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Review

Metabolite and protein shifts in mature erythrocyte under hypoxia

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SUMMARY

As the only cell type responsible for oxygen delivery, erythrocytes play a crucial role in supplying oxygen to hypoxic tissues, ensuring their normal functions. Hypoxia commonly occurs under physiological or pathological conditions, and understanding how erythrocytes adapt to hypoxia is fundamental for exploring the mechanisms of hypoxic diseases. Additionally, investigating acute and chronic mountain sickness caused by plateaus, which are naturally hypoxic environments, will aid in the study of hypoxic diseases. In recent years, increasingly developed proteomics and metabolomics technologies have become powerful tools for studying mature enucleated erythrocytes, which has significantly contributed to clarifying how hypoxia affects erythrocytes. The aim of this article is to summarize the composition of the cytoskeleton and cytoplasmic proteins of hypoxia-altered erythrocytes and explore the impact of hypoxia on their essential functions. Furthermore, we discuss the role of microRNAs in the adaptation of erythrocytes to hypoxia, providing new perspectives on hypoxia-related diseases.

INTRODUCTION

Oxygen is crucial for cell metabolism and energy production. Insufficient oxygen supply leads to a state of hypoxia, which is prevalent in various diseases, including anemia, cardiovascular disease, chronic obstructive pulmonary disease, neural degenerative disease, chronic kidney disease,[1–4] and even cancer.[5] When individuals travel to high-altitude areas rapidly, they will experience hypoxia and hypoxic adaptation processes, which may lead to acute mountain sickness (AMS) if they are maladapted. The clinical symptoms include headache, gastrointestinal symptoms, fatigue, dizziness, and sleep difficulties. Untreated AMS can progress to high-altitude cerebral edema and high-altitude pulmonary edema (HAPE).[6] Approximately 140 million people, including about 79 million in Asia, live in high-altitude areas above 2,500 m.[7] These plateau residents suffer from chronic mountain sickness (CMS), a clinical syndrome that occurs in highland areas and is characterized by excessive erythrocytosis (EE) and severe hypoxemia.[8] The incidence of CMS increases with altitude and age.[9–11] Research on hypoxia can not only promote the understanding of the development of various hypoxic diseases and therapeutic targets but also has potential applications in areas such as tourism, aerospace, and military deployment in the future.

Erythrocytes, the most abundant cells in the human body, play a vital role in hypoxic adaptation; they can absorb oxygen in the lungs and transport it to low-oxygen tissues, thus maintaining tissue functions and survival. Metabolomics and proteomics are ideal for studying mature erythrocytes due to their advancements and ability to bypass the research limitations of the lack of protein *de novo* synthesis in erythrocytes. In addition, microRNAs (miRNAs) contribute to the fine-tuning of adaptive responses in mature erythrocytes. We summarize the alterations in mature erythrocytes' protein composition and metabolites in high-altitude or hypoxic environments, as well as how hypoxia affects the function of erythrocytes. Moreover, we explore the effects of miRNAs on erythrocyte adaptation to hypoxia, offering new insights into hypoxia-related diseases.

COMPOSITION AND STRUCTURE OF THE HUMAN ERYTHROCYTE MEMBRANE

The human erythrocyte membrane comprises approximately 49% protein, 43% lipids, and 8% carbohydrates. Lipids form a phospholipid bilayer connected to the cytoskeleton of the erythrocytes. The insertion of cholesterol can regulate the fluidity of the human erythrocyte membrane.[12] In addition to lipids, proteins also play essential roles in maintaining the function and structure of erythrocytes. Erythrocyte membrane proteins are closely related to membrane deformability, the transport and release of oxygen molecules, the regulation of ion balance, and signal recognition and transduction.[13,14] The cytoskeletal structure of the erythrocyte membrane is composed mainly of a protein network consisting of spectrin, which can be assembled with actin oligomers and Protein 4.1 and connect to the membrane matrix through the band

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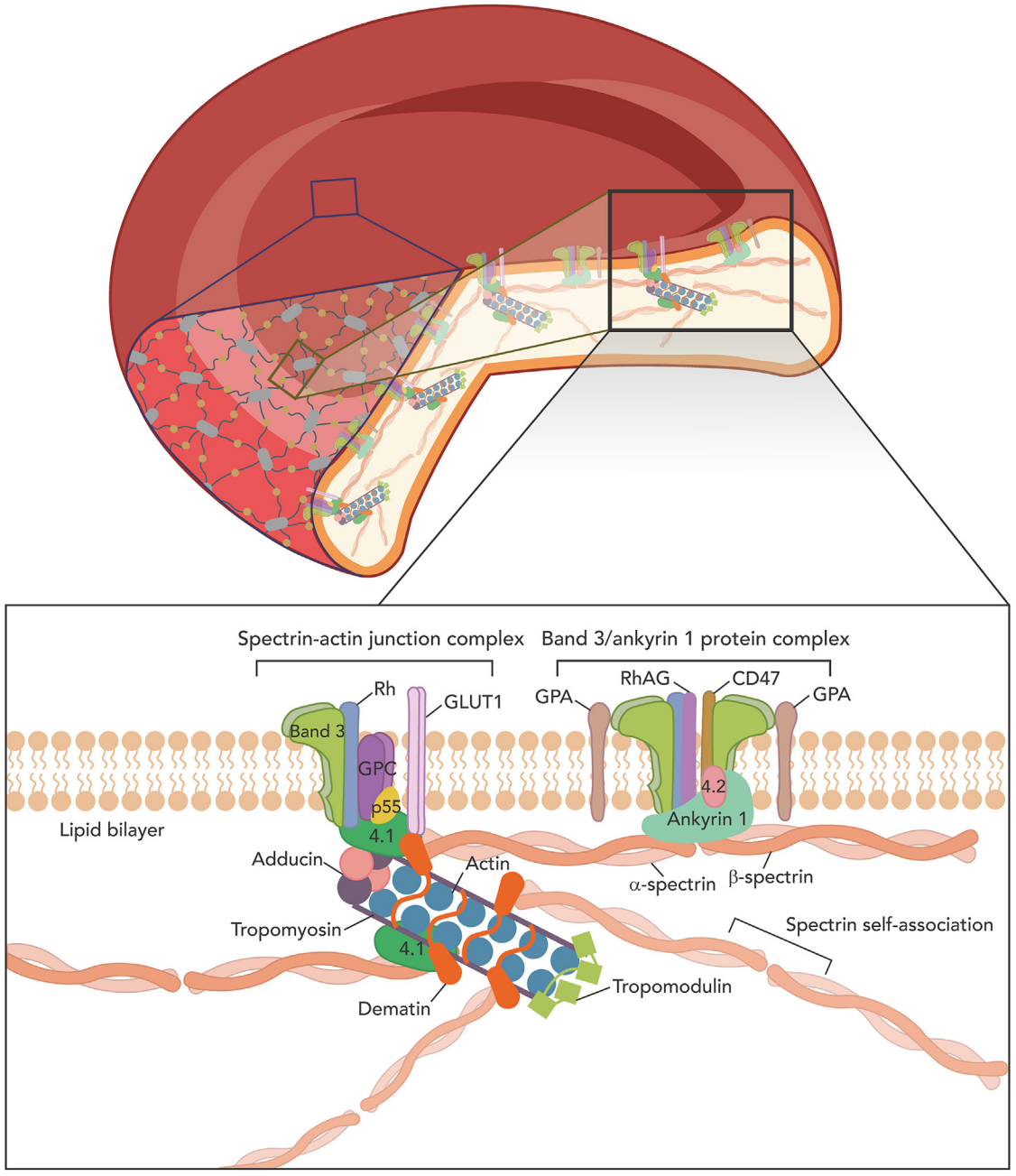

Figure 1. Skeleton structure of erythrocyte membrane

Magnifying the erythrocyte membrane from the area of the blue square, it can be seen that the surface of the erythrocyte membrane is supported by a hexagonal-like network skeleton. The sides of the hexagon are made up of spectrin tetramers, and the end and midpoints of each side are covered by gray ovals and semi-transparent yellow circles, respectively. When zooming in further, the gray oval, representing the spectrin-actin junction complex, anchors the ends of the spectrin tetramer to the cell membrane. The semi-transparent yellow circle, which represents the band 3/ankyrin 1 protein complex, can anchor the spectrin tetramer to the cell membrane from the middle.

3/ankyrin 1 protein complex.[15,16] The first important site for the connection between the erythrocyte cytoskeleton and the erythrocyte membrane is the interaction between the spectrin-actin junctional complex and Protein 4.1, adducin, and dematin[17–20] (Figure 1). Spectrin exists in the form of a heterotetramer. Specifically, the α chain and β chain of spectrin form a dimer with a length of approximately 100 nm, and two dimers are connected head-to-head to form a tetramer with a length of 200 nm. Protein 4.1 connects the end of the spectrin tetramer to the actin oligomer to establish a spectrin-actin junction complex, where the actin oligomer can bind to the six spectrin tetramers and weave a hexagonal meshwork based on spectrin.[16,21,22] The barbed end and pointed end of the actin oligomer are covered by adducin and

tropomodulin, respectively. Tropomyosin coiled coils are bound to both sides of the actin complex. The presence of all three kinds of proteins together determines the length of the junction complex.[19]

The second vital connection between the erythrocyte cytoskeleton and the erythrocyte membrane is the interaction between spectrin and the band 3/ankyrin1 protein complex (Figure 1). The spectrin tetramer can be anchored to the plasma membrane in the middle of the chain by the band 3/ankyrin 1 protein complex, an essential hub for regulating the erythrocyte cytoskeleton structure dynamics. Sae-Lee et al.[23] discovered ankyrin's spring-like behavior, essential for maintaining erythrocyte morphology to pass through the microvascular system. In addition to anchoring the cytoskeleton to the phospholipid bilayer, the band 3/ankyrin 1 protein complex is also the hub of erythrocyte metabolic activity.[23] The N-terminal domain of band 3 is located in the cytoplasm and connects to ankyrin, deoxyhemoglobin (deoxyHb) and metabolic enzymes. When the body experiences hypoxia or stress stimulation, the affinity of band 3 for these substances varies, which is discussed in detail later. The C-terminal domain of band 3 passes through the cell membrane, acting as an anion exchange channel for bicarbonate to help erythrocytes transport carbon dioxide (CO_2) from tissues to the lungs.[21,24,25] In addition, erythrocyte membrane surface proteins glycolipid transfer protein and signal peptide peptidase-like 2a, and the bioactive lipid molecule sphingosine-1-phosphate (S1P), also participate in the regulation of the stability, deformability, and adhesiveness of erythrocyte membranes.[26,27]

HYPOXIA AFFECTS ERYTHROCYTE MEMBRANE PROPERTIES

Hypoxia affects erythrocyte deformability

The impact of hypoxia on erythrocyte deformability is extensively studied in this field. The deformability of erythrocytes refers to the ability to reversibly change the morphology of erythrocytes to adapt to mechanical stress and maintain microcirculation blood flow. It is an indispensable characteristic of erythrocytes for adapting to hypoxic environments and is critical for supplying oxygen and nutrients to the microcirculation.[28,29] Erythrocyte deformability is primarily influenced by the following factors: maintenance of the biconcave disc shape by the membrane and cytoskeleton; the cytoplasmic ionic content determined by the mean corpuscular hemoglobin concentration (MCHC) and the surface-to-volume ratio; the cytoplasmic viscosity determined by adenosine triphosphate (ATP) and MCHC; and other factors such as nitric oxide (NO) and reactive oxygen or nitrogen species (ROS/RNS).[30–34]

Hypoxia affects erythrocyte deformability through the cytoskeleton. Band 3/ankyrin 1 protein complex, an important junction between the cytoskeleton and cell membrane, bears the brunt of the effects of hypoxia. DeoxyHb spatially and indirectly competes with ankyrin for band 3 without altering band 3's affinity for ankyrin, separating band 3 from ankyrin and thus separating the cell membrane from the cytoskeleton[35,36] (Figure 2A). Under *in vitro* hypoxic conditions, band 3, originally 77% bound and anchored to the cytoskeleton, reduced to 35%,[36] indicating that hypoxia affects the integrity of the erythrocyte cytoskeleton. Furthermore, acute hypobaric hypoxia tends to degrade band 3 and reduces its anion transport function after reoxygenation *in vivo*, decreasing its binding to glyceraldehyde-3P-dehydrogenase (GAPDH).[37] Hypoxia also influences the deformability of erythrocytes through the spectrin-actin junction complex. Hypoxia-reoxygenation diminishes band 3 protein functions in human erythrocytes, leading to actin rupture and cytoskeletal destabilization *in vivo*.[38]

NO prevents a decrease in erythrocyte deformability in hypoxic environments (Figure 2B). Red blood cells (RBC) have NO synthase (RBC-NOS) activity,[39] mediated by the PI3-kinase/Akt kinase pathway.[32,40] NO can activate guanylate cyclase, and the guanylate cyclase-catalyzed product guanosine monophosphate in turn affects NO synthesis.[22] When healthy adults are exposed to mildly hypoxic (12–16% O_2) conditions, RBC-NOS activation is restricted, the NO concentration decreases, and erythrocyte deformability is reduced.[41] Adding exogenous NO reduces hypoxia-induced clustered band 3 formation by attenuating membrane lipid peroxidation and protein tyrosine phosphorylation, affecting the affinity between the ankyrin and the band 3 N-terminus *in vitro*.[40,42] NO also protects protein function, stability, and location from further oxidative damage and improves RBC deformability by promoting reversible S-nitrosylation with erythrocyte cytoskeleton proteins such as α-spectrin and β-spectrin.[42,43] In severe hypoxia (<10% O_2), due to RBC-NOS inhibition, erythrocytes prefer to maintain the S-nitrosylation of spectrin by reducing nitrite via deoxyHb to form NO to prevent a decrease in RBC deformability.[41] Moreover, deoxyHb assists NO in regulating the mechanical properties of erythrocyte membranes, modulating erythrocyte deformability and oxygen-carrying-releasing ability. In tissues with higher levels of NO, deoxyHb binds to glutamate in the intracellular fraction of the band 3, increasing erythrocyte membrane permeability to NO.[42]

Intracellular ions also play a pivotal role in erythrocyte membrane deformability under hypoxia. Under physiological conditions, Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase maintain low intracellular concentrations of Na^+ and Ca^{2+} while consuming ATP. During acute hypoxia, ATP binds to Hb, reducing its oxygen affinity and supplying oxygen to tissues.[44] A decrease in cytosolic ATP concentration reduces the functions of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase on the erythrocyte membrane due to an insufficient energy supply, resulting in an imbalance in erythrocyte ionic transporters. Na^+ and Ca^{2+} in erythrocytes cannot be pumped out properly by the ion pump, which increases the intracellular osmotic pressure and leads to intracellular water retention in erythrocytes, making it difficult to maintain a normal morphology *in vivo*.[45] Although the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases after *in vitro* cyclic hypoxia, inhibiting mechanosensitive ion channels that transport Ca^{2+} does not have much of an effect on the mechanical degradation of erythrocytes.[46] Fluctuations in $[Ca^{2+}]_i$ also impair the binding of Hb to the membrane, resulting in a massive translocation of Hb into the cytoplasm.[47] With the deoxidation of erythrocytes, intracellular Mg^{2+} can alter erythrocyte deformability by modulating the cytoskeleton (Figure 2C). Under hypoxic conditions, the binding of 2,3-bisphosphoglycerate (2,3-BPG) and ATP to deoxyHb releases Mg^{2+} originally chelated with it into the cell, leading to increases in the concentration of free Mg^{2+} within the cell,[48] which promotes the binding of tropomyosin to actin[49] and the cross-linking of spectrin dimers,[50] stabilizing the erythrocyte membrane structure and improving its deformability. Moreover, an elevated intracellular Mg^{2+} concentration can affect band 3 phosphorylation.[51] Tyrosine phosphorylation of band 3 attenuates its binding to ankyrin and detaches it from the cytoskeleton, changing it

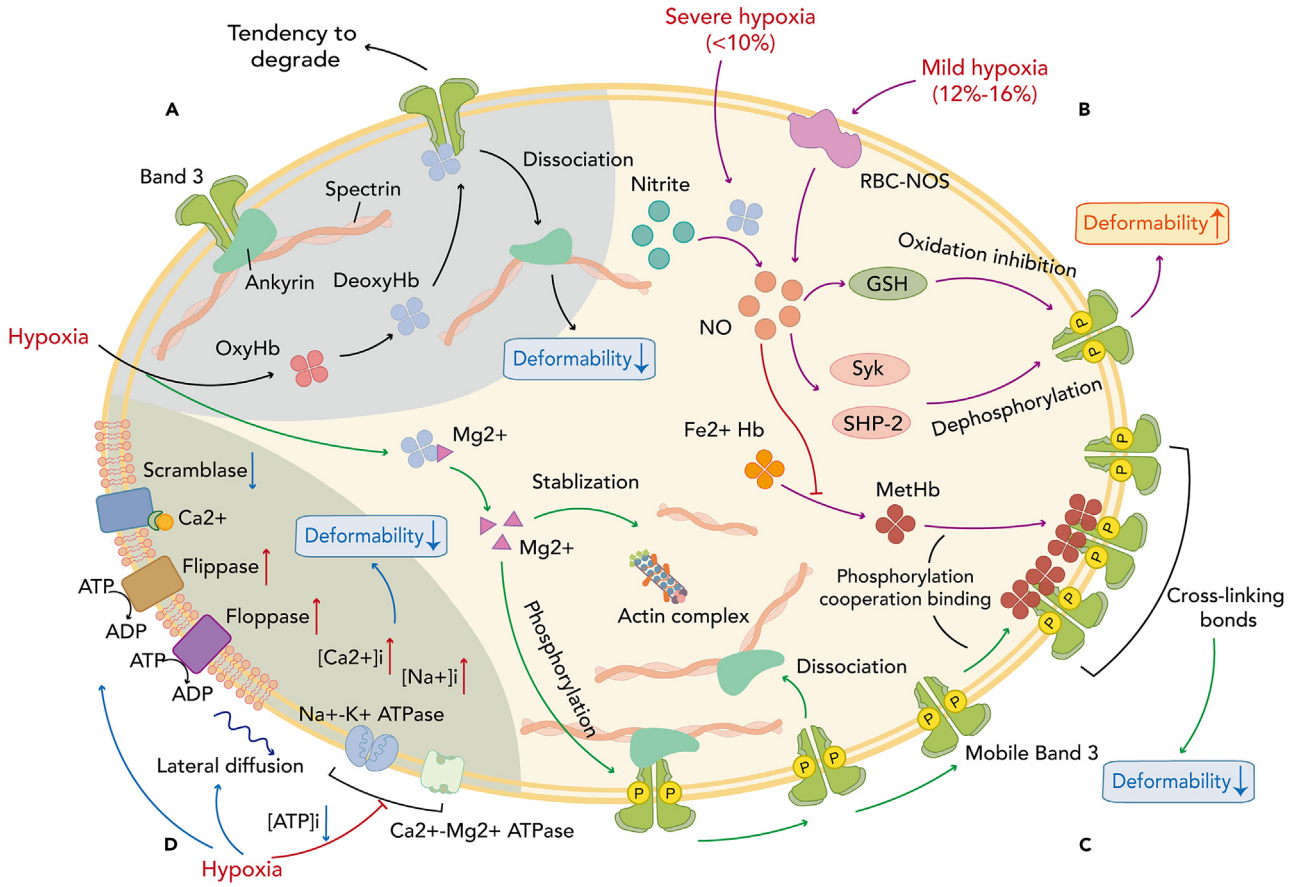

Figure 2. Hypoxia affects the deformability of erythrocyte membrane

(A) Competition of deoxyHb with ankyrin for band 3 reduces erythrocyte membrane deformability by separating the cytoskeleton from the membrane.

(B) The way of NO production under different hypoxia levels and its multiple effects on erythrocyte membrane deformability.

(C) Hypoxia leads to the release of Mg^{2+} bound to deoxyHb, which makes band 3 more susceptible to phosphorylation. The phosphorylated band 3 detaches from the cytoskeleton to form mobile band 3, which further aggregates into clusters, resulting in a decrease in erythrocyte membrane deformability.

(D) Hypoxia causes changes in the number and activity of enzymes on the cell membrane resulting in a decrease in the deformability of the erythrocyte membrane.

from a fixed to a mobile band 3. Mobile band 3 then combines with methemoglobin to form clusters, triggering cascade events that ultimately lead to hemolysis.[52–55] The influence of ions on the deformability of erythrocytes is complex and diverse; it adapts erythrocytes to hypoxia by altering the intra- and extracellular ion concentrations and affecting the cytoskeleton.

Lipids are principal components of the erythrocyte membrane, and alterations in lipid properties caused by hypoxia influence the maintenance of erythrocyte morphology. The outer phospholipids of the cell membrane are abundant in phosphatidylcholine (PC) and sphingomyelin (SM), while the inner lipids are mainly phosphatidylserine (PS) and phosphatidylethanolamine (PE). This asymmetric distribution is the basis for maintaining the structure and function of the erythrocyte membrane. After acute hypoxia, a significant change occurred in the phospholipid content of the erythrocyte membrane *in vivo*. The PS, SM, and SM/PC ratio markedly increased, while the PC decreased considerably. Additionally, the lateral diffusion rate of erythrocyte membrane lipids increases, potentially contribute to the reduced deformability of erythrocyte membranes.[45,56] During acute hypoxia, phospholipid scramblases promoting erythrocyte membrane phospholipid turnover are downregulated, thereby inhibiting lipid redistribution (Figure 2D). After hypoxic acclimation, phospholipids scramblases are similarly downregulated, but aminophospholipid translocases, which can flip PS and PE from the outer layer to the inner layer, are upregulated *in vivo*. ATP-dependent floppases, which are antagonistic to aminophospholipid translocases, are also upregulated. Taken together, the findings of these three enzymes suggest that the downregulation of phospholipid turnover in erythrocyte membrane lipids reduces the erythrocyte deformability after acute hypoxia, whereas inhibiting phospholipid turnover maintains this process after hypoxic acclimation.

The polyunsaturated fatty acids in the erythrocyte membrane are sensitive to free radicals in organisms. Patients with EE at high altitudes are chronically hypoxic and experience severe disorders of oxygen free radical metabolism. The erythrocyte membrane malondialdehyde content was notably greater in these patients than in normal controls, which reflects the rate and intensity of lipid peroxidation in tissues.[57] Under these conditions, erythrocyte membrane fluidity and deformability decrease, and the aggregation of erythrocytes increases, leading to

further hypoxia in the organism. However, other researchers found that the membrane fluidity of erythrocytes is unaltered in rats exposed to an anaerobic environment *in vitro* for 20 min.[58]

Despite numerous studies on the impact of hypoxia on erythrocyte membrane deformability, conflicting findings have emerged. Some studies report a decrease in erythrocyte deformability under acute hypoxic conditions,[45,51,59–62] while others indicate no impact on erythrocyte deformability,[60,63–65] and some suggest an elevation in erythrocyte deformability.[41,66,67] Recently, the deformability of erythrocytes was shown to be related to the duration of deoxygenation and the degree of hypoxia; it increased and then decreased with prolonged deoxygenation time *in vitro*.[68] Erythrocyte deformability was depressed in mildly hypoxia (12–16% O_2) but instead ascended in participants exposed to severe hypoxia (<10% O_2).[41] In patients with sickle cell anemia, hypoxia followed by reoxygenation significantly enhances the mechanical fragility of erythrocytes, leading to membrane damage.[29] The regulation of erythrocyte deformability by hypoxia is complicated, with varying effects depending on the degree of hypoxia, duration, and number of hypoxic reoxygenation cycles.

The regulation of deformability under hypoxia can be divided into four main components: the modulation of the cytoskeleton by hypoxia, the impact of hypoxia-induced NO on erythrocyte deformability, the effect of ions on deformability, and the influence of altered enzymes on the cell membrane. The reduced deformability of erythrocytes can lead to impaired perfusion, compromised oxygen delivery, and circulatory disturbances.[69] These outcomes can in turn cause hypoxia in the body. Therefore, it is crucial to investigate the mechanisms of erythrocyte deformability not only for hypoxia-related diseases but also for diseases that induce blood stasis.

Hypoxia affects erythrocyte aggregation and adhesion

Erythrocytes tend to aggregate under low-flow or static conditions to form rouleaux,[70,71] where erythrocytes accumulate and connect along the long axis. Healthy blood flow decomposes rouleaux and disperses erythrocytes. However, the continued presence of rouleaux can lead to microvascular occlusion in patients in a disease state.[72,73] Erythrocyte aggregation occurs in diseases like multiple myeloma and macroglobulinemia, as well as hypoxemia-associated disorders like stroke and myocardial infarction.[74] The external factors affecting erythrocyte adhesion and aggregation include the plasma composition, temperature, and oxygen saturation, while the internal factor on the erythrocyte membrane is band 3. Erythrocyte aggregation is faster and more severe under hypoxic conditions *in vitro* than under normoxic conditions, whereas the aggregation is drastically reduced by treatment with 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, an inhibitor of the anion exchange function of band 3.[75] This indicates that the erythrocyte aggregation under hypoxic conditions is related to band 3. In the high-altitude polycythemia (HAPC) rat model,[76] the erythrocyte aggregation index and whole blood viscosity were substantially greater than those in the control group. In contrast, a study on erythrocyte storage demonstrated that hypoxic storage inversely maintained erythrocyte stability, with no significant alterations in erythrocyte deformation and aggregation compared to normal storage conditions.[77]

Hypoxia exacerbates the abnormal adhesion of erythrocytes to sickle mature erythrocytes in sickle cell disease, a hereditary hemoglobin disorder where abnormal sickle hemoglobin (HbS) forms polymers during deoxygenation, leading to erythrocytes distortion and stagnation in the microcirculation.[78–80] Under hypoxic conditions, band 3 phosphorylation in sickle erythrocytes contributes to overall instability of the erythrocyte membrane, increasing adhesion.[53,81,82] A recent finding indicated that the polymerization of HbS added bending stress on the erythrocyte membrane.[83] Upregulated sphingomyelinase caused erythrocytes to produce higher-than-normal levels of sulfatides, promoting abnormally adherent. Meanwhile, the increase in plasma Hb and free heme during hypoxia activates the vascular endothelium, causing sickle erythrocyte adhesion[84] and increased adhesion to laminin.[85]

Changes in aggregation often accompany alterations in deformability, and both parameters are intimately linked to the morphology and function of circulating erythrocytes.[86] Reduced deformability diminishes erythrocyte oxygen-supplying capacity to tissues, while the formation of aggregates is associated with hemostasis and microcirculation. The chronic hypoxic symptoms in HAPC patients are likely related to these two parameters.

HYPOXIA ALTERS ERYTHROCYTE MEMBRANE PROTEINS AND CYTOPLASMIC PROTEINS

Hypoxia is a common phenomenon in physiological and pathological conditions. Tissues with increased cell renewal capacity often have the lowest oxygen content in the body. For instance, bone marrow contains 1–2% oxygen, the spleen contains 0.5–4.5%, and the thymus contains less than 1% oxygen.[87] As oxygen transporters, erythrocytes efficiently adapt to and shuttle within hypoxic environments to perform their core functions. When people go to high-altitude areas or experience diseases, erythrocytes are the main force assisting the body in systemic adaptation to hypoxia. Erythrocytes must cope well with hypoxic environments, or the function of various organs may be impaired.

A study on the effects of polyphenols and nitrogen oxide donors on human erythrocytes under hypoxic conditions *in vitro* revealed changes in erythrocyte organization at all levels, including lipid oxidation, erythrocyte cytoskeleton proteins, and the structure and morphology.[88] After 60 min in hypoxia, erythrocyte protein components significantly altered. Spectrin, ankyrin, and band 3, which constitute the cytoskeleton, reduced by 39.6%, 62.2%, and 76.0%, respectively. Band 4.1 and band 4.2 decreased to undetectable levels,[88] confirming the previous observation that band 3 was susceptible to acute hypobaric hypoxia *in vivo*.[56] The band 4.1a/4.1b ratio, considered as a molecular clock of erythrocyte age, decreased at altitude and gradually raised after volunteers returned to the plain.[89] However, the level of actin, which is also a component of the cytoskeleton, remained stable under hypoxia.[88] The expression of GAPDH, a metabolism-related protein, also tended to decrease by 50%.

Compared with normoxia group, *in vivo* expression of complement binding proteins, membrane attack complex inhibitory factors, lipid scramblases, and glucose transporters was reduced in erythrocytes from rats exposed to acute hypoxia.[45] In another study of hypoxic erythrocyte proteomics in rat blood *in vitro*,[58] hypoxia contributed to a 2-fold increase in erythrocyte hemoglobin (Hb) α and β, tetratricopeptide

repeat domain 39D, and extended synaptotagmin 1. The content of superoxide dismutase and membrane-bound 60S ribosomal protein L4 increased 1.5 times. In addition, the level of oxidative modification of proteins in hypoxic samples is lower than that in samples with standard oxygen content.[58] Thus, the authors proposed a model related to the Cullin-RING E3 ubiquitin ligase complex to explain this phenomenon. However, this model contradicts the conclusions of previous studies[56,88] that band 3 is more prone to degradation.

In addition to the changes in cytoskeletal proteins and cytoplasmic proteins, the ion channels on the erythrocyte membrane are also affected. The activities of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase reduced in the erythrocytes of rats in the acute hypoxia group.[45] These enzymes are associated with maintaining ion concentration differences in erythrocytes. However, after intermittent hypoxic training, the ATPase activity of the rats increased to a level close to that observed under normoxia *in vivo*,[45] indicating that the change in ATPase activity is reversible.

Acute hypoxia can lead to changes in metabolites. It is widely acknowledged that, under hypoxia, the affinity of band 3 for deoxyHb increases, resulting in the release of glycolysis-related enzymes initially bound to band 3. The shift from the pentose phosphate pathway to the glycolysis pathway in erythrocytes is enhanced under hypoxic conditions, promoting the metabolic pathways crucial for erythrocyte functions.[90–92] The effects of hypoxia on metabolism are explained in detail in the following section.

Since erythrocytes lack the ability to synthesize *de novo* proteins, posttranslational modifications play vital roles in maintaining the function of erythrocytes. After 42 days of storage at 4°C under hypoxia, human erythrocyte showed reduced methionine consumption, a factor promoting protein oxidative stress. Hypoxia also limits the methylation level of N-terminal residues in all proteins induced by storage or oxidative stress in erythrocytes *in vitro*, as observed in the band 3.[93] Additionally, cord blood erythrocytes cultured at 37°C for 16 h under hypoxia formed more methemoglobin than did those in the normoxia group.[94] This condition involves strong immune response proteins with their tyrosine sites phosphorylated. Moreover, the tyrosine phosphorylation of antioxidant proteins expands, which can protect erythrocytes from oxidative stress.[94] However, it should be noted that their research object was cord blood erythrocytes, which may not entirely represent the characteristics of mature erythrocytes in adults.

Hypoxia induces multifaceted changes in erythrocytes, including alterations in membrane proteins, cytoskeletal proteins, cytoplasmic proteins, and ion channels. These modifications impact the deformability of the erythrocyte membrane and the orientation of metabolic pathways. Furthermore, hypoxia influences protein posttranslational modifications, enhancing the antioxidant capacity of erythrocytes. The study of protein posttranslational modifications has deepened the understanding of the antioxidant capacity of erythrocytes, potentially providing clues for the development of novel antioxidant therapeutic strategies.

HYPOXIA IMPACTS ERYTHROCYTE microRNA

MicroRNAs (miRNAs), short noncoding RNAs approximately 18–23 nt in length, can inhibit the translation of transcripts through sequence complementarity. MiRNAs are involved in many cellular physiological processes, including cell proliferation, differentiation, senescence, death, and tumor formation.[95–98] Over a decade ago, researchers identified erythrocyte-derived miRNAs as the major source of miRNAs in the blood,[99] with erythrocytes playing a crucial role in regulating oxygen concentrations in the body when adapting to hypoxia. MiRNAs possess the ability to rapidly and reversibly alter gene expression, making them well-suited for responding to acute environmental changes like hypoxic stress.

Many studies have reported that miRNAs are affected by hypoxia,[96,100–102] and these miRNAs are termed hypoxia-regulated miRNAs (HRMs). Among the HRMs, miR-210 has been extensively studied, implicated in various biological processes throughout human tissues and organs. For instance, miR-210 involves in the metabolic regulation of neural stem cells,[103,104] the regulation of the angiogenic capacity of endothelial cells,[105–107] cell proliferation, and apoptosis.[108,109] A positive feedback loop exists between miR-210 and hypoxia-inducible factor-1 (*HIF-1*) under hypoxic conditions.[110] Upon hypoxia, *HIF-1* induces the production of miR-210, which inhibits the expression of glycerol-3-phosphate dehydrogenase 1-like (*GPD1L*). A reduction in the GPD1L protein attenuates the activity of prolyl-hydroxylase domain isoforms (PHDs) on HIF-1 proline hydroxylation, thereby reducing HIF-1 degradation and increasing HIF-1 protein stability.[110]

MiR-210 is inextricably linked to erythropoiesis. Its expression is progressively elevated during erythroid differentiation in mouse fetal hepatocytes.[111] In a study on hypoxic erythroid differentiation of peripheral blood $CD34^+$ cells from HAPC patients, hypoxia upregulated the level of GATA-binding protein 1 (GATA1) more in HAPC patients than in controls, promoting miR-451a and miR-210-3p expression and accelerating erythroid differentiation.[112] In contrast to those living on plains, the concentration of miR-210-3p in blood cells and plasma was significantly higher in Tibetan people at high altitudes, and it was positively correlated with erythrocyte counts and Hb levels.[113] In K562 cells, hypoxia also influences GATA1 to upregulate miR-451a and miR-210-3p, which repress 14-3-3ζ and SMAD2, respectively, to promote erythroid differentiation.[114–116] In addition to these two miRNAs, miR-363 participates in erythroid differentiation under hypoxia.[117] Together with GATA-1, it regulates the differentiation of K562 cells into erythroid cells via the HIF-1 pathway. These HRMs are likely to be key targets for mechanistic studies of HAPC therapy.

Hypoxia impacts microRNAs in blood circulation

High-altitude hypoxia influences miRNA profiles in plasma and erythrocytes.[118] A study by Yan et al.[118] provided the first definitive data on high-altitude hypoxia's impact on human plasma miRNA profiles. The authors observed significant differences in plasma miRNA patterns between Han Chinese migrating to Tibet and those residing at low altitudes. Additionally, they found that miR-130a-3p, miR-302b-5p, miR-572, and miR-629-5p were positively correlated with erythrocyte counts, Hb levels, and hematocrit. The first two miRNAs' target genes are predicted to be associated with erythropoietin and megakaryopoiesis, potentially influencing erythropoiesis during high-altitude acclimatization.

Another study on Tibetan and Han Chinese individuals living at high or low altitudes revealed that both altitude and ethnicity affect erythrocyte miRNA profiles.[100] Interestingly, the differential expressed miRNA levels in Tibetan people residing at high altitudes were more similar to those in the low-altitude population. In contrast, the difference between the miRNA levels in Han Chinese individuals migrating to high altitudes and those in low-altitude controls was somewhat broader. This suggests that Tibetan populations are genetically uniquely adapted to high altitudes. MiR-144-5p and miR-629-5p, two differential miRNAs mentioned in both studies,[100,118] the former could increase erythrocyte counts by repressing *RAB14* transcription,[119] and the latter may respond to hypoxia through its predicted target gene *HIF-3*.[120]

Both of these studies were conducted on healthy subjects.[100,118] After characterizing the peripheral blood miRNA profiles in the AMS and non-AMS groups acutely exposed to the plateau environment, researchers found that miR-369-3p, miR-449b-3p, and miR-136-3p as biomarkers for identifying AMS.[121] Additionally, HAPE susceptibility is correlated with miRNA levels in peripheral blood.[122] Overexpressing miR-124-3p in HAPE patients decreases plasma endothelial NO synthase (NOS), apelin, and V-Ets avian erythroblastosis virus E2 oncogene homolog 1 levels. A reduction in these substances inhibits peripheral vasodilation and may play a role in the pathophysiology of HAPE.

Besides plasma and blood cells, extracellular vesicles (EVs) also host miRNAs. EVs, released by cells into the external environment, are structures encapsulated by lipid bilayers and critical components of intercellular communication systems. Exosomes are EVs with diameters ranging between 30 and 150 nm that contain lipids, proteins, miRNAs, mRNAs, and DNA.[123] Exosomes regulate cellular functions primarily through association with miRNAs, whose variations in the expression have also been used as markers for disease monitoring. Comparing plasma exosomal miRNAs in HAPC patients with healthy controls, miR-122-5p, miR-4235p, miR-4433b-3p, miR-1291, miR-106b-5p, and miR-200c-3p were aberrantly expressed in HAPC patients.[101] Among them, miR-122-5p was the best predictor of HAPC, directly targeting *HIF-1A*.[124] These studies provide background on exosomal miRNAs for future HAPC research. The limitations of these studies of miRNAs in plateau samples include two commonalities. First, the studies are more biased toward describing sequencing results and lack further investigation of the subsequent mechanisms. Second, the disease-predicting biomarkers screened in small samples were not validated in larger cohorts. These two issues must be addressed to further explore the mechanism of plateau hypoxia in organisms.

Erythrocyte-derived EV (RBCEV) secretion is implicated in erythrocyte differentiation, disease, and adaptation to the external environment. RBCEVs involve in intracellular NO homeostasis, redox balance, procoagulant effects, and immunomodulation.[125,126] RBCEVs increase oxidative stress, disrupting NO homeostasis.[127,128] NO is an essential molecule for regulating erythrocyte metabolism under hypoxic conditions. However, it is unknown how RBCEVs regulate NO and oxidative stress under hypoxic conditions, which may constitute a complex regulatory network. Despite RBCEVs playing diverse roles in organisms, there is still a large lack of studies on proteins and miRNAs within RBCEVs under hypoxic conditions. This research may be instrumental in the development of novel therapies for HAPC in the future.

Metabolic effects of hypoxia-affected MicroRNAs

MiRNAs regulate metabolic reprogramming in disease. Most hypoxia-related disease research has focused on tumors with hypoxic microenvironment.[129–132] Hypoxia inhibits macrophage differentiation and functions by suppressing miR-30c expression, mTOR activity, and glycolytic pathways in gastric cancer.[130] MiR-186, an HRM, inhibits HIF-1A-induced tumor proliferation in gastric cancer,[133] and its downregulation enhances glycolysis in cancer-associated fibroblasts.[134] Regarding miRNA regulation of metabolism, miR-210 is vital and implicated in several metabolic processes, such as autophagy,[135] mitochondrial metabolism,[136] redox homeostasis, and supporting cancer cells' glycolysis.[137] Its metabolic regulation of mitochondria, ROS generation, and cancer cells has been reviewed in more detail elsewhere.[131,138]

However, studies on the impact of miRNAs on erythrocyte metabolism during hypoxia are lacking. Researchers have focused more on the metabolism of other tissues rather than erythrocytes, despite their intimate contact with various tissues. Two reasons may contribute to this sparsity of data: (1) in addition to classical metabolic pathways, erythrocytes may have nonclassical metabolic pathways during hypoxia, complicating the study of erythrocyte metabolic pathways regulation; (2) the characteristics of erythrocytes flowing in the circulation predispose them to respond to the condition of the whole organism, and the exchange of substances between erythrocytes and other tissues or organs further complicates their contents.

The reversible regulation, rapid targeting, broad range of action, and low cost of synthesizing miRNAs make them suitable modulators of metabolic reprogramming.[139] In conclusion, studying erythrocyte miRNAs in hypoxia enhances the understanding of the mechanisms of hypoxia-associated disorders like cardiovascular disease. Alterations in miRNAs contained in erythrocytes and RBCEVs during hypoxia may serve as targets for diagnosing, predicting, and treating diseases.

HYPOXIA CHANGES ERYTHROCYTE FUNCTIONS

Erythrocyte function is regulated by a large number of proteins, such as Hb, which regulates oxygen binding and release, and cytoskeletal proteins, which regulate elasticity and deformability. As aforementioned, under hypoxic conditions, the amount and modification of erythrocyte proteins and the deformability of membranes are altered, which has certain impacts on erythrocyte function. This review focuses on the effects of protein and metabolite changes on erythrocyte function under the influence of hypoxia, including the release and transport of oxygen molecules, erythrocyte metabolism, and eryptosis and survival.

Hypoxia changes the release of oxygen in erythrocytes

The core function of erythrocytes is the transport and release of oxygen. Erythrocytes are both carriers and sensors of oxygen, with Hb being the cruical protein for the primary function. Exposed to high-altitude hypoxia increases Hb levels variably as PaO_2 decreases, with its oxygen

affinity decreasing to maintain the body's oxygen supply.[4,140,141] Hb has two conformational states, a relaxed state (R-state) and a tense state (T-state), representing the primary forms of oxyhemoglobin (oxyHb) and deoxyHb, respectively. R-state Hb has a high affinity for oxygen and is relatively less likely to release oxygen, while T-state Hb has a low affinity for oxygen and is prone to releasing oxygen to supply tissue cells, which is instrumental when PaO_2 reduced.[142,143] The allosterism of Hb requires the regulation of 2,3-BPG, a glycolysis pathway product, whose level increases under high-altitude hypoxia.[144] 2,3-BPG is more inclined to bind deoxyHb and forms ionic bonds to promote the Hb transition from the R- to the T-state, thereby reducing the affinity of Hb for oxygen.[145–147]

Moreover, this process is inextricably linked to band 3, acting as an anion exchanger to sense tissue hypoxia by conducting chloride-bicarbonate exchanges within and outside the erythrocyte membrane.[148] It can also interact with glycolic enzymes. The increase in deoxyHb combined with band 3 during hypoxia leads to an increase in glycolysis-related enzyme dissociation from band 3 into the cytoplasm,[22] as band 3 has a greater affinity for deoxyHb.[36] The release of glycolysis-related enzymes enhances the glycolysis pathway and increases 2,3-BPG production. The detailed mechanism of hypoxia's explicit regulation of the 2,3-BPG will be described in detail in the section on erythrocyte metabolism. However, hypoxia-induced respiratory alkalosis neutralizes the effect of 2,3-BPG on Hb through the Bohr effect. The Bohr effect refers to the phenomenon where a reduction in blood pH or an increase in pCO_2 decreases the affinity of Hb. Conversely, elevated blood pH breaks of Hb ionic bonds, shifting Hb from the T-state to the R-state, increasing Hb affinity for oxygen.[149,150] The oxygen dissociation curves measured in 1984 confirmed that the effect of 2,3-BPG is eliminated by the increase in Hb affinity for oxygen due to respiratory alkalosis in the hypoxic plateau environment.[149] The regulation of Hb under hypoxic conditions is the consequence of a balance of multiple factors. However, how to determine the direction of their balance has yet to be explored.

Hypoxia changes erythrocyte metabolism

High-altitude hypoxia induces considerable metabolic alterations in erythrocytes. Studying these metabolic adaptations may not only enhance the mechanistic exploration and treatment of hypoxia-related diseases, but also benefits the quality of life for high-altitude residents around the world. Metabolic changes under hypoxia are categorized into six main pathways: glycolysis, the pentose phosphate pathway (PPP), glutathione metabolism, nitrogen metabolism, arginine and sulfur metabolism, and carboxylic acid metabolism.

Glycolysis and the pentose phosphate pathway

Due to the lack of mitochondria, mature erythrocytes derive energy from anaerobic glycolysis, distinct from most cells that rely on aerobic respiration. There are two pathways linked to the glycolytic pathway closely related to erythrocyte metabolism: the Rapoport-Luebering shunt and the PPP. The Rapoport-Luebering shunt transforms 1,3-bisphosphoglycerate (1,3-BPG) in the glycolysis pathway into 2,3-BPG under the action of bisphosphoglycerate mutase (BPGM) and then into 3-phosphoglycerate with the help of 2,3-BPG phosphatase. The 2,3-BPG produced by this pathway is an allosteric modulator and a critical metabolite that regulates the release of oxygen from Hb. As mentioned earlier, 2,3-BPG can bind to the T-state of Hb to form ionic bonds, promoting a rightward shift of the oxygen dissociation curve[143] (Figure 3). The other bypass is the PPP, which is a process from glucose 6-phosphate shunting through 6-phosphogluconolactone and 6-phosphogluconate to ribose 5-phosphate and then through a series of ketone and aldehyde transfer to re-synthesize fructose 6-phosphate and glyceraldehyde 3-phosphate, returning to the glycolytic pathway. The nicotinamide adenine dinucleotide phosphate (NADPH) produced by the PPP interacts with glutathione to provide a defense system against ROS, while another product produced by the PPP, ribose 5-phosphate, can participate in nucleotide biosynthesis.

Compared to high-altitude volunteers' erythrocytes, mature erythrocytes at sea level exhibit increased shunting to the PPP under normoxic conditions, with the glycolysis pathway relatively suppressed. This phenomenon is considered to promote the production of NADPH to combat ROS.[90] Under high-altitude hypoxia, increased deoxyHb directly competes with glycolysis-related enzymes on the membrane for residues 1–30 at the N-terminus of the band 3 polypeptide.[151] This process results in the dissociation of glycolysis-related enzymes like GAPDH and fructose-bisphosphate aldolase from band 3, which enter the cytoplasm to promote the glycolysis pathway, and produce more 1,3-BPG and 2,3-BPG, thus facilitating the supply of oxygen to tissues.[91,92,152–155]

Recently, many studies have concentrated on the factors determining the production of 2,3-BPG in erythrocytes at high altitude or under hypoxia. These factors include the adenosine monophosphate (AMP)-activated protein kinase (AMPK)-BPGM and the ADORA2B-PKA signaling pathway, along with the research on glycolytic enzymes translocation (Figure 3). In high-altitude hypoxia, soluble CD73 activity and plasma adenosine levels elevate. CD73 transform AMP to adenosine (ADO), which acts on the adenosine A2b receptor (ADORA2B), a G protein-coupled receptor activated at high ADO levels. Activated ADORA2B phosphorylates AMPK, which subsequently activates BPGM to increase 2,3-BPG production and induce oxygen release[156] (Figure 3A). A recent study on chronic kidney disease identified an erythrocyte hypoxia sensor, the ENT1-AMPD3-AMPK-BPGM signaling network.[157] Hypoxia suppresses AMP deaminase 3 (AMPD3) activity, allowing rapid conversion of extracellular ADO transduced by equilibrium nucleoside transporter 1 (ENT1) to AMP instead of IMP, which promotes the AMPK-BPGM pathway. The levels of 2,3-BPG correlated with the expression levels of the Rhesus blood group RHCE, possibly related to the promotion of cellular alkalinization by the Rh group complex in favor of increased BPGM activity.[158]

Furthermore, ADORA2B receptor activation can activate the cAMP-dependent protein kinase A (PKA) pathway (Figure 3B). PKA phosphorylates ENT1, which is then ubiquitinated for degradation. As ADO rises at high altitudes or during secondary hypoxic exposure, extracellular ADO accumulates rapidly due to its inability to enter erythrocytes, thus promoting ADOA2B-AMPK signaling. This increases the level of 2,3-BPG, facilitating faster adaptation to hypoxia.[159] The ADORA2B-PKA signaling pathway also intersects with glycolytic enzyme translocation. The PKA signaling pathway activates sphingosine kinase 1 through the ERK1/2 cascade, generating S1P, a multifunctional signaling lipid.[160]

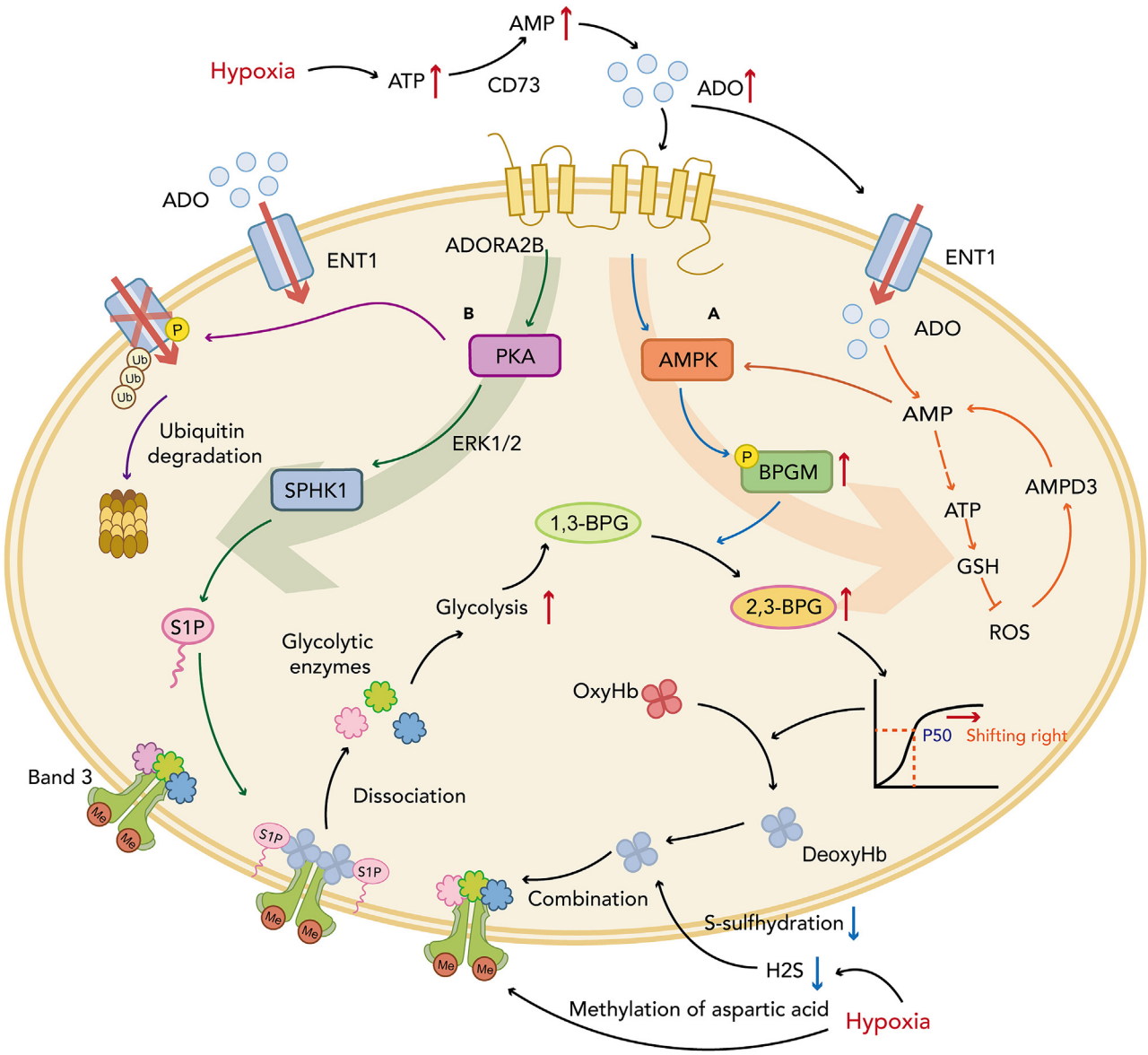

Figure 3. Hypoxia affects adenosine signaling pathways in erythrocytes

(A) AMPK-BPGM signaling pathway.

(B) ADOA2B-PKA signaling pathway.

Elevated S1P under hypoxia binds directly to deoxyHb, promoting glycolytic enzyme translocation from the membrane to the cytoplasm.[161] Additionally, S1P induces the release of the ubiquitinated p97, a component of proteasome machinery, into the cytoplasm for proteasome functions.[162] Hypoxia increases band 3 aspartic acid methylation and decreases S-sulfuration of deoxyHb, which facilitates the translocation of glycolytic enzymes and anchoring of deoxyHb on band 3, respectively.[93,163]

Glutathione metabolism

The glutathione system, crucial for clearing intracellular ROS, is the most abundant antioxidant system. Glutathione (GSH) is synthesized from cysteine, glycine, and γ-glutamyl amino acids catalyzed by γ-glutamylcysteine synthase and GSH synthetase. GSH can be converted into glutathione disulfide (GSSG) in the presence of glutathione peroxidase (GPx) while scavenging intracellular ROS. GSSG is then reduced to GSH by glutathione reductase (GR) using hydrogen supplied by NADPH from the PPP.

D'Alessandro et al.[152] found an increase in GSH and a decrease in GSSG in human mature erythrocytes under hypoxic conditions. The depletion of glutamic acid and cysteine, the raw materials for GSH synthesis, may indicate increased GSH *de novo* synthesis or decreased

oxidative stress. However, the reduced shunt to the PPP under hypoxia implies less NADPH, hindering GSSG to GSH conversion, which does not fully explain the altered levels of GSSG and GSH in erythrocytes. Paradoxically, it has been shown that organisms are more likely to produce ROS under hypoxic conditions than under normoxic conditions,[164,165] and erythrocyte membrane proteins and lipids are more vulnerable.[56] Reduced PPP hampers ROS defense, while the upregulation of the plasma erythrocyte enzyme antioxidant defense system improves the erythrocyte antioxidant capacity. Magalhães et al.[165] showed the increase in plasma superoxide dismutase and GR activities and the decrease in GPx activity, explaining the possible reasons for the GSSH decrease in erythrocytes. Moreover, the growth tyrosine phosphorylation of several antioxidant proteins, including peroxiredoxin, thioredoxin peroxidase, and catalase, also enhances the antioxidant capacity of erythrocytes. Overall, the erythrocyte antioxidant system may be regulated by multiple factors, warranting further exploration.

Nitrogen metabolism

The nitrogen metabolism of erythrocytes is associated with NO synthesis, purine metabolism, and the urea cycle. Arginine and oxygen can generate citrulline and NO through the action of NOS. Inorganic anions NO_3^- and NO_2^- can be transformed into NO by deoxyHb in erythrocytes.[166] Under hypoxia, the accumulation of asymmetric dimethylarginine, an NOS inhibitor, leads to the reduction of NO synthesis in erythrocytes. However, the levels of NO_2^- and NO_3^-, can be used as alternative sources of increased NO.[152] Nitrogen metabolism can provide nitrogen for purine synthesis. The degree of change in purine homeostasis in erythrocytes is proportional to the duration of exposure to higher altitudes, with extracellular ATP, AMP, and adenosine accumulation promoting the glycolysis pathway.[156] The regulation of purine metabolism during hypoxia cannot be achieved without the availability of amino acids or the involvement of other nitrogen sources.

The synthesis of carbamoyl phosphate from CO_2 and NH3 in the urea cycle and the synthesis of citrulline from carbamoyl phosphate and ornithine are both accomplished in the mitochondria. After leaving the mitochondria, citrulline converts into urea and ornithine through enzymatic reactions, with ornithine returning to the mitochondria—known as the complete ornithine cycle. Mature erythrocytes have already expelled mitochondria, and the depletion of arginine and accumulation of polyamines in the ornithine cycle under high altitude hypoxia suggest active communication between erythrocytes and the extracellular environment, or it may be the result of undiscovered metabolic patterns unique to erythrocytes. The biological significance of these fundings remain to be investigated.

Arginine and sulfur metabolism

Arginine is relevant to nitrogen metabolism, sulfur metabolism, and creatine production, so variations in arginine concentration may result from coregulation of multiple metabolic pathways. Guanidinoacetate, synthesized from arginine and glycine, generates creatine and S-adenosyl homocysteine (SAH) via the catalysis of guanidinoacetate N-methyltransferase with S-adenosyl methionine. SAH converts into cysteine for H2S and taurine generation. Arginine consumption during hypoxia may be related to increased creatine synthesis, but the declines in cysteine, H2S and taurine levels lack explanation.[152] H2S, a vasodilation molecule to regulate blood flow and blood vessel pressure, reduced under high-altitude hypoxia and would restrain the sulfuration of the protein in erythrocytes.[163] Creatine synthesis primarily occurs in the kidneys and liver.[167] Erythrocytes may simply be transporters of creatinine, partially reflecting the body's overall state.

Carboxylic acid metabolism

Citric acid metabolism relies on several enzymes located in mitochondria. However, recent applications of proteomics technology, isotope tracing technology[168–170] and computational evidence[171] have suggested the presence of cytoplasmic isomers of active Krebs cycle enzymes in mature red blood cells lacking mitochondria. After hypoxia exposure at high altitudes, the levels of several citric acid metabolites (carboxylic acid, α-ketoglutarate, and 2-hydroxyglutarate) gradually decrease in human erythrocytes, while the levels of fumaric acid and malate initially decline and then gradually climb.[152,170–172] In the AltitudeOmics study,[152] as the participants acclimatized to high altitude reduced oxygen levels, succinate, a biomarker of tissue hypoxia,[173] gradually decreased in their erythrocytes. Another study of rapid ascent to high altitude showed elevated plasma succinate in the early stages of acute hypoxia, implying that the change in succinate concentration is a continuous process.[174] Elevated succinate concentrations at the beginning of acute hypoxia indicate increased tissue hypoxia, diminishing with gradual acclimatization to plateau hypoxia. However, further studies are needed to determine if altered erythrocyte succinate concentrations can be used as an indicator of acclimatization success to plateaus. Although hypoxia-exposed erythrocytes exhibit increased citric acid uptake *in vitro*, there was a decrease in citric acid catabolism to malate and an increase in the conversion to glutamic acid and 5-oxoproline.[152] This phenomenon suggested that hypoxia leads to a shift in citrate metabolism toward GSH synthesis, which was confirmed by the increase in GSH/GSSG ratios in the *in vivo* data, as seen in a recent study on hypoxic storage of blood in mice.[154] More research is needed in the future to reveal how mature erythrocytes without mitochondria achieve this particular regulatory network of carboxylic acid metabolism.

Metabolic pathway connections in hypoxic erythrocytes

During hypoxia, the most closely related metabolic pathways in erythrocytes are glycolysis, the PPP, and glutathione metabolism. The glycolytic pathway is the primary energy source for erythrocytes and connects to the Rapoport-Luebering shunt, whose product 2,3-BPG aids the release of oxygen from Hb. The glycolytic pathway is also linked to the PPP, which supports the oxidation reduction of glutathione through NADPH production. Erythrocytes, aiming to enhance oxygen release, shift their metabolic pathway from a bias toward the PPP during

normoxia to a bias toward the glycolytic pathway during hypoxia. This change reduces NADPH production, thereby interfering with the defense of glutathione against oxidative stress.

Arginine is crucial for nitrogen and sulfur metabolism. It's a vital source for both pathways. NO, produced from arginine by NOS, protects the deformability of the erythrocyte membrane from damage under hypoxic conditions. Arginine and glycine synthesize SAH. Cysteine, which is converted by SAH, can serve as a raw material for the synthesis of GSH, which in turn is linked to glutathione metabolism. Glycolysis connects to nonclassical carboxylic acid metabolism in erythrocytes via pyruvate, and the ribose produced by the PPP can feed purine synthesis.

A complex regulatory network allows erythrocytes to adapt to various physiological conditions. Adjusting these metabolic pathways under hypoxic conditions maintains erythrocyte function and structure. In-depth study of these metabolic pathways may be beneficial in the treatment of hypoxic diseases as well as oxidative stress-related disorders.

Hypoxia affects eryptosis and survival

Eryptosis is the apoptosis of mature erythrocytes, whose morphological characteristics are similar to those of apoptotic cells except for the absence of changes in chromatin and organelles.[175] Eryptosis is primarily triggered by increased $[Ca^{2+}]_i$ through activation of Ca^{2+} channels, followed by activation of flippase, calpain and other protein hydrolases leading to programmed erythrocyte death.[176] Ma et al.[177] examined the apoptosis of erythroblasts in bone marrow mononuclear cells from patients with CMS and found that the Bcl-2 family plays an influential role in regulating the reduced apoptosis of erythroblasts, which may be one of the pathogenic mechanisms of EE in CMS patients. Hypoxic exposure increases the susceptibility of human erythrocytes to damage from ROS.[58] Salidroside can protect human erythrocytes from H_2O_2-induced apoptosis through its antioxidant activity, caspase-3 inhibition and stress-induced $[Ca^{2+}]_i$ elevation.[178] However, whether this can be served as a therapeutic target against high-altitude diseases still needs verification.

Erythrocyte counts decrease upon returning from the plateau, but it is unclear whether this phenomenon is related to erythrocyte apoptosis. Klein et al.[89] found that the reduction in erythrocytes counts in volunteers who returned to the plain after a 3-week stay at high altitude was not achieved by removing newly formed erythrocytes generated at the plateau but rather by reducing the rate of erythropoiesis and by maintaining the clearance rate of senescent erythrocytes. Ascending to the plateau and returning to the plain constitute a process of hypoxia and reoxygenation of the entire body. In circulation, erythrocytes face constant oxygen tension change. Cyclic hypoxia study proposes a biophysical mechanism for erythrocyte senescence: repeated hypoxia alters erythrocyte membrane cytoskeleton, impairing erythrocyte deformability and causing mechanical degradation with fatigue damage accumulation.[179] Long-term deoxygenation also elevates the tendency toward membrane rupture due to the weakened cytoskeleton.[180]

Conclusions

Erythrocytes undergo numerous hypoxia-induced changes, affecting membrane structure and composition, membrane properties, miRNAs, oxygen release capacity, metabolic pathway switching, and eryptosis and survival. Band 3 is central to these alterations, sensing tissue hypoxia, regulating metabolism direction with glycolytic enzymes or deoxyHb, influencing deformability through ankyrin or deoxyHb, and affecting aggregation and adhesion via anion exchange function. In addition to band 3, NO also has multiple functions. NO diastoles blood vessels, inhibits hypoxia-induced deformable injury of erythrocytes by reducing the cross-linking of band 3, and stabilizes proteins via S-nitrosylation. Additionally, mutual balance and priority of effects from the same metabolite in different pathways remain unclear. For example, the shunt of the glycolysis pathway in oxygen transport is also involved in erythrocyte metabolism, and the deformability of erythrocytes also influences erythrocyte aggregation and adhesion.

There are still many unanswered questions in this field. The impact of hypoxia on erythrocyte deformability and how to precisely regulate erythrocyte ROS production remain debated. Hypoxia shunts the PPP in erythrocytes toward the glycolysis pathway, thus ensuring 2,3-BPG production for oxygen release from oxyHb. Moreover, the PPP generates NADPH to maintain the antioxidant capacity of glutathione. Since hypoxia causes an increase in ROS in erythrocytes, how can this increase in ROS be counteracted while reducing the PPP? Although erythrocytes modify antioxidant-related enzymes to boost activity against hypoxia,[94] their effectiveness in mitigating oxidative damage requires further investigation. It is also inconclusive whether the clearance of proteins from erythrocyte membranes damaged by hypoxia oxidation depends on hydrolytic enzymes in the membrane or intracellular ubiquitination degradation or to what extent it depends on EV transport.

Under hypoxia, the intracellular redox processes in red blood cells form a complex network.[181] However, the role of RBCEVs within this network remains unexplored. The impact of miRNAs in RBCEVs or inherent to red blood cells on metabolism also warrants further investigation. Although lacking mitochondria, erythrocytes undergo alterations in many mitochondria-dependent metabolic pathways during hypoxia. Whether this represents a unique metabolic pathway in erythrocytes requires extensive experimental verification. Furthermore, current research mostly concentrates on acute hypoxia, and studies on chronic hypoxia need to be complemented.

Proteomics and metabolomics are potent tools for studying membrane and cytoplasmic alterations in erythrocytes under plateau or hypoxic conditions, providing new insights into disease research and treatment.[182] In recent years, plasma proteomics and metabolomics studies of HAPC have proliferated, but few studies have focused on mature erythrocytes themselves. Studying the proteome and metabolome of mature erythrocytes is essential. Proteomic analysis of the erythrocyte membrane and cytoplasm can reveal variations in protein composition in erythrocytosis, aiding disease exploration and prediction. Proteomics helps identify proteins influencing HAPC and discover therapeutic targets. Moreover, mapping erythrocyte protein expression patterns in HAPC patients can facilitate the development of personalized treatment plans. Erythrocyte metabolomics can also unveil metabolism-related biomarkers for predicting disease occurrence and status,

improving intervention. Multiomics analysis integrates the erythrocyte metabolome, proteome, and transcriptome for disease modeling and a more comprehensive dynamic deciphering of the biological complexity of the disease.[183]

EE is a characteristic symptom of CMS. Late stages of CMS develop systemic organ involvement, closely related to erythrocytes. However, proteomic and metabolomic studies focus more on plasma from AMS patients than CMS patients, leaving a gap in the proteomic and metabolomic data available for erythrocytes from CMS patients, which may be due to limitations in the technology. High Hb levels in the erythrocyte cytoplasm can mask the detection of low-abundance proteins, especially in diseases such as CMS, where Hb is elevated. Erythrocyte proteomic studies of CMS are of intriguing, and the study of CMS can be extended to chronic hypoxic diseases. As erythrocytes are constantly present in circulation to supply oxygen to various organs, targeting erythrocytes to explore mechanisms and therapeutic means can also provide more ideas for some systemic diseases.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.S., J.G., J.T., and X.J.; writing the original draft, X.J.; review and editing, L.S.; figure conception and preparing: X.J., Y.Z., D.W., and Xiaoru Z.; manuscript revision, L.S., X.J., Y.Z., Xiaoru Z., Y.L., and D.W.; figure revision, X.J., Y.L., J.W., and L.Z.; literature collection, X.J., H.S., Xu Z., J.L., and J.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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