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Beneficial Role of Erythrocyte Adenosine A2B Receptor-Mediated AMPK Activation in High Altitude Hypoxia

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Abstract

Background—High altitude is a challenging condition caused by insufficient oxygen (O_2) supply. Inability to adjust to hypoxia may lead to pulmonary edema, stroke, cardiovascular dysfunction and even death. Thus, understanding the molecular basis of adaptation to high altitude may reveal novel therapeutics to counteract the detrimental consequences of hypoxia.

Methods—Using high throughput unbiased metabolomic profiling, we report that the metabolic pathway responsible for production of erythrocyte 2,3-bisphosphoglycerate (2,3-BPG), a negative allosteric regulator of hemoglobin-O_2 binding affinity, was significantly induced in 21 healthy humans within two hours of arrival at 5260m, and further increased following 16 days at 5260m.

Results—This finding led us to uncover discover that plasma adenosine concentrations and soluble CD73 (sCD73) activity rapidly increased at high altitude and were associated with

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Disclosures

None.

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elevated erythrocyte 2,3-BPG levels and O_2 releasing capacity. Mouse genetic studies demonstrated that elevated CD73 contributed to hypoxia-induced adenosine accumulation and that elevated adenosine-mediated erythrocyte A2B adenosine receptor (ADORA2B) activation was beneficial by inducing 2,3-BPG production, triggering O_2 release to prevent multiple tissue hypoxia, inflammation and pulmonary vascular leakage. Mechanistically, we demonstrated that erythrocyte AMP-activated protein kinase (AMPK) was activated in humans at high altitude and that AMPK is a key protein functioning downstream of ADORA2B, phosphorylating and activating BPG mutase and in this way inducing 2,3-BPG production and O_2 release from erythrocytes. Significantly, preclinical studies demonstrated that activation of AMPK enhanced BPG mutase activation, 2,3-BPG production and O_2 release capacity in CD73-deficient mice, in erythrocyte specific ADORA2B knockouts, and in wild type mice and in turn reduced tissue hypoxia, and inflammation.

Conclusions—Altogether, both human and mouse studies reveal novel mechanisms of hypoxia adaptation and potential therapeutic approaches for counteracting hypoxia-induced tissue damage.

Keywords

hypoxia; adenosine; AMP-activated protein kinase signal transduction; oxygen; adenosine receptor; erythrocyte; high-altitude acclimatization; altitudeOmics

Hypoxia is defined as inadequate oxygen (O_2) supply to the whole body or a region of body. Hypoxia is commonly seen in patients with cardiovascular[1–3], respiratory[4, 5] and hemolytic[6] diseases. Hypoxic conditions associated with these diseases are dangerous as decreased O_2 availability promotes end organ damage and failure. Hypoxia also frequently occurs in healthy individuals when they travel to high altitude, or other low O_2 content environments[7, 8]. It is widely observed that people differ in their ability to adapt to high altitude hypoxia[8–11]. Inability to adapt quickly to altitude hypoxia can result in pulmonary edema, stroke, cardiovascular dysfunction and even death[3, 7, 12, 13]. Thus, hypoxia is a highly dangerous condition for both normal individuals exposed to high altitude hypoxia and for patients with cardiovascular, respiratory and hemolytic diseases. However, current strategies to counteract hypoxia are limited due to a lack of fundamental understanding of molecular mechanisms underlying adaptation to hypoxia.

The erythrocyte is the most abundant circulating cell type and responsible for delivery of O_2 to peripheral tissues. To function effectively in O_2 uptake, transport and delivery, especially under hypoxia conditions, erythrocytes rely on sophisticated regulation of Hb-O_2 affinity by endogenous allosteric modulators. One of the best known allosteric modulators is 2,3-BPG, a metabolic byproduct of glycolysis synthesized primarily in erythrocytes for the purpose of regulating Hb-O_2 affinity[14]. It has been known for more than four decades that when humans ascend to high altitude the concentration of 2,3-BPG in erythrocytes increases significantly and its elevation is associated with an increased capacity of O_2 release from hemoglobin[15]. Erythrocyte 2,3-BPG levels are also elevated in many cardiovascular diseases (CVD) associated with hypoxia[16–18]. However, the molecular basis underlying high altitude and disease-related hypoxia-induced erythrocyte 2,3-BPG remains unknown.

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To address these questions, we recruited 21 young healthy human volunteers, and conducted high altitude studies. Our human studies showed that circulating adenosine levels and the activity of soluble ecto-5′-nucleotidase (CD73), a key enzyme to generate extracellular adenosine, were significantly induced at high altitude, and were associated with increased erythrocyte 2,3-BPG and O_2 releasing capacity. *In vivo* genetic mouse studies coupled with *in vitro* human and mouse studies demonstrated that increased extracellular adenosine signaling via erythrocyte ADORA2B led to the sequential activation of AMPK (a well-known cellular energy sensor) and 2,3-BPG mutase, resulting in increased 2,3-BPG production and enhanced O_2 release capacity to peripheral tissues and in turn reduced tissue hypoxia, inflammation and pulmonary injury. Thus, our findings highlight the importance of this newly identified erythrocyte adenosine signaling pathway via AMPK in human adaptation to high altitude and identify several attractive targets to counteract hypoxia in humans.

Methods

For expanded Methods sections, please refer to the Supplemental Materials.

Human Subjects

This study was conducted as part of the Altitudeomics project examining the integrative physiology of human responses to hypoxia[19]. In brief, all procedures conformed to the Declaration of Helsinki and were approved by the Universities of Colorado and Oregon Institutional Review Boards and the US Department of Defense Human Research Protection office. After informed written consent, recreationally active sea level (SL) habitants participated in the high altitude study. The participants were non-smokers, free from cardiorespiratory disease, born and raised at <1500 m, and had not travelled to elevations >1000 m in the 3 months prior to investigation.

Each subject was studied near SL (130 m, average PB=749 mmHg) and on the first and sixteenth days at Mt Chacaltaya, Bolivia (5260 m; average PB=406 mmHg; ALT1, ALT16). Baseline studies at SL were conducted over a two-week period in Eugene, OR, USA. Approximately one month after the SL studies, subjects traveled to Bolivia in pairs on successive days. Upon arrival at El Alto (4050m) after an overnight flight, subjects immediately descended to Coroico, Bolivia (1525 m; PB=639 mmHg). Subjects rested for 48 hours in Coroico to limit the effects of jet lag and were then driven over three hours to 5260 m. To provide an acute change in inspired PO_2 from 1525 m to 5260 m, subjects breathed supplemental oxygen (2 L/min, nasal cannula or mask) during the drive. On arrival at 5260 m, the first subject immediately began the experimental protocol as previously described[19].

Mouse Subjects

Eight to ten-week-old C57BL/6 wild-type (WT) mice were purchased from Harlan Laboratories (Indianapolis, IN). CD73-deficient (*Cd73*$^{-/-}$) mice and A1, A2A, A2B and A3 adenosine receptor (ADORA1, ADORA2A, ADORA2B, and ADORA3)-deficient mice with C57BL/6 background were generated and genotyped as described before[20, 21]. A novel

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line of mice with erythrocyte-specific deletion of *Adora2b* was generated by crossing mice homozygous for a floxed *Adora2b* allele with mice expressing Cre recombinase under the control of erythropoietin receptor (EpoR) gene regulatory elements[22]. All protocols involving animal studies were reviewed and approved by the Institutional Animal Welfare Committee of the University of Texas Health Science Center at Houston.

Blood collection and preparation

Human and mouse blood were collected and stored as described before[20, 23]. Approximately four milliliter human blood were collected with heparin as an anti-coagulant for 2,3-bisphosphoglycerate (2,3-BPG) measurement using a commercial assay (Roche, USA) as previous described[20]. For plasma adenosine assay, one ml of EDTA collected blood was aliquoted to tubes containing 10 μM dipyridamole (an inhibitor of equilibrative nucleoside transporters) and 10 μM deoxycoformycin (an inhibitor of adenosine deaminase). Approximately one ml of mouse blood was collected and used for 2,3-BPG and plasma adenosine measurement similar to human blood as described above[20, 23]. High altitude human blood was collected and stored at −80°C after collection and during shipment from the field until assay.

Measurement of soluble CD73 activity in lowland volunteers

sCD73 enzyme activity in humans was measured by quantifying the rate of conversion of [^{3}H]-AMP to [^{3}H]-adenosine as described previously[24, 25]. Briefly, sCD73 activity was measured after incubation of plasma with 300 uM [^{3}H]AMP (Quotient Bioresearch; GE Healthcare, Rushden, UK) for 60 min at 37°C. Premixture was then applied onto Alugram SIL G/UV254 sheets (Macherey-Nagel, Germany). Radiolabelled substrates and their products were separated by thin layer chromatography (TLC) and quantified by scintillation β-counting.

Plasma adenosine measurement

Plasma adenosine concentration was measured by reversed phase HPLC as previously described[20]. Briefly, 500 μl plasma was used to isolate adenosine by sequentially adding 0.6 M cold perchloric acid, 3 M $KHCO_3$/3.6 N KOH, 1.8 M ammonium dihydrogen phosphate (pH 5.1) and phosphoric acid (30%). Next, the sample was centrifuged at 20,000 × g for 5 min and the supernatant was transferred to a new tube for reversed phase HPLC analysis. Adenosine content was normalized to volume and expressed as a concentration.

Measurement of P50 in lowland volunteers

Arterial bloods of high altitude human subjects were collected and used for P50 measurement. Blood PaO_2 and pH were measured by blood gas analyzer, and SaO_2 was measured by Co-Oximeter. The P50 was then calculated by using a Hill constant of 2.7 and the standard equation as described before[26].

Statistics

All data are expressed as the mean ± SEM. Data were analyzed for statistical significance by using GraphPad Prism 5 software. Nonparametric equivalent Wilcoxon Rank-Sum tests were

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applied in two-group analysis. Differences between the means of multiple groups were compared by one-way ANOVA, followed by a Tukey's multiple comparisons test. Comparison of the data obtained at different time points was analyzed by two-way repeated-measures, followed by the Tukey post hoc test. P value < 0.05 was considered significant.

Results

Metabolomic screening reveals that high altitude hypoxia activates the Rapoport-Luebering Shunt in erythrocytes resulting in increased production of 2,3-BPG and elevated oxygen release from hemoglobin

To identify metabolic alterations in erythrocytes in response to hypoxia, we conducted nonbiased high throughput metabolomic profiling of erythrocytes from humans at SL and at high altitude. Specifically, we recruited 21 young healthy lowland volunteers who were examined at SL and within two hours of arrival at an altitude of approximately 5260 meters (ALT1) and on day 16 at high altitude (ALT16) (Figure 1A). Overall, 233 metabolites were confidently identified and relatively quantified out of over 9000 detected features (mass to charge ratios) in erythrocytes under SL conditions and at high altitude (Supplementary Table 1).

Analysis of these metabolic changes revealed that the erythrocyte-specific Rapoport-Luebering shunt, occurring at a branch point in the pathway of glycolysis for production of 2,3-BPG from 1,3-bisphoglycerate (1,3-BPG) (Figure 1B), was rapidly activated by high-altitude hypoxia. Specifically, levels of BPG and its upstream glycolytic intermediate, glyceraldehyde-3-phosphate (G3P), were increased at ALT1 and were further elevated at ALT16 (Figure 1C and D). In contrast, the levels of the erythrocyte monophosphoglycerates (3-PG and 2-PG) and phospoenolpyruvate (PEP), the three immediate downstream products of 2,3-BPG, were significantly reduced at ALT1 and further reduced at ALT16 compared to SL (Figure 1E and F). To validate our metabolomic screening, we quantified erythrocyte 2,3-BPG concentrations in human samples. Consistently, we found that the erythrocyte 2,3-BPG concentration was significantly induced at ALT1 and further elevated at ALT16 (Figure 1G). Thus, our results indicate that in response to high altitude hypoxia the Rapoport-Luebering Shunt is activated resulting in a significant portion of G3P being utilized to produce 2,3-BPG rather than directly being metabolized to the glycolytic intermediate, 3-PG (Figure 1B).

Because 2,3-BPG is an allosteric modulator that decreases Hb-O_2 binding affinity and thereby promotes O_2 release[14], it is possible that hypoxia-induced 2,3-BPG production in erythrocytes triggers O_2 release. To test this possibility, we quantified the O_2 release capacity of erythrocytes by measuring P50, the partial pressure of O_2 required to achieve 50% Hb-O_2 saturation, from the human subjects at high altitude. We found that erythrocyte P50 was significantly induced at ALT16 compared to ALT1, reflecting reduced Hb-O_2 binding affinity and enhanced erythrocyte O_2 release capacity at high altitude (Figure 1H). No significant differences were noted between male and female subjects (Supplementary Figure 1A and B). These findings provide evidence that high altitude hypoxia coordinately induces erythrocyte 2,3-BPG production and P50 levels in humans adapting to high altitude (Figure 1B).

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Extracellular adenosine concentrations and soluble CD73 activity increase in humans at high altitude

Specific factors and signaling pathways involved in 2,3-BPG induction and subsequent O_2 release from erythrocytes under high altitude have not been previously identified. Recent studies show that increased circulating adenosine is involved in 2,3-BPG induction in erythrocytes of individuals with sickle cell disease (SCD)[20]. However, whether adenosine signaling contributes to normal erythrocyte 2,3-BPG induction and subsequent O_2 release underlying high altitude hypoxia adaptation remains unknown. To test this hypothesis, we used high performance liquid chromatography (HPLC) to measure plasma adenosine levels in the 21 lowland volunteers at SL and following ascent to high altitude. We found that the circulating adenosine levels were elevated at ALT1 compared to SL and were further elevated at ALT16 (Figure 1I). No significant differences in elevated plasma adenosine levels were observed between males and females (Supplementary Figure 1C).

CD73 is an ectoenzyme anchored to the cell surface that plays a key role in the synthesis of extracellular adenosine from AMP[27]. Under certain circumstances, the ectoenzyme can be cleaved from the cell surface and emerge in the circulation as a soluble nucleotidase[28]. Therefore, we measured soluble CD73 (sCD73) activity in normal individuals at SL and at high altitude. sCD73 activity was significantly increased in the human subjects at ALT1 compared to SL and further increased at ALT16 (Figure 1J). There was no significant differences in elevated sCD73 activity between males and females in response to high altitude hypoxia (Supplementary Figure 1D). Taken together, these results show that plasma adenosine concentration and sCD73 activity increase rapidly in response to high altitude hypoxia and that their induction is associated with increased erythrocyte 2,3-BPG concentrations and P50 levels.

Soluble CD73 activity is induced by hypoxia and elevated CD73 is essential for hypoxia-induced plasma adenosine, erythrocyte 2,3-BPG and oxygen release capacity

Although extracellular adenosine is well-known to orchestrate a physiological response to tissue injury[29], nothing is known about the functional role of adenosine signaling in normal erythrocytes under hypoxia. Our human studies presented above raise an intriguing hypothesis that activated CD73-mediated elevated plasma adenosine has a previously unrecognized role in high altitude adaption by regulating 2,3-BPG production and Hb-O_2 binding affinity in erythrocytes. To test this hypothesis, we exposed WT mice and *Cd73*$^{-/-}$ mice to normobaric hypoxia (10% oxygen, similar to that at 5260 m) for one week. Similar to the human high altitude studies, we found that CD73 activity, plasma adenosine, erythrocyte 2,3-BPG and O_2 releasing capacity were significantly increased in WT mice following 1 week of hypoxia compared to normoxia (Figure 2A–D). In contrast, hypoxia-mediated increased plasma adenosine, erythrocyte 2,3-BPG and O_2 releasing capacity were significantly impaired in *Cd73*$^{-/-}$ mice (Figure 2A–D). These results indicate that in mice CD73 activity is induced by hypoxia, as seen in human volunteers at high altitude, and that CD73 is required for hypoxia-induced plasma adenosine production and elevated erythrocyte 2,3-BPG concentration and O_2 release capacity.

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CD73-mediated elevated plasma adenosine prevents tissue hypoxia, inflammation and lung injury in mice

To assess the functional importance of CD73-mediated increased plasma adenosine in tissue response to hypoxia, we measured tissue hypoxia levels using an immunohistochemical (IHC) analysis with hypoxyprobe in WT mice and C*d73*$^{-/-}$ mice under normoxia and following one week of hypoxia as described above. No hypoxia signals were seen in tissue sections of C*d73*$^{-/-}$ or WT mice under normoxia (Figure 2E). However, under hypoxia, hypoxyprobe revealed that multiple organs including kidneys, lungs and hearts displayed a mild hypoxia signal in WT mice (Figure 2E). In contrast, 1 week of 10% O_2 led to severe hypoxia in various organs including kidneys, lungs and hearts of C*d73*$^{-/-}$ mice (Figure 2E). Image quantification analysis demonstrated that the intensity of hypoxyprobe in the kidneys, lungs and hearts was significantly elevated in C*d73*$^{-/-}$ mice compared to WT mice (Supplementary Figure 2B–D).

Besides analyzing tissue hypoxia, we further quantified neutrophils in multiple tissues since it was reported that hypoxia rapidly induces infiltration of neutrophils to tissue[30, 31]. Specifically, we measured the activity of myeloperoxidase (MPO), a specific enzyme expressed in neutrophils, in multiple tissues under normoxia and hypoxia in WT and C*d73*$^{-/-}$ mice. Similar to tissue hypoxia staining (Figure 2E), we found that MPO activity was mildly induced by hypoxia in multiple tissues of WT mice, and was significantly induced further by hypoxia in multiple tissues of C*d73*$^{-/-}$ mice compared to WT mice (Figure 2F). Thus, we conclude that CD73-mediated elevation of plasma adenosine is beneficial to prevent tissue hypoxia and inflammation.

Hypoxia is known to induce lung injury including pulmonary vascular leakage and inflammation[19]. Thus, we collected bronchoalveolar lavage fluid (BALF) of WT and C*d73*$^{-/-}$ mice for evaluation of pulmonary damage and inflammation. Specifically, we quantified cell count, albumin concentration, and interleukin 6 (IL-6) concentration in BALF of WT and C*d73*$^{-/-}$ mice under normoxia and hypoxia. Consistent with our tissue hypoxia staining and MPO activity measurements in the lung (Figure 2E–F), cell count, albumin and IL-6 concentrations in BALF were slightly induced by hypoxia in WT mice and their levels were significantly further increased in C*d73*$^{-/-}$ mice (Figure 2G–I). Thus, these results show that CD73-mediated elevation of plasma adenosine plays a beneficial role in preventing hypoxia-induced lung damage and inflammation in mice.

ADORA2B underlies hypoxia-induced 2,3-BPG production and oxygen release capacity in *in vitro* cultured mouse erythrocytes

It is known that increased extracellular adenosine elicits physiological or pathological functions by engaging its four adenosine receptors including ADORA1, ADORA2A, ADORA2B, and ADORA3[32]. Each adenosine receptor has a distinct cellular or tissue distribution and affinity for adenosine[33]. To identify which adenosine receptor is responsible for hypoxia-induced 2,3-BPG production and O_2 release capacity in erythrocytes, we conducted genetic studies in four adenosine receptor-deficient mice. We found that hypoxia significantly induced 2,3-BPG production and O_2 release capacity in cultured erythrocytes isolated from WT mice, *Adora1*-deficient mice, *Adora2a*-deficient mice and *Adora3*-

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deficient mice. However, hypoxia-mediated effects were absent in cultured erythrocytes isolated from *Adora2b*-deficient mice (Figure 3A and B), indicating that hypoxia-induced erythrocyte 2,3-BPG production and O_2 release capacity is dependent on ADORA2B.

Genetic deletion of erythrocyte ADORA2B attenuates hypoxia-induced 2,3-BPG production and oxygen release capacity *in vivo*

To precisely determine the importance of erythrocyte ADORA2B-mediated elevated 2,3-BPG production and O_2 release in hypoxia, we generated mice with erythrocyte lineage-specific ablation of *Adora2b* genes by mating mice with floxed *Adora2b* alleles ($Adora2b^{f/f}$) with mice containing a transgene expressing Cre recombinase only in the erythroid lineage ($EpoR\text{-}Cre^{+}$) (Supplementary Figure 3A). First, we validated the specificity and efficiency of ablation of *Adora2b* in erythroid cells by EpoR-Cre using bone marrow (BM) cells isolated from $Epo\text{-}Cre^{+}$, $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ and $Adora2b^{-/-}$ mice. The BM-derived hematopoietic progenitor cells (HPCs) were enriched by negative selection, followed by induction to erythroid progenitor cells in erythroid-differentiation medium containing erythropoietin (Supplementary Figure 3B). After two days, we found that over 96% of harvested HPCs isolated and enriched from $Epo\text{-}Cre^{+}$, $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ and $Adora2b^{-/-}$ mice were differentiated into $CD71^{+}$ erythroid progenitor cells, which is consistent with a previous publication (Supplementary Figure 3C)[34]. Moreover, qRT-PCR analysis showed that erythrocyte lineage *Adora2b* mRNA levels of $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ were reduced to levels similar to that of $Adora2b^{-/-}$ mice, whereas pulmonary *Adora2b* mRNA levels in $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ were still similar to control mice ($EpoR\text{-}Cre^{+}$) (Supplementary Figure 3D and E). These results demonstrated that we had successfully generated mice with erythrocyte lineage specific deletion of *Adora2b.*

To test the functional role of erythrocyte ADORA2B signaling in response to hypoxia, we exposed *EpoR-Cre* mice and $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ mice to normoxia or hypoxia (10% O_2) for one week. The levels of plasma adenosine, erythrocyte 2,3-BPG and P50 were similar in $EpoR\text{-}Cre^{+}$ mice and $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ mice under normoxia (Figure 3C–E). Following one week of hypoxia exposure, plasma adenosine concentrations increased to similar levels in $EpoR\text{-}Cre^{+}$ mice and $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ mice (Figure 3C). However, the hypoxia-induced erythrocyte 2,3-BPG production and P50 levels observed in the $EpoR\text{-}Cre^{+}$ mice were significantly attenuated in $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ mice (Figure 3D and E). Thus, our studies provide strong genetic evidence that mouse erythrocyte ADORA2B is essential for elevated adenosine-mediated induction of erythrocyte 2,3-BPG and O_2 releasing capacity under hypoxia.

Erythrocyte ADORA2B is essential to protect against tissue hypoxia, inflammation and lung damage

To further determine if erythrocyte ADORA2B signaling is tissue protective under hypoxia, we assessed tissue hypoxia by hypoxyprobe in $EpoR\text{-}Cre^{+}$ mice and $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ mice following one week of hypoxia (10% O_2). We found that hypoxia treatment led to severe tissue hypoxia in various organs including kidneys, lungs and hearts in $Adora2b^{f/f}/EpoR\text{-}Cre^{+}$ mice, while only a mild hypoxia signal was present in those tissues of $EpoR\text{-}Cre^{+}$ mice (Figure 3F). Image quantification analysis demonstrated that the intensity of

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hypoxyprobe signals in kidneys, lungs and hearts were significantly elevated in *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice compared to *EpoR-Cre*$^{+}$ (Supplementary Figure 4B–D) following hypoxia challenge. We also observed greater elevation of MPO activity in kidney, lung and heart of *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice compared to *EpoR-Cre*$^{+}$ mice after hypoxia exposure (Figure 3F). Furthermore, greater lung injury reflected by increased cell counts, albumin and IL-6 concentrations in BALF were detected in *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice after hypoxia challenge (Figure 3H–J). These findings demonstrate that erythrocyte ADORA2B is essential for tissue protection during hypoxia exposure.

AMPK functions downstream of erythrocyte ADORA2B in mice and underlies hypoxia-induced 2,3-BPG production by phosphorylation and activation of 2,3-BPG mutase

Erythrocyte 2,3-BPG is synthesized from the glycolytic intermediate 1,3-BPG by 2,3-BPG mutase (Figure 1B). Factors regulating 2,3-BPG production are poorly understood. A previous report identified that 2,3-BPG mutase is one of the substrates of AMPK in erythrocytes, and follow-up studies validated that purified AMPK directly phosphorylates purified 2,3-BPG mutase in a cell free system[35]. Additional studies showed that ADORA2B signaling activates AMPK in other cellular systems[36]. Like adenosine, AMPK plays an essential role in multiple cellular functions especially under conditions of energy depletion or limited O_2 availability[37]. However, whether AMPK functions downstream of ADORA2B as a key enzyme responsible for hypoxia-induced 2,3-BPG production in erythrocytes has not been previously studied. To test this possibility, we exposed *EpoR-Cre* mice and *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice to hypoxia (10% oxygen) for different times. We found that erythrocyte *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG concentration and P50 level were significantly induced in *EpoR-Cre* mice under hypoxia in a time-dependent manner. However, hypoxia-induced time-dependent elevation of *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG and P50 were significantly attenuated in *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice (Figure 4A–D). Next, we conducted experiments to determine whether hypoxia activated AMPK interacts with and phosphorylates 2,3-BPG mutase in erythrocytes. Specifically, we assessed AMPK activation by monitoring its phosphorylation at T172 of the catalytic α subunit in erythrocytes. We found that basal erythrocyte *p*-AMPKα at T172 levels were similar in *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice and *EpoR-Cre*$^{+}$ mice under normoxia (Figure 4E), while *p*-AMPKα at T172 was significantly induced in erythrocytes of *EpoR-Cre*$^{+}$ mice following 1 week of hypoxia (10% O_2). In contrast, hypoxia-induced *p*-AMPK α at T172 was significantly attenuated in the erythrocytes of *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice (Figure 4E). Then, using an antibody that recognizes *p*-AMPK substrates we showed that *p*-AMPK specifically phosphorylates 2,3-BPG mutase and that under normoxia the levels of AMPK-phosphorylated 2,3-BPG mutase were similar in A*dora2b*$^{f/f}$/E*poR-Cre*$^{+}$ mice and *EpoR-Cre*$^{+}$ mice (Figure 4F). Subsequently, we found that hypoxia significantly induced *p*-AMPK-phosphorylated 2,3-BPG mutase in erythrocytes of *EpoR-Cre*$^{+}$ mice and that this phosphorylation was significantly attenuated in A*dora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice (Figure 4F).

Finally, to determine the essential role of AMPK activation in adenosine ADORA2B-mediated induction of 2,3-BPG, P50 and subsequent tissue protection, we treated *Cd73*$^{-/-}$ and *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice with AICAR (5-aminoimidazole-4-carboxamide ribonucleotide), a cell permeable AMPK activator[38]. Specifically, we treated these mice with

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AICAR under hypoxia (10% O_2) for 3 days. Our results (Figure 4G–J) show that AICAR treatment restored erythrocyte AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG and P50 in both *Cd73*$^{-/-}$ and *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice under hypoxia. Thus, our results demonstrate that AICAR can override a genetic block to hypoxia-induced adenosine production (CD73 deficiency) or erythrocyte ADORA2B signaling (erythrocyte-specific ADORA2B deficiency) to stimulate erythrocyte 2,3-BPG production and O_2 release capacity by the pharmacologic activation of AMPK. Taken together, we provide *in vivo* evidence that AMPK is a critical intermediate that functions downstream of erythrocyte ADORA2B underlying hypoxia-induced 2,3-BPG production by phosphorylation and activation of 2,3-BPG mutase.

AMPK activation induces erythrocyte 2,3-BPG mutase activity, 2,3-BPG production and oxygen release capacity in mice under normoxia

Our discovery that erythrocyte AMPK is activated and subsequently phosphorylates 2,3-BPG mutase in response to adenosine ADORA2B signaling suggests that activation of AMPK may be sufficient to induce 2,3-BPG production by activating 2,3-BPG mutase. To test this possibility, we conducted *in vitro* studies in cultured WT mouse erythrocytes with AICAR. Initially, we found that AICAR treatment significantly induced phosphorylation of AMPK and activity of 2,3-BPG mutase, 2,3-BPG levels and P50 in a time-dependent manner (Supplementary Figure 5A–D). To further test whether AICAR-mediated effects in cultured erythrocytes were dependent on AMPK, we utilized Compound C (dorsomorphin), a cell permeable inhibitor of AMPK[38–40]. Our results showed that Compound C co-treatment significantly attenuated AICAR-induced *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG and O_2 releasing capacity in cultured mouse erythrocytes (Supplementary 5A-D). Thus, our *in vitro* studies demonstrated that AMPK activation is sufficient to induce 2,3-BPG production and enhanced O_2 release capacity in mouse erythrocytes.

Next, we extended *in vitro* studies to *in vivo* to test if AICAR treatment in WT mice under normoxia is capable of inducing AMPK, 2,3-BPG mutase activity and 2,3-BPG production. As expected, we found that AICAR treatment directly induced phosphorylation of AMPK, 2,3-BPG mutase activity, 2,3-BPG production and P50 levels in WT mice under normoxia (Supplementary Figure 5 E–H).

In vivo beneficial effects of AMPK activation in hypoxia adaptation in mouse

To determine the *in vivo* effect of AMPK activation in hypoxia adaptation in WT mice, we pre-treated WT mouse with AICAR prior to a hypoxic challenge. For these experiments, a lower O_2 content (8% O_2) was utilized for a shorter period of hypoxia exposure up to 24 hours for evaluating the therapeutic potential of AMPK activation in WT mice. We found that AICAR treatment induced greater elevation of erythrocyte 2,3-BPG and O_2 releasing capacity by further stimulating erythrocyte *p*-AMPK and 2,3-BPG mutase activity in WT mice compared to the saline-treated group under hypoxia (Figure 5A–D). IHC with hypoxyprobe and MPO activity measurement showed that AICAR treatment significantly attenuated tissue hypoxia and inflammation in lung, kidney and heart in WT mice compared to the saline-treated group under hypoxia (Figure 5E–H and Supplementary Figure 6A–C).

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Next, to validate AMPK activation is essential for hypoxia adaptation, we treated WT mice with Compound C under hypoxia. We found that Compound C treatment significantly suppressed hypoxia-induced *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG production and O_2 releasing capacity in WT mouse erythrocytes, thereby resulting in enhanced tissue hypoxia and inflammation in lungs, kidneys and hearts compared to the saline-treated group under hypoxia (Figure 5E–H and Supplementary Figure 6A–C). These studies indicate that erythrocyte *p*-AMPK is critical for enhanced hypoxia adaptation in WT mice by rapidly inducing 2,3-BPG mutase activity, 2,3-BPG and subsequent O_2 release to peripheral tissues.

Erythrocyte *p*-AMPK and 2,3-BPG mutase activity are induced in humans at high altitude, and AMPK activation induces erythrocyte 2,3-BPG mutase activity, 2,3-BPG production and oxygen release in cultured human erythrocytes

To translate our mouse findings to humans, we measured erythrocyte *p*-AMPK by western blot and ELISAs in human subjects at SL and at high altitude on ALT1 and ALT16. We found that erythrocyte *p*-AMPK levels were increased at ALT1 and were further elevated at ALT16 (Figure 6A and B). No significant differences in *p*-AMPK levels were observed between male and female volunteers in response to high altitude (Supplementary Figure 1E). Additionally, we found that erythrocyte 2,3-BPG mutase activity was induced at ALT1 and was further elevated at ALT16 (Figure 6C). Consistently, we observed that levels of *p*-AMPK-phosphorylated 2,3-BPG mutase in erythrocytes were significantly increased at ALT1, and were further enhanced at ALT16 (Figure 6D), implicating AMPK activation as associated with high altitude hypoxia-induced activation of erythrocyte 2,3-BPG in humans.

To translate our mouse findings regarding the significance of AMPK activation in erythrocytes to humans, we conducted a series of *in vitro* studies by culturing normal human erythrocytes. First, we found that AICAR treatment induced *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG and subsequent O_2 release in cultured normal human erythrocytes in a time-dependent manner (Figure 6E–H). To further test whether AICAR-mediated effects in cultured human erythrocytes are dependent on AMPK, we incubated AICAR-treated human normal erythrocytes with or without Compound C. We found that Compound C significantly attenuated AICAR-induced *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG levels and O_2 release capacity in cultured human erythrocytes (Figure 6E–H). Taken together, these data show that high altitude hypoxia induces human erythrocyte *p*-AMPK and 2,3-BPG mutase activity in a time-dependent manner. *In vitro* experiments with cultured human erythrocytes showed that activation of AMPK induces 2,3-BPG mutase activity, 2,3-BPG production and subsequent O_2 release in cultured human erythrocytes. Compound C inhibits AMPK activation and subsequently suppresses the induction of 2,3-BPG mutase activity, 2,3-BPG production and O_2 release in cultured human erythrocytes. Thereby, we provide human evidence that AMPK activation triggers erythrocyte O_2 release by promoting 2,3-BPG mutase activity and 2,3-BPG production.

Discussion

It has been known for more than four decades that when humans ascend to high altitudes the affinity of Hb for O_2 decreases to make more O_2 available to hypoxic peripheral

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tissues[15, 41]. It was also recognized early on that the reduced O_2 affinity was due in part to the increase in erythrocyte 2,3-BPG which functioned as a negative allosteric regulator of Hb-O_2 affinity[14, 42, 43]. The molecular mechanisms accounting for the high altitude-mediated regulation of erythrocyte 2,3-BPG concentration were not understood until we conducted non-biased metabolomic screening of erythrocytes in humans and mouse genetic studies. Both human and mouse studies reported here provide strong evidence that CD73-mediated adenosine signaling via ADORA2B in erythrocytes, plays a beneficial role in preventing hypoxia-induced tissue hypoxia, inflammation and lung damage by inducing 2,3-BPG production and triggering O_2 release in an AMPK-dependent manner (Figure 6I). Erythrocyte 2,3-BPG levels are increased by high altitude hypoxia in normal individuals[15] and in patients with CVD frequently facing hypoxia[17–19]. Here we found that adenosine is induced by high altitude and that our newly identified erythrocyte adenosine signaling cascade promotes O_2 delivery to prevent tissue hypoxia. Thus, our findings are highly significant and highlight multiple new therapeutic avenues to prevent and treat hypoxia-induced tissue injury in both normal individuals at high altitude and CVD patients facing hypoxia.

Although extracellular adenosine is known to be induced under hypoxia, no prior study has reported that plasma adenosine levels are increased at high altitude, and a role for elevated plasma adenosine in high altitude adaptation has never been recognized prior to our study with young healthy human volunteers. Here we show for the first time that plasma adenosine levels were induced at ALT1 and increased further at ALT16. Consistent with the high altitude-dependent increase in plasma adenosine, we also found that the activity of sCD73, likely released from endothelial cells, was also significantly induced by high altitude hypoxia. Moreover, we confirmed early studies showing that erythrocyte 2,3-BPG and O_2 releasing capacity were significantly elevated in humans at high altitude. The elevated plasma adenosine concentration is associated with increased erythrocyte 2,3-BPG concentration and O_2 releasing capacity. Similar to humans at high altitude, we found that sCD73 activity, plasma adenosine levels, erythrocyte 2,3-BPG concentration and O_2 release capacity were significantly elevated in WT mice under hypoxia (10% O_2) for 1 week. However, genetic deletion of CD73 significantly reduced hypoxia-induced plasma adenosine, erythrocyte 2,3-BPG and O_2 releasing capacity in mice. The decreased O_2 availability to peripheral tissues resulted in multiple tissue hypoxia, enhanced inflammation and pulmonary damage, which is consistent with early studies showing that genetic deletion of CD73 leads to vascular leakage and increased immune cell infiltration in the lungs under hypoxia.[27, 44] Erythrocyte specific ADORA2B knockouts allowed us to demonstrate for the first time that ADORA2B is essential for elevated plasma adenosine induced-2,3-BPG production and O_2 releasing capacity from erythrocytes. The ablation of erythrocyte ADORA2B in mice results in severe tissue hypoxia, inflammation and lung injury, similar to CD73-deficient mice. Thus, we have determined that elevated plasma adenosine signaling via erythrocyte ADORA2B plays an important role in preventing multiple tissue hypoxia and injury by inducing erythrocyte 2,3-BPG concentration and triggering O_2 release to local tissues. The discovery of this adenosine erythrocyte activated signaling pathway reveals new dimensions to the importance of adenosine signaling in adaptation to hypoxia and

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immediately suggest novel therapeutics to counteract tissue hypoxia in normal individuals at high altitude and CVD patients frequently suffering from hypoxia.

AMPK is a well-known metabolic stress-sensing kinase, which plays an essential role in regulating cellular energy metabolism.[45, 46] In erythrocytes, AMPK plays critical roles in regulating oxidative stress and maintaining the integrity and life span of the cell[47–50]. A previous study used a proteomics approach to identify 2,3-BPG-mutase as a potential AMPK target in erythrocytes[35]. Our current work revealed that AMPK, functioning downstream of ADORA2B, phosphorylates and activates 2,3-BPG-mutase resulting in increased 2,3-BPG production, and elevated O_2 release from erythrocytes. Moreover, we showed that AICAR induced phosphorylation of AMPK and the activation of 2,3-BPG mutase, 2,3-BPG production and O_2 release capacity in cultured WT mouse erythrocytes. In contrast, the AMPK inhibitor, Compound C, significantly attenuated the effects of AICAR in cultured WT mouse erythrocytes. Because of the importance of AMPK in the induction of 2,3-BPG production in erythrocytes and broad usage of AICAR as an AMPK activator previously[18], we conducted preclinical studies and found that pretreatment with AICAR induced erythrocyte 2,3-BPG concentration and O_2 availability to local tissue both in *Cd73*$^{-/-}$ mice and *Adora2b*$^{f/f}$*/EpoR-Cre*$^{+}$ mice under hypoxia. Furthermore, we demonstrated that AICAR enhanced hypoxia adaptation by rapidly inducing erythrocyte AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG levels, and triggering O_2 release to local tissue in WT mice. Similar to mouse findings, we showed that *p*-AMPK and 2,3-BPG mutase activity were significantly elevated in erythrocytes of humans facing high altitude hypoxia. The significance of our mouse finding is further supported by human translational studies showing that AICAR-induced *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG production and O_2 releasing capacity in cultured normal human erythrocytes. In contrast, Compound C attenuates AICAR-induced *p*-AMPK, 2,3-BPG mutase activation, 2,3-BPG production and O_2 releasing capacity in cultured normal human erythrocytes. Thus, preclinical studies in mice and translational studies with human volunteers provide a strong foundation for future clinical trials in humans to test the ability of AMPK activators to increase O_2 availability in humans adapting to high altitude or facing other hypoxia challenges (Figure 6I).

In conclusion, we have identified a functional role of CD73-dependent elevated extracellular adenosine signaling in O_2 release from Hb in erythrocytes in response to hypoxia. This discovery has revealed a previously unrecognized role of adenosine signaling in erythrocyte physiology and provides a novel molecular mechanism underlying adaptation to hypoxia. Moreover, our finding that AMPK, functioning downstream of ADORA2B in erythrocytes, activates 2,3-BPG mutase, subsequently induces 2,3-BPG production and O_2 releasing capacity, has important clinical implications. Overall, our current studies have added significant insight to the molecular mechanisms underlying human adaptation to hypoxia to counteract tissue hypoxia, inflammation and injury and thereby have highlighted novel therapeutic possibilities for prevention and treatment of hypoxia-related conditions and diseases.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Perspective

What is new?

- Plasma adenosine concentrations and sCD73 activity are rapidly increased at high altitude and are associated with elevated erythrocyte 2,3-BPG levels and O_2 releasing capacity.
- Elevated CD73 contributes to hypoxia-induced adenosine and that elevated adenosine-mediated erythrocyte ADORA2B activation is beneficial by inducing 2,3-BPG production and triggering O_2 release to prevent multiple tissue hypoxia, inflammation and lung vascular leakage.
- Erythrocyte AMP-activated protein kinase (AMPK) is activated in humans at high altitude.
- AMPK functions downstream of ADORA2B to phosphorylate and activate BPG mutase and in this way induces 2,3-BPG production and O_2 release from erythrocytes.

What are the clinical implications?

- ***Rapidly*** elevated adenosine signaling via ADORA2B coupled with AMPK activation-induced O_2 deleivery is not limited to high altitude hypoxia but it is relevant to many pathological hypoxia conditions including cardiovascular, hemolytic and respiratory diseases.
- Targeting ADOAR2B-AMPK may be beneficial to counteract tissue hypoxia by enhancing O_2 availability in both normal individuals ascending to high altitude and patients with cardiovascular, hemolytic and respiratory diseases constantly facing hypoxia.

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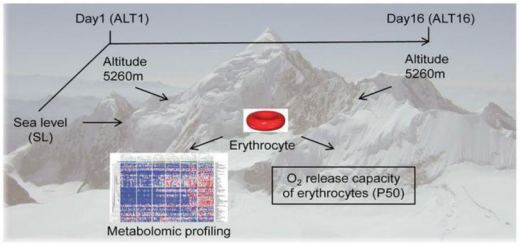
A

	Male	Female
Number	12	9
Age(Years)	21.08±1.24	20.44±1.51
HT(cm)	181.30±4.06	168.48±5.18
WT(kg)	75.29±6.26	62.31±6.43
BMI(kg/m^2)	22.85±1.67	21.88±1.84



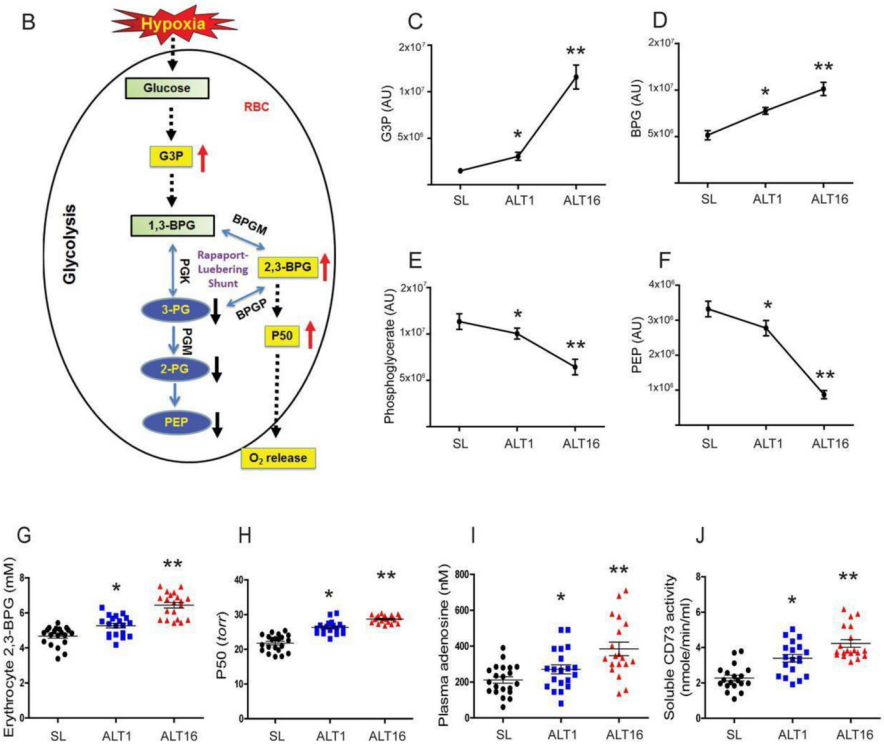


Figure 1. Metabolomic profiling reveals high altitude hypoxia increases erythrocyte specific Rapoport-Luebering Shunt to induce 2,3-BPG production and P50 levels in humans

(**A**) Table of human volunteer characteristics including age, height (HT), weight (WT), body mass index (BMI), and schema for high altitude human studies. (n=21) (**B**) Schematic drawing of erythrocyte-specific Rapoport-Luebering shunt occurring at a branch point in the pathway of anaerobic glycolysis for 2,3-bisphophoglycerate (2,3-BPG) production. (**C–F**) Metabolomic profiling reveals the significant changes in the relative erythrocyte concentration of glyceraldehyde-3-phosphate (G3P) (**C**), bisphosphoglycerate (BPG) (**D**), the monophosphoglycerates, 2-phosphoglycerate (2-PG) and 3-phosphoglycerate (3-PG) (**E**) and phophoenopyruvate (PEP) (**F**) at sea level (SL) and at high altitude on day 1 and day 16. (**G–J**) Erythrocyte 2,3-BPG concentration, P50 level, plasma adenosine concentration and soluble CD73 activity (sCD73) were elevated at high altitude hypoxia on ALT1 and ALT16 over SL. Erythrocyte 2,3-BPG concentration (**G**), P50 level (**H**), plasma adenosine concentration (**I**), and sCD73 (**J**). Data are expressed as mean ± SEM; *P<0.05 vs SL; **P<0.05 vs ALT1. AU (area under the peak)

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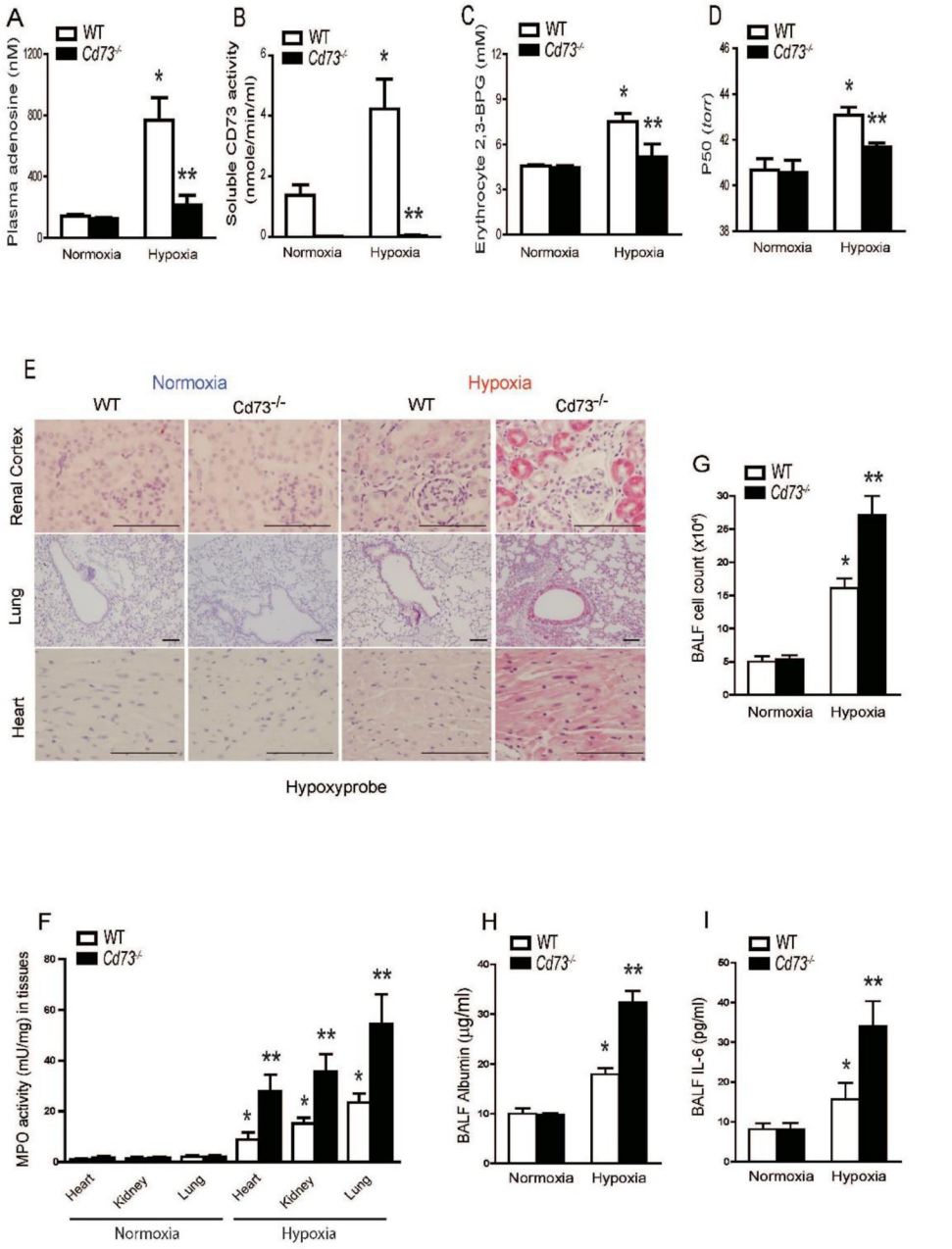

Figure 2. Elevated CD73 is essential for hypoxia-induced plasma adenosine, erythrocyte 2,3-BPG and oxygen release capacity to prevent tissue hypoxia, inflammation and lung damage in mice

(**A–D**) CD73 is essential for hypoxia-induced plasma adenosine, erythrocyte 2,3-BPG concentration and P50 levels in mice. Plasma adenosine concentration (**A**), soluble CD73 activity (**B**), Erythrocyte 2,3-BPG (**C**), and P50 (**D**) in WT mice and *Cd73*$^{-/-}$ mice under normoxia or hypoxia (10% O_2 1 week). Data are expressed as mean ± SEM; *P<0.05 vs WT under normoxia; **P<0.05 vs WT under hypoxia (n=10). (**E–J**) Deletion of C*d73*$^{-/-}$ results in elevated tissue hypoxia and inflammation infiltration in multiple tissues, as well as pulmonary dysfunction under hypoxia (10% O_2 1 week). Immunohistochemical analysis of tissue hypoxia by hypoxyprobe (**E)** and Myeloperoxidase activity (**F**) in kidney, lung and

heart. Bronchoalveolar lavage fluid (BALF) total cell count (**G**), BALF albumin concentration (**H**), BALF interleukin 6 concentration (**I**) in WT mice and *Cd73*$^{-/-}$ mice. Data are expressed as mean ± SEM; *P<0.05 vs WT under normoxia; **P<0.05 vs WT under hypoxia (n=10; Scale bar=200μm).

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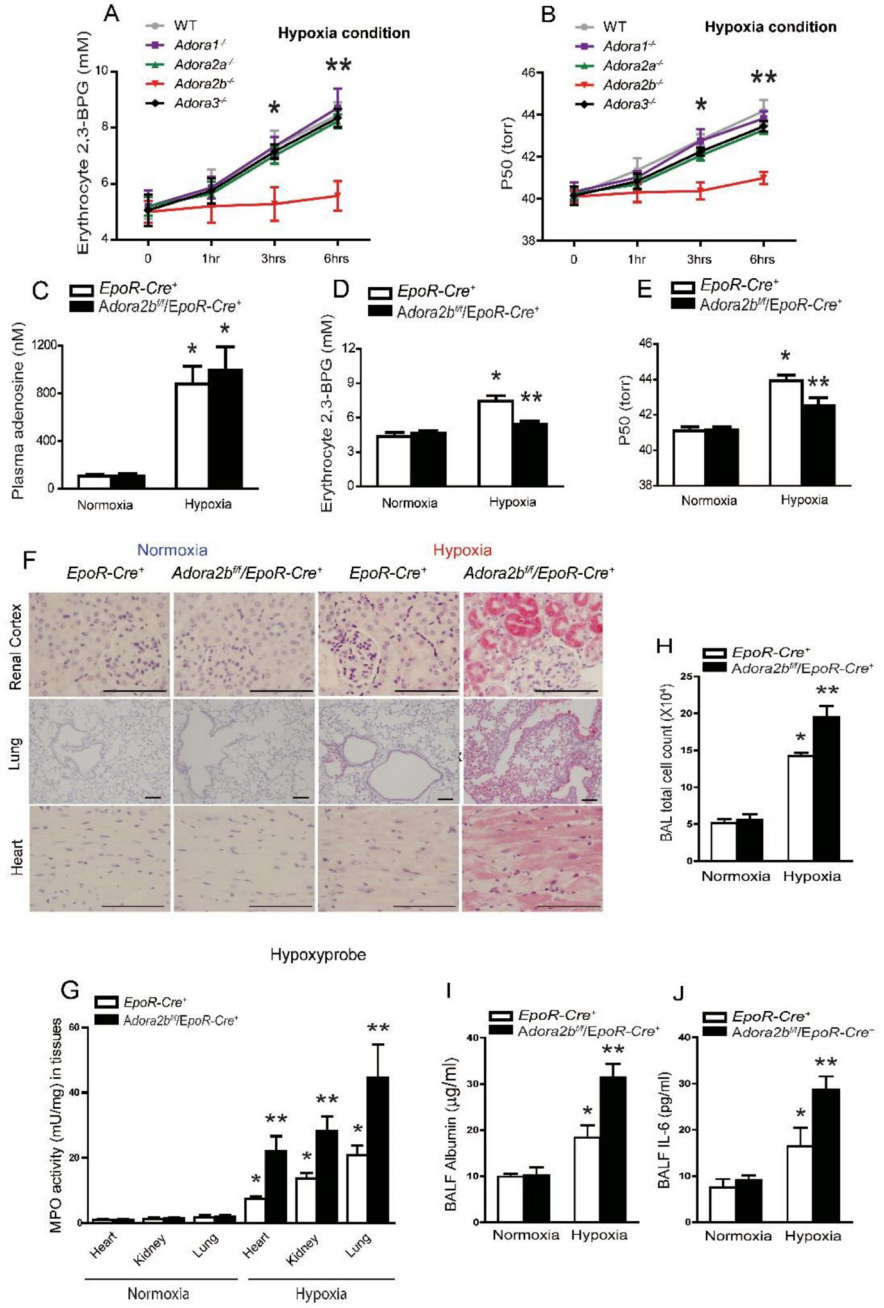

Figure 3. Erythrocyte ADORA2B activation induces erythrocyte 2,3-BPG production and oxygen release capacity to counteract tissue hypoxia, inflammation and lung damage in mice

(**A** and **B**) Hypoxia induces 2,3-BPG production (**A**) and P50 (**B**) levels in cultured WT, *Adora1*$^{-/-}$, *Adora2a*$^{-/-}$, *Adora3*$^{-/-}$ but not *Adora2b*$^{-/-}$ mouse erythrocytes in a time-dependent manner. *P<0.05 for 3 hours hypoxia vs normoxia; **P<0.05 for 6 hours hypoxia vs 3 hours hypoxia (n=8). (**C**–**E**) Erythrocyte ADORA2B contributes to hypoxia-induced 2,3-BPG production and P50 levels. Plasma adenosine (**C**), erythrocyte 2,3-BPG (**D**) and P50 (**E**) of E*poR-Cre*$^{+}$ mice and *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice under normoxia or hypoxia (10% O_2 1 week). Data are expressed as mean ± SEM; *P<0.05 vs E*poR-Cre*$^{+}$ mice under

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normoxia; $^{**}P<0.05$ vs E*poR-Cre*$^{+}$ mice under hypoxia (n=10). Targeted deletion of erythrocyte ADORA2B results in elevated tissue hypoxia and inflammation infiltration in multiple tissues, as well as pulmonary dysfunction under hypoxia (10% O_2, 1 week). Immunohistochemical analysis of tissue hypoxia by hypoxyprobe (**F)** and Myeloperoxidase activity (**G**) in kidney, lung and heart. Bronchoalveolar lavage fluid (BALF) total cell count (**H**), BALF albumin concentration (**I**), BALF interleukin 6 concentration (**J**) in E*poR-Cre*$^{+}$ mice and *Adora2b*$^{f/f}$/*EpoR-Cre*$^{+}$ mice. Data are expressed as mean ± SEM; $^{*}P<0.05$ vs E*poR-Cre*$^{+}$ mice under normoxia; $^{**}P<0.05$ vs E*poR-Cre*$^{+}$ mice under hypoxia (n=10; Scale bar=200μm).

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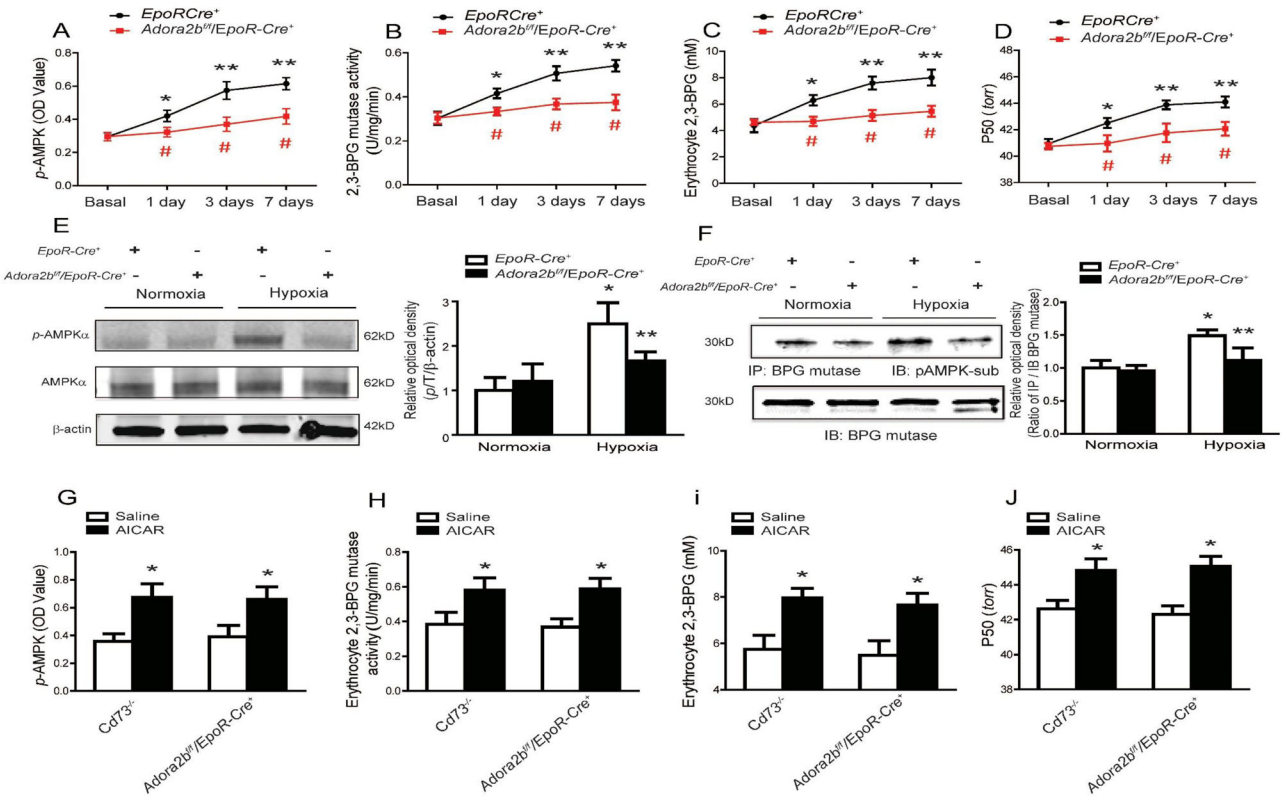

Figure 4. AMPK functions downstream of erythrocyte ADORA2B and underlies hypoxia-induced 2,3-BPG production by phosphorylation and activation of 2,3-BPG mutase in mice

(**A**–**D)** Erythrocyte ADORA2B is essential for hypoxia-induced *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG concentration and P50 levels *in vivo*. Erythrocyte *p*-AMPKα (quantified by ELISA) (**A**), 2,3-BPG mutase activity (**B**), 2,3-BPG concentration (**C**) and P50 (**D**) of *EpoR-Cre*$^{+}$ mice and *Adora2b*$^{f/f}$*/EpoR-Cre*$^{+}$ mice under normoxia or hypoxia (10% O_2, 90% N_2) for 1 day, 3 days, and 7 days. Data are expressed as mean ± SEM; **P*<0.05 vs E*poR-Cre*$^{+}$ mice under normoxia; ***P*<0.05 vs E*poR-Cre*$^{+}$ mice under hypoxia for 1 day; $^{\#}$*P*<0.05 *Adora2b*$^{f/f}$*/EpoR-Cre*$^{+}$ mice vs E*poR-Cre*$^{+}$ mice at the same time point (n=10). (**E**) Representative western blot and relative image quantification analysis of *p*-AMPKα in erythrocytes of *EpoR-Cre*$^{+}$ mice and *Adora2b*$^{f/f}$*/EpoR-Cre*$^{+}$ mice under normoxia or hypoxia (10% O_2, 90% N_2) for 1 week. (**F**) Representative western blot and relative image quantification analysis of *p*-AMPK phosphorylated 2,3-BPG mutase levels in the erythrocyte lysates in *EpoR-Cre*$^{+}$ mice and *Adora2b*$^{f/f}$*/EpoR-Cre*$^{+}$ mice under normoxia or hypoxia (n=3). Data are expressed as mean ± SEM; **P*<0.05 vs E*poR-Cre*$^{+}$ mice under normoxia; ***P*<0.05 vs E*poR-Cre*$^{+}$ mice under hypoxia. (**G**–**J**) AICAR treatment significantly stimulated hypoxia-induced erythrocyte AMPK phosphorylation (**G**), 2,3-BPG mutase activity (**H**), 2,3-BPG production (**I**) and P50 levels (**J**) compared to saline-treated group in *Cd73*$^{-/-}$ mice and *Adora2b*$^{f/f}$*/EpoR-Cre*$^{+}$ mice under hypoxia (10% O_2, 90% N_2) for 3 days. Data are expressed as mean ± SEM; **P*<0.05 for AICAR-treated mice vs saline-treated mice (n=8).

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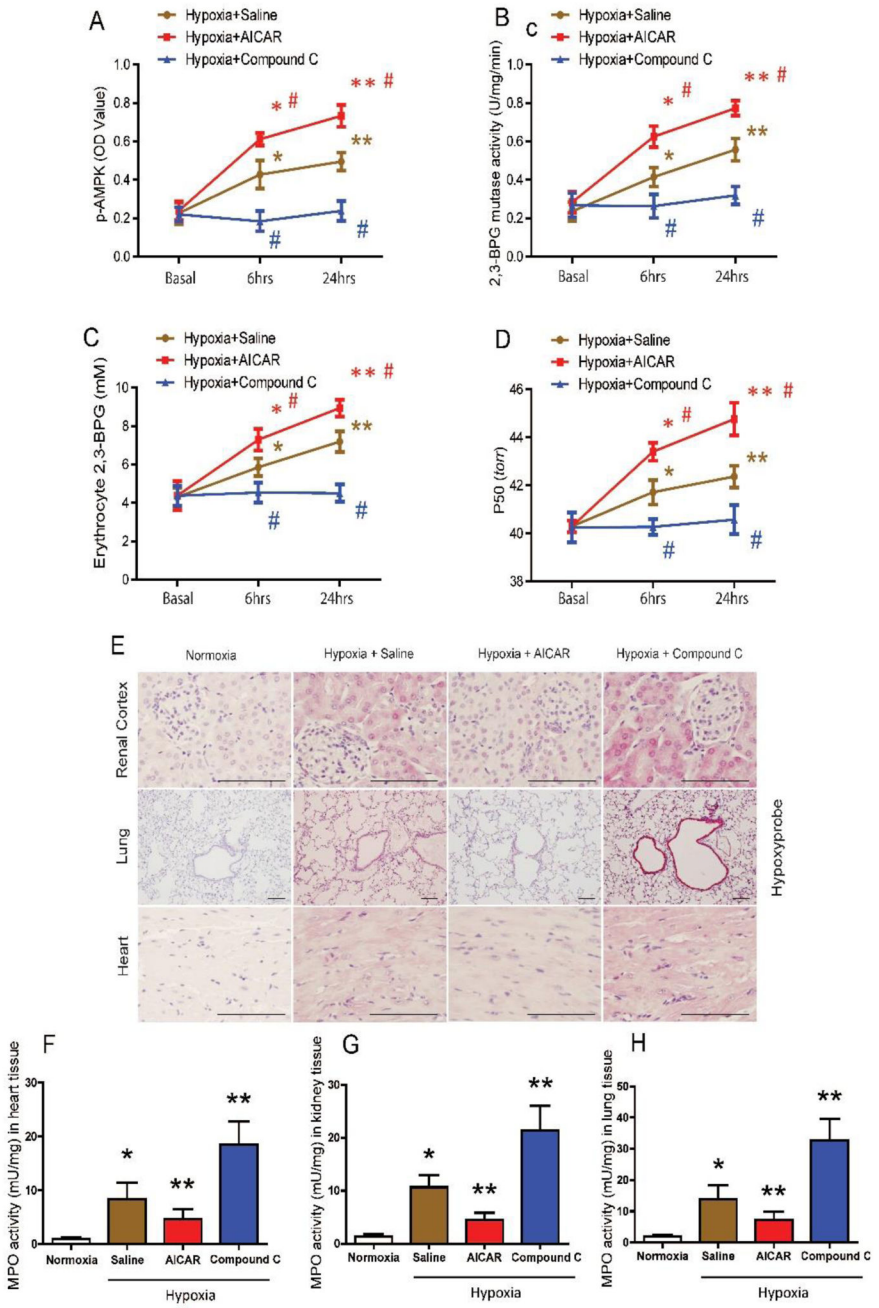

Figure 5. ***In vivo*** **effects of AICAR and Compound C treatment under hypoxia**

(**A**–**D**) AICAR treatment significantly stimulated erythrocyte *p*-AMPK (**A**), 2,3-BPG mutase activity (**B**), 2,3-BPG production (**C**) and P50 levels (**D**) in WT mice compared to saline-treated WT mice under hypoxia (8% O_2) in a time-dependent manner, while Compound C treatment significantly attenuated erythrocyte *p*-AMPK (**A**), 2,3-BPG mutase activity (**B**), 2,3-BPG production (**C**) and P50 levels (**D**) in WT mice compared to saline-treated WT mice under hypoxia (8% O_2). Data are expressed as mean ± SEM; $^{*}P<0.05$ for 6-hour vs basal level, $^{**}P<0.05$ for 24-hour vs 6-hour. $^{\#}P<0.05$ for AICAR-treated group vs saline group, and Compound C-treated group vs saline group at the same time point (n=8). (**E**–**H**)

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AICAR treatment prevented tissue hypoxia and inflammation infiltration, while Compound C treatment aggravated tissue hypoxia and MPO activity in WT mice under hypoxia for 24 hours. IHC analysis of tissue hypoxia by hypoxyprobe in kidney, lung and heart (**E**) (Scale bar=200μm). MPO activity in heart (**F**), kidney (**G**) and lung (**H**). AICAR or Compound C-treated WT mice after 24 hours hypoxia treatment (n=8). Data are expressed as mean ± SEM; **P*<0.05 vs WT mice under normoxia; ***P*<0.05 vs WT mice with saline treatment under hypoxia.

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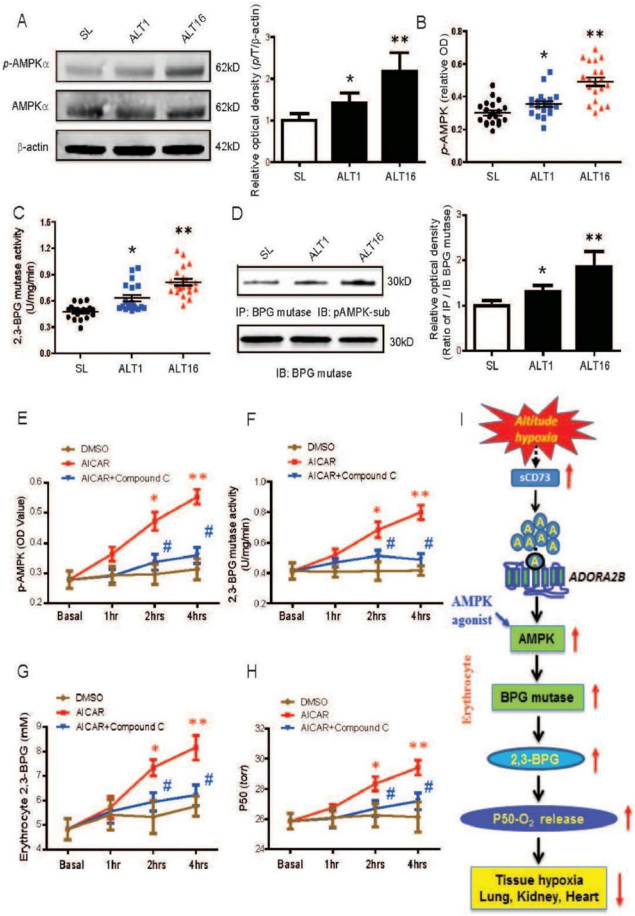

Figure 6. Erythrocyte *p*-AMPK and 2,3-BPG mutase activity are induced in humans at high altitude, and AMPK activation induces erythrocyte 2,3-BPG mutase activity, 2,3-BPG production and oxygen release in cultured human erythrocytes

(**A**) Representative western blot and relative image quantification analysis (n=3 per group) of *p*-AMPKα subunit phosphorylated at threonine 172 (T172), total AMPKα subunit, and β-actin in the erythrocytes of humans at sea level (SL) and high altitude on day 1 and day 16. Erythrocyte *p*-AMPK levels quantified by ELISA (**B**) and 2,3-BPG mutase activity (**C**) at high altitude ALT1 and ALT16 over SL. (**D**) Representative western blot and relative image quantification analysis (n=3 per group) of *p*-AMPK phosphorylated 2,3-BPG mutase at SL and high altitude on day 1 and day 16 in erythrocyte lysates. Data are expressed as mean ± SEM; **P*<0.05 for high altitude on day 1 vs SL; ***P*<0.05 for high altitude on day 16 vs day 1. (**E**–**H**) AMPK activator AICAR-induced phosphorylation of AMPK (**E**), 2,3-BPG mutase activity (**F**), 2,3-BPG concentration (**G**) and P50 levels (**H**) in cultured normal human erythrocytes under normoxia in a time-dependent manner. Compound C significantly attenuated the effect of AICAR treatment. Data are expressed as mean ± SEM; **P*<0.05 for AICAR-treated 2-hour group vs 1-hour group, and for AICAR-treated 2-hour group vs DMSO-treated 2-hour group, ***P*<0.05 for AICAR-treated 4-hour group vs 2-hour group, and for AICAR-treated 4-hour group vs DMSO-treated 4-hour group. #P<0.05 for AICAR plus Compound C vs AICAR at the same time point (n=8). (**I**) Working model: CD73 is essential for high altitude hypoxia-induced plasma adenosine production. Elevated plasma

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adenosine prevents hypoxia-induced tissue inflammation and damage by activating ADORA2B on erythrocytes to induce 2,3-BPG mutase activity, 2,3-BPG production, and subsequently increased P50 and increased O_2 release to peripheral hypoxic tissues. AMPK is a key enzyme that functions downstream of ADORA2B to activate 2,3-BPG mutase, promote 2,3-BPG production and O_2 release from erythrocytes. Thus, enhancing the CD73-ADORA2B-AMPK signaling pathway is a promising therapeutic strategy to treat or prevent hypoxia-induced tissue damage.