functional characterisation of subtypes of the lung barosensitive C-fibres conducting afferentation to NTS through the nodose ganglion [121, 122]. It is known that apnoea is often preceded by the classic reflex of C-fibres (frequent shallow breathing, bradycardia and hypotension) [65, 122, 123]. We substantiated that the cholinergic C-fibres could act on nAChRs affecting theirs through secondary cholinergic barosensitive neurons and the weakening of their influences led to the suppression of parasympathetic reflexes occurring in NTS and thereby to the augmentation of resistance to SHBH.

5.1.2. In the high-resistant rats

In the high-resistant rats, in the caudal brainstem (**Figure 5b**), HBH provoked inhibition of the water-soluble ChAT activity in the pre-synapses of light synaptosomal fraction. We substantiated that the nerve endings of this rat group may be outside NTS. Additionally, a correlation was found between the HBH-induced changes in activity of water-soluble ChAT in caudal brainstem and membrane-bound ChAT in pre-synapses of cortical projection neurons (**Figure 6b**, the cortical light synaptosomal fraction [124, 125]) ($r = +0.911$, $p < 0.02$, $n = 6$, Pearson's correlative test). This allowed us to assume that the inhibition of the water-soluble ChAT activity under HBH conditions in this group of rats occurred in pre-synapses of the projection neurons from laterodorsal (LDT) and/or pedunculopontine (PPT) tegmental cholinergic nuclei of the middle brain. LDT and more intensively PPT send plurality of the fibres to both the pontine and the

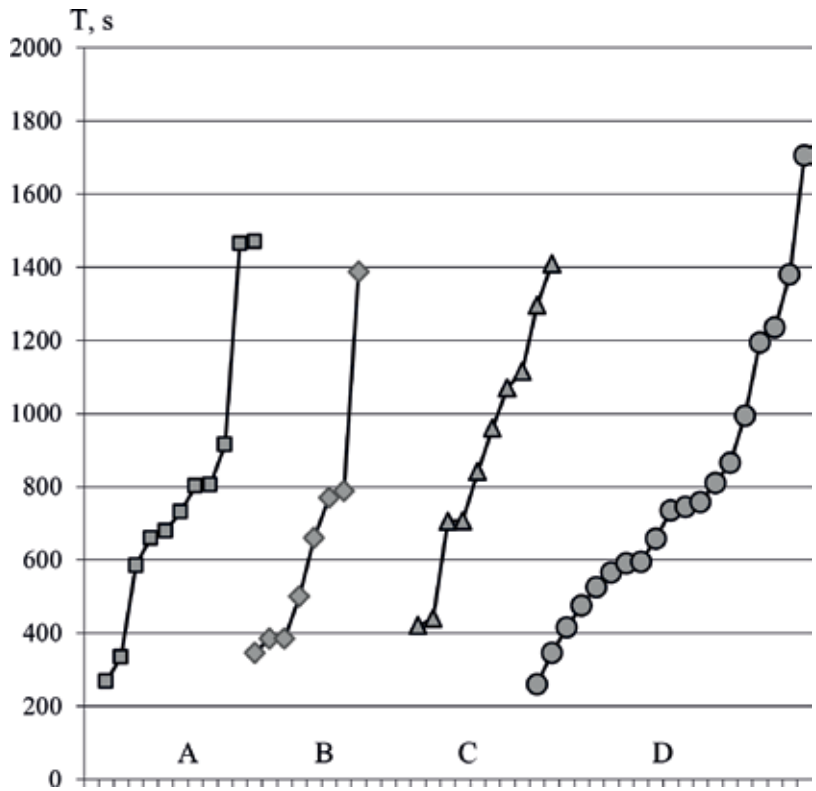

Figure 4. Individual *T* values of the rat resistance to SHBH after HBH in the low-resistant (A), intermediate-resistant (B), high-resistant (C) and intact (D) rat groups. Resistance to SHBH demonstrates that *T* values of all HBH groups formed the same variational series. n = 11, 8, 10 and 19 in A, B, C and D as in the corresponding HBH groups in **Figure 3**.

medulla oblongata nuclei [101, 102, 126] and also to the cortical cholinergic projection neurons of the basal forebrain nuclei [126].

In the high-resistant rats, in the caudal brainstem (**Figure 5b**), a simultaneous decrease in the content of synaptic c- and m-proteins (r = +0.871, $p < 0.05$, n = 6) in the heavy fraction in non-cholinergic pre-synapses (correlation between cChAT activity and c-protein content is absent) suggests the possibility of reduction of the number of synapses in non-cholinergic neurons.

According to the literary data in sub-chapter 3, in the first place, the serotoninergic system is reported to be involved in the provocation of apnoea in all of the studied key areas of the autonomic regulation of the cardiorespiratory functions. At the same time, the reduction of the influences of any analysed neurotransmitter systems at corresponding sites may be involved in the mechanisms of the hypoxic preconditioning. It was therefore necessary to analyse the presence of pre-synapses of these neurotransmitter systems in the heavy fraction of synaptosomes, and their representation in the fraction must be enough to identify their reduction by means of such non-specific parameters as protein content.

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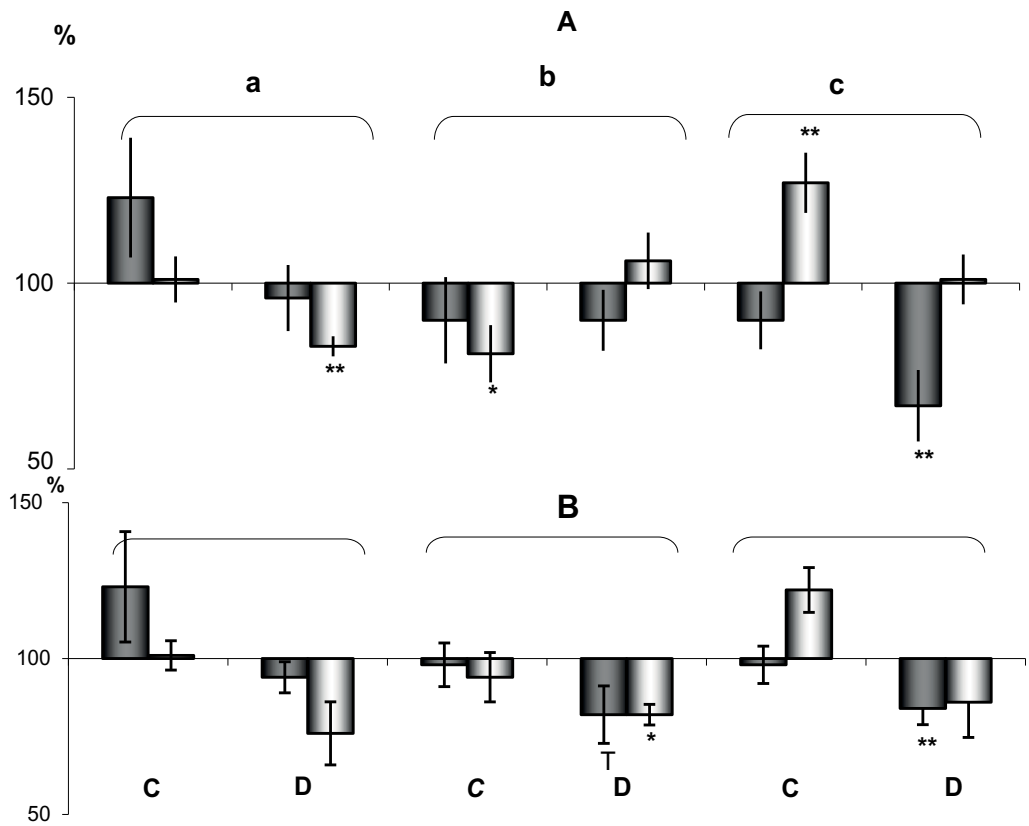

Figure 5. The effect of a single HBH session on the ChAT activity (A) and protein content (B) in the sub-synaptic fractions of the caudal brainstem in the pre-tested low-resistant (a) and high-resistant (b) rat groups and in the intact rat group (c). The values of ChAT activity and protein content are expressed as means ± SE. (C) Sub-fractions of light synaptosomes; (D) sub-fractions of heavy synaptosomes. In each pair of bars: left (dark) bar, sub-fraction of synaptic membranes; right (light) bar, sub-fraction of synaptoplasm. The data are shown as percentages as compared to the control, which was taken as 100%. $^{*}p < 0.05$; $^{**}p < 0.025$, Fisher's exact test..

5.1.2.1. Mediator composition of the heavy fraction of synaptosomes

Analysis of the synaptosomes with the above mediator specificity showed that, as expected, synaptosomes with any mediatory specificity have a wide range of density and sizes. This is shown for glycine, glutamate, serotonin and GABAergic pre-synapses under fractionation in continuous sucrose density gradient [127–129]. Also, in discontinuous sucrose density gradient, serotonin, glutamate and GABAergic pre-synapses were revealed in the light and heavy fractions of synaptosomes [130–135].

In sucrose-percoll gradient, the adenosinergic pre-synapses were isolated in the percoll interlayer 10–16% [86]. This percoll fraction apparently corresponds to the heavy fraction of synaptosomes in the sucrose gradient. This is indicated by a similarity in size of synaptosomes ([136, 137] compared with our data [124]) and a significant percentage of the free mitochondria

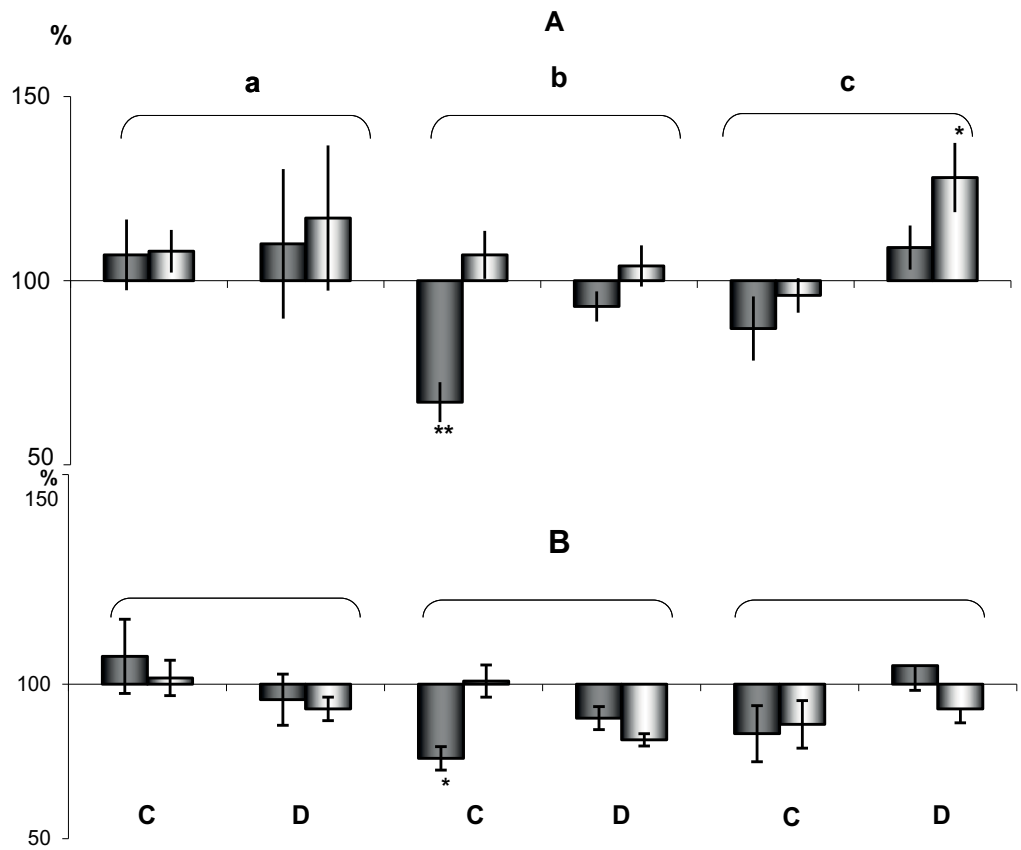

Figure 6. The effect of a single HBH session on the ChAT activity (A) and protein content (B) in the sub-synaptic fractions of the cortex in the pre-tested low-resistant (a) and high-resistant (b) rat groups and in the intact rat group (c). Designations are as shown in **Figure 5**.

in the fractions. It is known that the density of a substantial part of the free mitochondria coincides with the density of synaptosomes with an expressed vector of the concentration of the mitochondrial organelles towards denser layers of sucrose [128]. As a result, the mitochondria are present in large numbers in the heavy fraction of synaptosomes (20–40% and more) [124, 134], while they were revealed only as the single irregular inclusions in the light fraction [124, 134, 138]. The similar pattern is observed in the percoll gradient [137].

Therefore, the pre-synapses of all listed mediatory systems are present in the heavy fraction of synaptosomes with the exception of the opioid system, which synaptic transmission is absent in the caudal brainstem.

In accordance with the above literature, any of them dominate the heavy fraction or constitute at least half of the quantity/activity of the corresponding mediatory marker in the light fraction of synaptosomes. However, it is turned out in our case that the ratios between mediators within the light and heavy fractions are not as important when compared with their ratios

within the heavy fraction. It is well known that glutamate and GABA are the prevailing neurotransmitters in any brain formation. This is manifested in synaptosomal fractions. For example, according to a comparative study of Johnson and Roberts [134], in the heavy fraction of the whole mouse brain, the content of glutamate-GABA-glycine-5-HT in the percentage distribution was 52-36-4-9%.

The relationships in the fraction between the protein content of the pre-synapses with different mediators would be similar. Therefore, the percentage of the sought-for mediator in the heavy fraction should be more than 18%, that is, above the fall in the protein content in our research. This requirement is consistent only with glutamate and GABA. According to the strictest calculation, they will dominate and be more than 33–23% in the heavy synaptosomal fraction when you consider that the entire brain glycine level is concentrated in the caudal brain stem [129] and that the heavy fraction also includes pre-synapses with some other "small "mediators (adenosinergic, cholinergic, etc.).

However, if GABA is the main inhibitory neurotransmitter of the brain, glutamate is the major excitatory neurotransmitter, including their physiological effects. Therefore, in apnoea, mechanisms may be involved only in a small part of a total pool of glutamate neurotransmission. Nevertheless, it is reasonable to assume that the pre-synapses of the powerful glutamatergic afferents in NTS are concentrated in the heavy fraction of synaptosomes by similarity with the pre-synapses of the cholinergic C-fibres, and thus, their concentration may be sufficiently representative in the heavy fraction of the caudal brainstem.

Taken together, it seems that GABAergic or glutamatergic neurons are the principal candidates in the hypoxic preconditioning mechanism of the reduction of the pre-synapses in the corresponding apnoeic sites of the caudal brainstem of the high-resistant rats. Perhaps HBH initiates signalling to pre-synapse reduction in the multiple mediatory neuronal populations (and in this case, the greatest interest represents the 5-HTergic system) but with the obligatory participation of GABAergic or glutamatergic neurons.

5.1.3. In the intact rats

In the intact rats, in the caudal brainstem (**Figure 5c**), HBH provoked an interconnected increase in the cChAT activity and c-protein content in the light synaptosomal fraction (r = +0.928, $p < 0.02$, n = 6) and a decrease in the mChAT activity and m-protein content (r = +0.933, $p < 0.02$, n = 6) in the heavy fraction. Changes in the activity of mChAT and content of m-protein in the heavy fraction were inversely proportional to the changes in cChAT activity and c-protein content in the light fraction (n = 6: mChAT-cChAT, $r = -0.962$, $p < 0.02$; m-protein–c-protein, $r = -0.921$, $p < 0.05$). Note that significant interfraction correlations between the light and heavy synaptosomal fractions were not found after HBH in the pre-testing rat groups.

We believe that in the intact rats, HBH initiated the transformation of cholinergic pre-synapses from the heavy fraction of synaptosomes, which altered their density characteristics, and during gradient fractionation, the transformed presynaptic population appeared in the light fraction. Moreover, cChAT activated in the transformed pre-synapses [112].

Activation of acetylcholine synthesis and non-quantum leakage in response to HBH points to the direct involvement of the relevant neurons in the preconditioning mechanisms in the intact caudal brainstem. Several respiratory-related sites exist in the VLM in which acetylcholine stimulated breathing and maintained an inspiration through mChR and/or nAChRs. Also, innervation of DFA by acetylcholine through nAChRs initiated the elevation of cerebral blood flow [108, 109].

In the intact rats, cChAT was activated in the cortical interneurons (**Figure 6c**, the heavy fraction of synaptosomes [124, 125]). There was no correlation between cChAT activity in the caudal brainstem and cortex in this rat group because of the absence of a direct link between the brain stem neurons and the cortical cholinergic interneurons.

Acetylcholine synthesis activation under HBH in the cortical interneurons could be related to their function of redistribution of the blood flow towards the brain. With respect to cerebral vessels, direct contacts with small cortical vessels and vasodilator effects of both the cholinergic projective neurons and interneurons were detected [139–141]. Thereby in intact brain, the cortical cholinergic interneurons might be involved in the local mechanisms to maintain the cerebral blood flow.

Thus, the intact rats had a synaptic response to HBH, the opposite of that of pre-tested rats: the activation of cardiorespiratory functions dominated in the intact rats, while the inhibition of pathways initiating apnoea appeared in the pre-tested rats. Apparently, the single pre-testing under SHBH altered synaptic and neuronal preconditioning mechanisms. The variety of neuronal pathways to achieve the same physiological effect demonstrates a great adaptive potential of brain. It seems, such adaptive possibilities are mortgaged by the composite, netted organisation of the respiratory centre. But it is not known whether all these mechanisms will go in the intact rats if theirs to activate, for example, pharmacologically.

In the total, HBH preconditioning eliminates the differences in resistance to SHBH between the intact, high- and low-resistant groups of rats with different innate resistance to severe hypoxia and prior hypoxic experiences. The same preconditioning effects of HBH in the intact rats and pre-tested under SHBH can be explained only by the fact that HBH preconditioning is realised by its own mechanisms, which do not depend on innate resistance to SHBH and prior hypoxic experiences.

At the same time, the resistance to SHBH initiated by HBH showed high rat-to-rat variability. So, the problem appeared to be the absence of methods for prediction of efficiency of hypoxic preconditioning.

Recently, such test was detected. It was a pre-pulse inhibition (PPI) estimated in the model of the acoustic startle reaction.

6. Experiments on the protocol number 2

It was found a correspondence between the values of PPI and T initiated by HBH, and the HBH efficiency was reliably and negatively correlated with PPI (**Figure 7**). The PPI in acoustic sensorimotor startle reaction is a well-known model that was developed in the second half of

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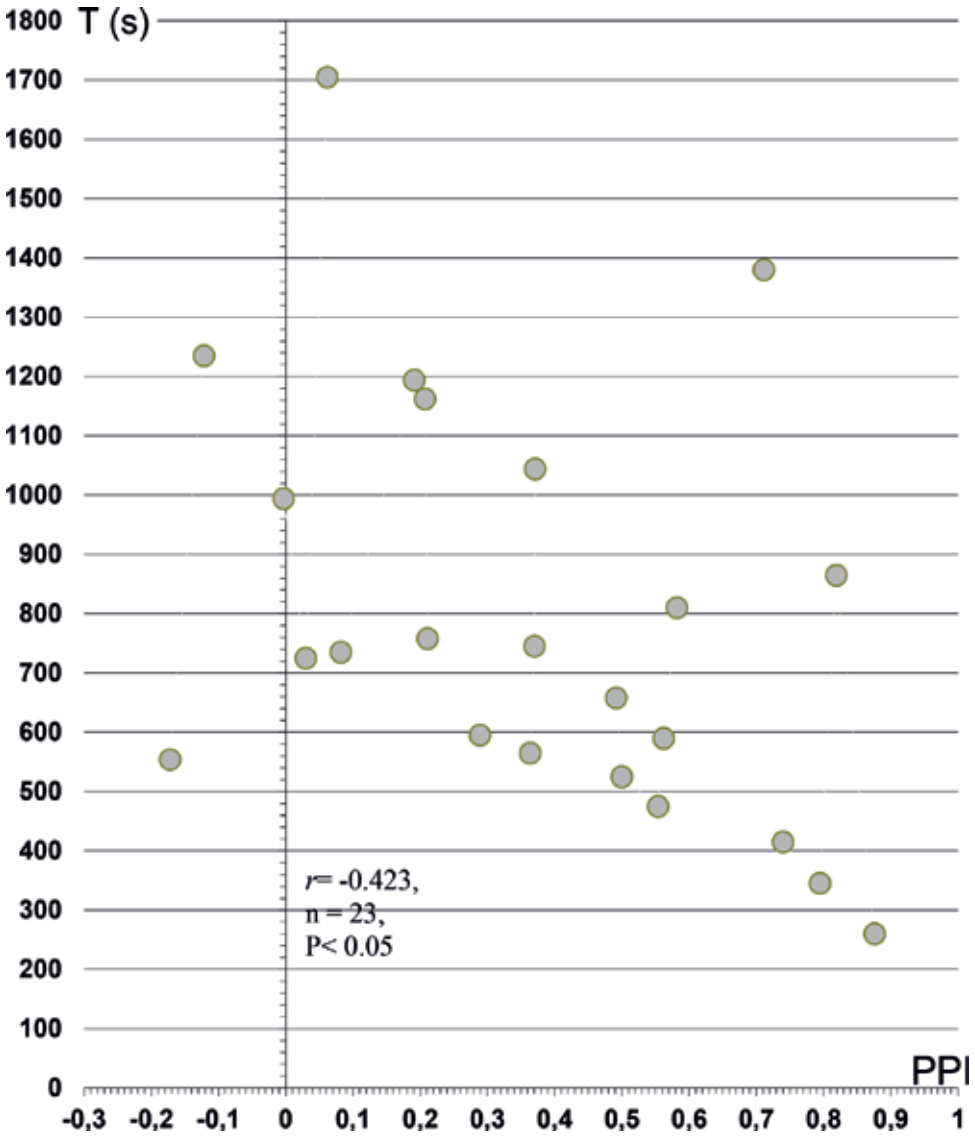

Figure 7. The graph of dependence of the HBH preconditioning efficiency (T) on the rate of PPI. *T*, a time before apnoea. Grey marks, individual values of the rat resistance to SHBH after HBH. The significant negative correlation takes place between T and PPI values, Pearson's *r*-criterion test.

twentieth century for neurobiology, especially psychiatry [142], and these were the first direct experimental data to report the relationship between PPI and hypoxic preconditioning pathways (RF patent 2571603).

In our recent publication [114] using literary data, we substantiated that acetylcholine, via nAChRs and especially via α7 nAChRs, is involved in hypoxic and ischaemic preconditioning and that an interconnection exists between α7 nAChRs, hypoxic preconditioning and PPI. Thereby in the pharmacological experiments, we investigated the effects of selective agonists

of $\alpha 4\beta 4$ and $\alpha 7$ nAChRs RJR and PNU, respectively, and PNU solvent DMSO on the HBH preconditioning. PPI measures were compared with the HBH-initiated preconditioning (resistance to SHBH) and with the effects of drugs on the HBH preconditioning efficiency (**Figure 2**).

RJR had no effect on the adaptive action of HBH. All the values of resistance in this group of rats ideally fitted into the variation series of *T* values of the HBH group (**Figure 8**).

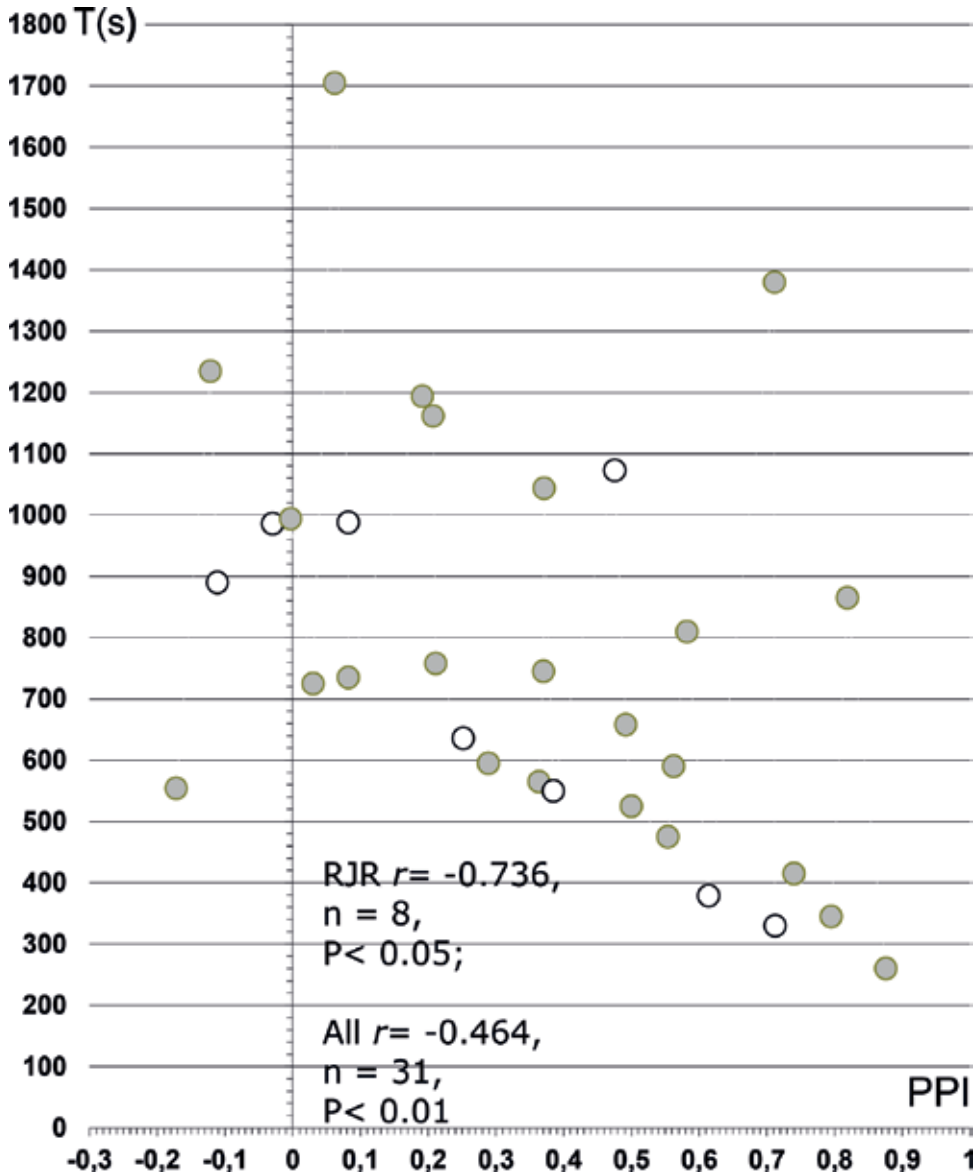

Figure 8. The influence on HBH preconditioning of the selective agonist $\alpha 4\beta 2$ nAChRs metanicotine RJR 2304 (RJR). Grey marks, a time before apnoea after HBH as shown in **Figure 7**; light marks, a time before apnoea after RJR + HBH, Pearson's *r*-criterion test.

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Unlike RJR, PNU inversed the effects of HBH (**Figure 9B**), and it was especially clearly observed in the DMSO group (**Figure 9C**). Moreover, when the graphs of HBH and DMSO groups were combined, the interval of PPI = 0.36–0.40 (36–40%) was found (**Figure 10**). Above these values of PPI, DMSO potentiated the effects of HBH, and lower these values of PPI, DMSO, on the contrary, inhibited them. On the same PPI interval, the directionality of the action of PNU on DMSO effects was divided (**Figure 11**).

Analysis of the literature data revealed the following: (1) PNU in the low doses used (about 2 and 20 nM in the brain) had a desensitising effect on $\alpha 7$ nAChRs, that is, acted as an antagonist [143, 144]; (2) DMSO has the various biological activities, but in the low concentrations used (hundredths or thousandths of a per cent in the brain), it had only anticholinesterase action, that is, activating effect on the cholinergic system [145], and it was found that the anticholinesterase

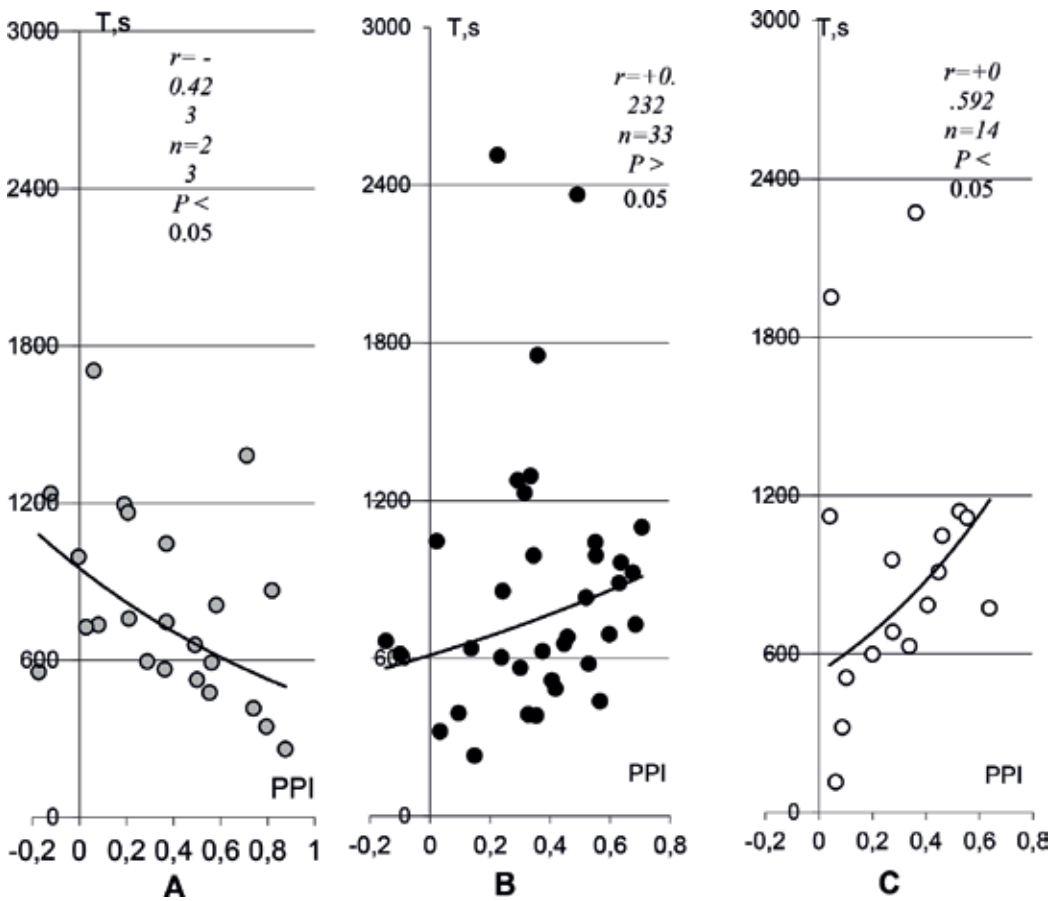

Figure 9. The influence on HBH preconditioning of the selective agonist $\alpha 7$ nAChRs PNU-282,987 (PNU) and its solvent dimethyl sulfoxide (DMSO). Grey marks (A), *T* values in the HBH rat group as shown in **Figure 7**; black marks (B), *T* values in the PNU rat group; light marks (C), *T* values in the DMSO rat group. The significant negative correlation between PPI and T values after HBH inversed into the positive correlation under influence of PNU and significantly under DMSO influence, Pearson's *r*-criterion test.

(neuroprotective) action is realised through the modulation of expression of nAChR genes that were shown for $\alpha 7$ and $\alpha 4$ subunits of nAChRs [146–148]. For details, see [114].

These data indicate the involvement of $\alpha 7$ nAChRs in the mechanisms of HBH preconditioning and explain the antagonism of PNU and DMSO actions. But the literature data do not explain the oppositely directed effects of DMSO and PNU on HBH preconditioning at the PPI boundary = 36–40%. Nevertheless, the existence of the interface does not seem random. Recently, it was found that the predisposed and resistant rats to convulsions in the hippocampal partial kindling model were differed in PPI: the resistant to convulsion rats had PPI of 36–58%, and the unstable to convulsion rats had in all experiments PPI larger, which was selectively susceptible to pronounced variability [149].

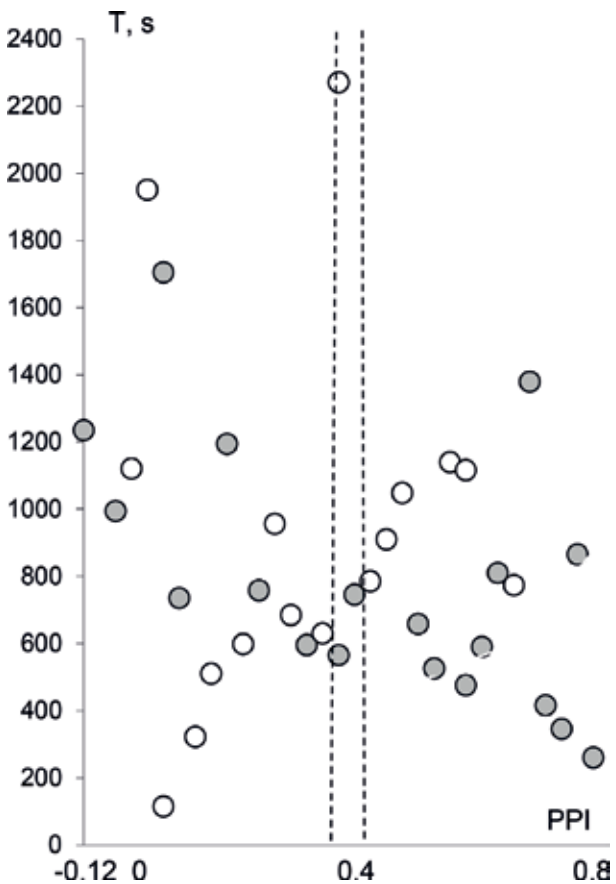

Figure 10. Combined graphs of the dependence of the HBH preconditioning efficiency (*T*) on the rate of PPI in the HBH and DMSO groups. Grey marks, *T* values in the HBH rat group as shown in **Figure 7**; light marks, *T* values in the DMSO rat group. Vertical dotted lines indicate the values of PPI 0.36 and 0.40. The figures under the *x*-axis are given for orientation and denote the location of the corresponding values of PPI on the axis. The relationship between *T* values in the compared groups differs on the opposite sides of PPI = 0.36–0.40.

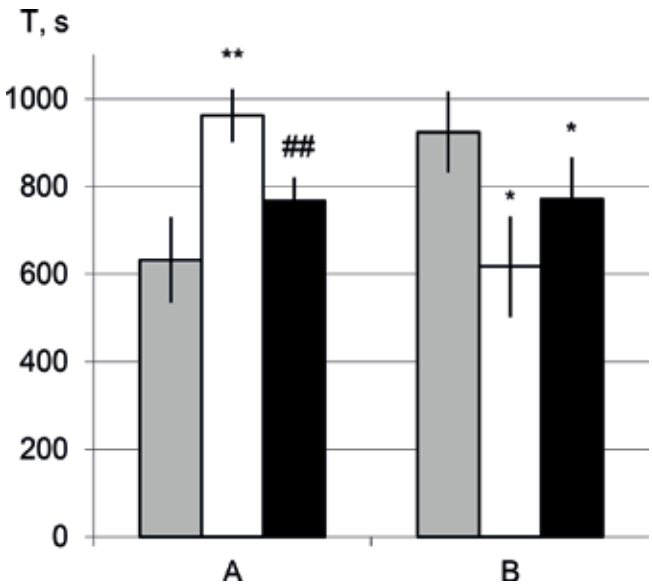

Figure 11. Influence of DMSO and PNU on the HBH preconditioning efficiency in subgroups with PPI > 0.4 (A) and PPI < 0.4 (B). Values are expressed as means ± SE. Grey bars, HBH rat group; light bars, DMSO group; black bars, PNU group. $^{*}p < 0.05$ and $^{**}p < 0.025$ compared with *T* values in the relevant HBH groups, $^{\#\#}p < 0.025$ compared with *T* values in the relevant DMSO group, Fisher's exact test.

Also, key brain structures involved in the innate mechanisms of hypoxic preconditioning are unknown. We suppose the obligatory participation of caudal brainstem. In studies related to PPI, the key structure is the hippocampus [149–152]. In our study, in the intact rat group with not arranged related to PPI, no reaction was shown in the hippocampus. We hope to clarify this problem somewhat in the planned neurochemical studies of synaptic pool in the intact rats pre-tested with PPI.

7. Conclusion

1. A search study of the effects and neuronal mechanisms of hypoxic preconditioning were carried out, and the certain results in this direction were obtained using the HBH model.
2. It has been revealed that the model of acoustic start-reaction can be used to predict the efficiency of hypoxic preconditioning and the study of their innate mechanisms because the magnitude of criterion for this model of PPI has the reverse dependence related to the HBH-initiated resistance to SHBH in the intact rats.
3. The pre-testing of intact rats at the PPI revealed the presence of oppositely directed cholinergic mechanisms of hypoxic preconditioning, separated at the border of PPI = 36–40%, and the α7 nAChRs participation in both the mechanisms.

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