Hypoxia Modulates Platelet Purinergic Signalling Pathways

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Keywords
► hypoxia
► platelet physiology
► anti-platelet agents
► ADP receptors
► high altitude

Abstract

Background Hypoxia resulting from ascent to high-altitude or pathological states at sea level is known to increase platelet reactivity. Previous work from our group has suggested that this may be adenosine diphosphate (ADP)-specific. Given the clinical importance of drugs targeting ADP pathways, research into the impact of hypoxia on platelet ADP pathways is highly important.

Methods Optimul aggregometry was performed on plasma from 29 lowland residents ascending to 4,700 m, allowing systematic assessment of platelet reactivity in response to several platelet agonists. Aggregometry was also performed in response to ADP in the presence of inhibitors of the two main ADP receptors, P2Y1 and P2Y12 (MRS2500 and cangrelor, respectively). Phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a key determinant of platelet aggregation, was analysed using the VASPFix assay.

Results Hypobaric hypoxia significantly reduced the ability of a fixed concentration of cangrelor to inhibit ADP-induced aggregation and increased basal VASP phosphorylation. However, in the absence of P2Y receptor inhibitors, we did not find evidence of increased platelet sensitivity to any of the agonists tested and found reduced sensitivity to thrombin receptor-activating peptide-6 amide.

Conclusion Our results provide evidence of increased P2Y1 receptor activity at high altitude and suggest down-regulation of the P2Y12 pathway through increased VASP phosphorylation. These changes in ADP pathway activity are of potential therapeutic significance to high-altitude sojourners and hypoxic sea level patients prescribed platelet inhibitors and warrant further investigation.

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Introduction

Acute hypobaric hypoxia, such as that induced by ascent to high altitude, has long been considered to produce a thrombogenic phenotype.\(^1\) Consistent with this, epidemiological studies report a markedly increased risk of strokes at high altitude: up to 30 times that at sea level.\(^2\) Furthermore, these events are reported to occur in younger patients with fewer cardiovascular risk factors.\(^3\) Although some studies examining the effect of acute hypoxia on coagulation have reported minimal changes in coagulation, they were investigating the ‘economy class syndrome’ and thus exposure to hypoxia was brief.\(^4,5\) On the contrary, studies examining a longer exposure to hypoxia have summarised in – Fig. 1. We chose to perform aggregometry assays on day 6 (one day following ascent to 4,700 m to examine effects of sub-acute hypoxia) and day 11 (the time point we had previously observed changes in Multiplate aggregation with ADP\(^6\)). Venepuncture was performed using 21G needles (Williams Medical, Rhymney, United Kingdom) into citrated tubes. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 175 \(\times\) g for 15 minutes (EBA 280, Hettich, Tuttingen, Germany). Platelet-poor plasma (PPP) was generated by further centrifugation at 3,624 \(\times\) g for 2 minutes. Peripheral oxygen saturation (SpO\(_2\)) was measured using a pulse oximeter (SM-100, Santamedical, Tustin, United States) at baseline and every day of the expedition.

Optimal Aggregometry

Modified optimal aggregometry plates were prepared as previously described by Chan and Warner.\(^6\) In brief, pre-diluted platelet agonists were added to individual wells of a gelatin-coated 96-well plate and the plates were lyophilised. Plates were vacuum-sealed and protected from light before use. Each 96-well plate contained lyophilised concentration ranges of arachidonic acid (0.03–1 mM), ADP (0.005–40 \(\mu\)M),

Methods

Data were collected from 29 participants before and during the APEX 5 Expedition. All participants had no known cardiovascular or respiratory conditions and no pre-existing coagulopathy. Participants were asked to refrain from alcohol and anti-platelet medications during the week prior to sampling. This study was approved by the ACCORD Research Ethics Committee (17-HV-030) and all participants gave informed consent as per the Declaration of Helsinki. Reagents were supplied by Sigma-Aldrich (Irvine, United Kingdom) unless otherwise specified.

Ascent Profile and Sample Collection

Participants were resident at < 250 m above sea level and had not travelled to high altitude (> 2,500 m) in the 2 months prior to the study. The ascent profile and study timeline are summarised in – Fig. 1. We chose to perform aggregometry assays on day 6 (one day following ascent to 4,700 m to examine effects of sub-acute hypoxia) and day 11 (the time point we had previously observed changes in Multiplate aggregation with ADP\(^6\)). Venepuncture was performed using 21G needles (Williams Medical, Rhymney, United Kingdom) into citrated tubes. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 175 \(\times\) g for 15 minutes (EBA 280, Hettich, Tuttingen, Germany). Platelet-poor plasma (PPP) was generated by further centrifugation at 3,624 \(\times\) g for 2 minutes. Peripheral oxygen saturation (SpO\(_2\)) was measured using a pulse oximeter (SM-100, Santamedical, Tustin, United States) at baseline and every day of the expedition.

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collagen (0.01–40 µg/mL), epinephrine (0.0004–10 µM), TRAP-6 amide (0.03–40 µM), and U46619 (thromboxane mimetic, 0.005–40µM).

PRP was aliquoted and incubated for 30 minutes (at 37°C) alone or in the presence of the P2Y1 inhibitor MRS2500 (1 µM, Tocris Bioscience, Bristol, United Kingdom) or the P2Y12 inhibitor cangrelor (100 nM, The Medicines Company, New Jersey, United States). After incubation, 40 µL of PRP (plus or minus inhibitor) was quickly added to wells containing the lyophilised platelet agonists. PPP was added to four agonist-free wells to provide a signal equivalent to 100% aggregation. PRP was also added to agonist-free wells and wells containing vehicle alone as a 0% aggregation control. Plates were then placed on a thermal shaker (BioShake iQ, QInstruments, Jena, Germany) for 5 minutes (37°C, 1,200 revolutions per minute). Light absorbance was read at 595 nm in a 96-well plate reader (SPECTROstar Nano, BMG LABTECH, Aylesbury, United Kingdom).

Percentage aggregation was calculated using absorbance values for PPP (100%) and PRP (0%) in agonist-free wells as reference values. A visual inspection of concentration–response curves was conducted to remove any clearly erroneous curves (deviating from a sigmoid shape). Data were also removed if they showed no response, with response defined as greater than 30% aggregation in response to two doses of agonist.

**Vasodilator-Stimulated Phosphoprotein Phosphorylation**

Vasodilator-stimulated phosphoprotein (VASP) is a platelet protein whose phosphorylation is modulated by cyclic adenosine monophosphate (cAMP).\(^{17}\) cAMP production is inhibited by the action of cGMP on adenylyl cyclase secondary to activation of P2Y12 receptors.\(^{18}\) The degree of VASP phosphorylation can thus be used as a marker of P2Y12 activity.\(^{19}\) The VASPFix assay\(^{20}\) was used to quantify VASP phosphorylation.

Aliquots of PRP were incubated for 6 minutes in one of three conditions: phosphate-buffered saline (PBS), a prostacyclin analogue iloprost (1 nM), or ADP (5 µM) + iloprost (1 nM) and mixed in a 1:5 ratio with VASPFix (Platelet Solutions Ltd., Nottingham, United Kingdom), vortexed, and snap frozen on dry ice. Samples from high altitude were transported to the United Kingdom for analysis on dry ice by a specialist company.

Flow cytometry was performed and the median fluorescence (mF) recorded for each condition. Iloprost induces maximal VASP phosphorylation with PBS acting as a negative control.

If iloprost did not induce phosphorylation (i.e., if mf (iloprost) < mf (saline)), it was assumed that there was a technical failure and these samples removed from the analysis. Similarly, if mf (iloprost + ADP) was greater than mf (iloprost), the sample was also removed from analysis.

**Full Blood Count**

Three millilitres of blood were collected into an ethylenediaminetetraacetic acid blood tube (Sarstedt Ltd., Leicester, United Kingdom) and samples analysed within 24 hours by clinical haematology laboratories (NHS Lothian Laboratories, Edinburgh, United Kingdom, and SELADIS, Universidad Mayor de San Andrés, La Paz, Bolivia).

**Statistics**

Optimul aggregometry data were fit to Eq. (1) by least squares, non-linear regression using the scipy.optimize library (for Python 2.7.2). Eq. (1): Agonist-response equation where y is the percentage aggregation, x is log[agonist], \(EC_{50}\) the concentration resulting in half maximal aggregation, Top/Bottom the maximum/minimum aggregation and the Hill Slope the steepness of the curve. For studies examining the dose–response curve of cangrelor, \(IC_{50}\) replaces \(EC_{50}\).

\[
y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{(\log EC_{50} - x) / \text{Hill Slope}}}
\]

The concentration resulting in half-maximal aggregation (\(EC_{50}\)) was used to compare curves from each time point. For comparisons between ADP concentration–response curves in the presence of a fixed antagonist, maximum response (\(R_{\text{max}}\)) to ADP was also calculated.

Outlier identification was based on difference of fits analysis.\(^{21}\) The effect of each point on \(EC_{50}\) (\(\Delta EC_{50}\)) and Hill Slope (\(\Delta \text{Hill}\)) was calculated by iterative removal of points and refitting the curves. \(\Delta EC_{50}\) and \(\Delta \text{Hill}\) values were reviewed for comparable data (all points in all concentration–response curves for one agonist at one time point) and any points falling out with two standard deviations (\(\Delta EC_{50}\)) or four standard deviations (\(\Delta \text{Hill}\)) were considered outliers. Additionally, for concentration–response curves in the presence of inhibitors, if the final point was less than 75% of the point preceding it, it too was considered an outlier, to ensure validity of the \(R_{\text{max}}\) parameter.

Wherever possible, paired statistics were used; however, the optimul aggregometry data were unpaired. For paired comparisons of two time points, paired Student’s t-tests were performed using the scipy.stats library.\(^{22}\) For paired data compared over three time points (SpO2), a one-way repeated-measure analysis of variance (ANOVA) was conducted with a Tukey’s HSD post hoc test using Prism 5.0 (GraphPad, La Jolla, California, United States). For optimul aggregometry data, comparisons between three time points were analysed using one-way ANOVA with Tukey’s HSD post hoc test using R 3.4.2.\(^{23}\) Whenever multiple comparisons were made, between multiple agonists or conditions, p-values reported were adjusted using a Bonferroni correction. Statistical significance was set as 0.05.

**Results**

Baseline characteristics of subjects are summarised in Table 1. All but one subject completed the study, and data have been included until dropout for unpaired analyses. The sojourn at high altitude induced a marked hypoxaemia (Fig. 2A), which was slightly more pronounced on day 11 than day 6. Platelet counts were also significantly elevated by...
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Table 1 Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>APEX 5 cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>9/20</td>
</tr>
<tr>
<td>Mean BMI (range)</td>
<td>22.3 (17.6–28.8)</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>20.7 (18–26)</td>
</tr>
</tbody>
</table>

Note: Body mass index (BMI) has units kg/m², and age is measured in years.

There is a growing body of evidence that hypoxia induces hyper-reactivity in platelets, a phenomenon important to both altitude physiology and sea-level pathophysiology. We found that hypoxia increases maximal aggregation to ADP in the presence of cangrelor and down-regulates platelet sensitivity to TRAP-6 amide. However, we did not demonstrate sensitisation to any of the platelet activators tested. Finally, and more strikingly, we have demonstrated modulation of basal VASP phosphorylation, a key step in the P2Y₁₂ signalling pathway.

Our results contrast with a previous study from our group which reported increased platelet sensitivity to ADP.³⁵ Our previous findings were based on Mutiple analysis and discrepancies could be related to the impact of platelet count on the two different assays: Multiple is more sensitive to platelet count than optimul aggregometry.²⁴,²⁵ This, however, would not explain why our previous findings were specific to ADP. Perhaps more likely is that differences relate to the parameter of platelet function that was tested. Mutiple is based on electrical-impedance aggregometry measured in real-time over 6 minutes, with the area under the impedance time curve as the final outcome.²⁶ This outcome is therefore dependent on both the final amplitude of the response and the rate at which this is reached. Optimul aggregometry, however, is based on one measurement at 5 minutes. Conceivably, differences in the ADP sensitivity could be time dependent, given the time sensitivity of phosphorylation²⁷ and changes in purinergic receptor expression²⁸ induced by ADP stimulation.

While we have not resolved the uncertainty surrounding ADP sensitivity, our study did reveal interesting changes in purinergic signalling. The $R_{\text{max}}$ induced by ADP in the presence of 100 nM cangrelor was significantly higher at altitude (Figs. 4B, Table 2). This could be due to increased P2Y₁ receptor activity. An increase in P2Y₁ activity may imply a difference in receptor expression or modulation of receptor activity since G-protein-coupled receptor activity can be regulated by multiple mechanisms.²⁹ Increased P2Y₁ activity would provide an interesting link to the proteomic data of Tyagi et al who described increased calpain expression and activity in rats exposed to hypoxia for 6 hours,³¹ since a downstream consequence of P2Y₁ activation is increased intracellular calcium.³⁰

Well-controlled, high-altitude expeditions are excellent models of acute hypoxia³² and their findings are often relevant to hypoxic patients at sea level. In this regard, patients with chronic hypoxic diseases such as chronic obstructive pulmonary disease and obstructive sleep apnoea (OSA) have an increased risk of thrombotic events such as myocardial infarction and stroke.³²,³³ Furthermore, these conditions are also associated with increased platelet reactivity, which in the case of OSA is reversed on correction of hypoxia.³⁴–³⁶ However, the
### Table 2
Summary results of optimul aggregometry data

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day 6</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EC$_{50}$ values</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>$-3.695 (0.053)$</td>
<td>$-3.696 (0.039)$</td>
<td>$-3.715 (0.064)$</td>
</tr>
<tr>
<td>ADP</td>
<td>$-5.990 (0.096)$</td>
<td>$-6.037 (0.122)$</td>
<td>$-5.912 (0.078)$</td>
</tr>
<tr>
<td>Collagen</td>
<td>$-6.776 (0.111)$</td>
<td>$-7.009 (0.107)$</td>
<td>$-6.853 (0.088)$</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>$-6.339 (0.194)$</td>
<td>$-6.805 (0.144)$</td>
<td>$-6.584 (0.140)$</td>
</tr>
<tr>
<td>TRAP-6 amide</td>
<td>$-6.314 (0.105)$</td>
<td>$-6.221 (0.064)$</td>
<td>$-5.759 (0.084)^a$</td>
</tr>
<tr>
<td>U46619</td>
<td>$-6.979 (0.122)$</td>
<td>$-6.652 (0.099)$</td>
<td>$-6.545 (0.122)$</td>
</tr>
<tr>
<td>ADP + MRS2500</td>
<td>$-5.280 (0.082)$</td>
<td>$-5.222 (0.063)$</td>
<td>$-5.244 (0.064)$</td>
</tr>
<tr>
<td>ADP + Cangrelor</td>
<td>$-5.186 (0.062)$</td>
<td>$-5.318 (0.068)$</td>
<td>$-5.335 (0.079)$</td>
</tr>
<tr>
<td><strong>$R_{\text{max}}$ values</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP + MRS2500</td>
<td>95.230 (2.835)</td>
<td>100.413 (2.159)</td>
<td>96.638 (2.788)</td>
</tr>
<tr>
<td>ADP + Cangrelor</td>
<td>80.005 (4.177)</td>
<td>97.927 (2.557)$^a$</td>
<td>97.186 (2.398)$^a$</td>
</tr>
</tbody>
</table>

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; PRP, platelet-rich plasma; TRAP, thrombin receptor-activating peptide.

Note: Dose–response curves of platelet aggregation were performed in response to AA, ADP, collagen, epinephrine, TRAP-6 amide and U46619. Aggregometry was also performed on PRP incubated for 30 minutes with 1 µM MRS2500 (P2Y$_1$ inhibitor) or 100 nM cangrelor (P2Y$_{12}$ inhibitor) with ADP as the agonist. For experiments with inhibitors, the maximal response ($R_{\text{max}}$) was also calculated. EC$_{50}$ and $R_{\text{max}}$ values were compared by one-way analysis of variance (ANOVA) followed by Tukey’s HSD post hoc tests where appropriate. $p$-Values reported were adjusted to account for family-wise error rate using Bonferroni corrections. $^a$ $p < 0.01$ vs. baseline.
clinical relevance of isolated, increased P2Y\textsubscript{1} activity is unclear since overall sensitivity to ADP was unaltered (►Fig. 3B, ▶Table 2). Nonetheless, increased P2Y\textsubscript{1} receptor activity has been postulated to contribute to P2Y\textsubscript{12} inhibitor resistance,\textsuperscript{37,38} although evidence for this is lacking and to our knowledge no study has investigated the impact of hypoxia on P2Y\textsubscript{12} inhibitor resistance.

An alternative explanation for the change in $R_{\text{max}}$ with cangrelor is an increase in P2Y\textsubscript{12} expression at altitude. Interestingly, a recent whole blood microarray study showed a twofold increase in P2Y\textsubscript{12} expression in well acclimatised sojourners at high altitude.\textsuperscript{35} These findings were not validated in isolated platelets or by quantitative polymerase chain reaction. With either an increase in P2Y\textsubscript{1} or P2Y\textsubscript{12} expression, a change in sensitivity to ADP might be expected in the absence of inhibitors. However, as we detected no change in sensitivity to ADP, we propose that our finding of increased basal VASP phosphorylation is a compensatory mechanism that is also activated by hypoxia. Together with a lack of change in maximum phosphorylation induced by iloprost (►Fig. 5B) and a reduction in the percentage increase in phosphorylation induced by iloprost (►Fig. 5C), this suggests that the basal ratio of VASP:VASP-P is altered by hypoxia. Although we did not observe reduced P2Y\textsubscript{12} activity in response to ADP (►Fig. 5A, C), we only studied a single high concentration of ADP (5 μM), a concentration which induces near-maximal aggregation (►Fig. 3B). It may be that lower concentrations of ADP are unable to overcome increased basal VASP phosphorylation. If this is the case, it could represent a compensatory response to increased P2Y\textsubscript{1} receptor activity or P2Y\textsubscript{12} expression, the explanations proposed for the reduction in cangrelor efficacy. VASP-P is regulated by several mediators that are altered in hypoxic conditions. For example, exhaled nitric oxide (NO) levels increased over 48 hours in healthy subjects exposed acutely to 4,559 m,\textsuperscript{30} while raised circulating cyclic guanosine monophosphate (cGMP) levels and NO metabolites (nitrite and nitrate) were reported following gradual ascent to 5,200 m.\textsuperscript{41} NO inhibits platelet aggregation, at least in part via cGMP-mediated VASP phosphorylation as platelet adhesion to injured vessel walls could not be inhibited by NO in VASP-deficient mice.\textsuperscript{42} Interestingly, recent work demonstrated that nitrite led to VASP phosphorylation in isolated platelets in the presence of deoxygenated red blood cells.\textsuperscript{43} Thus, either increased NO synthase or nitrite reductase activity could explain our finding of increased basal VASP phosphorylation at altitude.\textsuperscript{44} ►Fig. 6 summarises the proposed changes to purinergic signalling induced by hypoxia; however, this model will require further investigation to confirm its validity.

Kiouptsì et al examined the effects of brief exposure (30 minutes) of washed human platelets to extreme and moderate hypoxia (1 and 8% oxygen, respectively) on ex vivo expression of activated $\alpha$IIb$\beta$3 (a key integrin in platelet aggregation\textsuperscript{45}) and aggregation. They reported that platelets exposed to extreme hypoxia had reduced expression of activated $\alpha$IIb$\beta$3 in response to ADP stimulation.\textsuperscript{46} Our proposed model of hypoxia-induced alterations to purinergic signalling may offer a mechanistic explanation to these findings since an increase in VASP phosphorylation would attenuate $\alpha$IIb$\beta$3 in response to ADP stimulation.\textsuperscript{46} Our proposed model of hypoxia-induced alterations to purinergic signalling may offer a mechanistic explanation to these findings since an increase in VASP phosphorylation would attenuate $\alpha$IIb$\beta$3 in response to ADP stimulation.\textsuperscript{46} Our proposed model of hypoxia-induced alterations to purinergic signalling may offer a mechanistic explanation to these findings since an increase in VASP phosphorylation would attenuate $\alpha$IIb$\beta$3 in response to ADP stimulation.\textsuperscript{46}
Fig. 6 Proposed model of hypoxia-induced changes to purinergic signalling. Our findings are in red, while those of other studies examining the impact of hypoxia on platelet signalling are in blue. Our data suggest that hypoxia up-regulates the basal level of vasodilator-stimulated phosphoprotein (VASP) phosphorylation, a key determinant of platelet aggregation downstream of the P2Y12 receptor. Other work demonstrating increased nitric oxide (NO) levels at altitude provides a possible mechanism for this finding. Phosphorylation of VASP (VASP-P) inhibits the expression of active αIIbβ3 expression, which links to Koumpti et al’s finding that hypoxic platelets have lower expression of active αIIbβ3 in response to adenosine diphosphate (ADP). Increased P2Y1 pathway activity would be consistent with Tyagi et al’s finding that calpains are up-regulated by hypoxia. Since we found no change in overall sensitivity to ADP, it may be that the changes produced by hypoxia in these two pathways counteract one another. AC, adenylate cyclase; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanine monophosphate; GC, guanylate cyclase; PI3K, phosphoinositide 3-kinase.

What is known about this topic?

- Exposure to hypobaric hypoxia is thought to induce a thrombogenic phenotype, with increased risk of stroke in those residing at high altitude.
- The platelet proteome is altered by hypoxia and previous work has suggested platelet sensitivity to ADP is increased at altitude.

What does this paper add?

- Using optimal aggregometry, we show no significant changes in the dose–response of several platelet receptor agonists.
- Hypoxia suppressed the extent to which cangrelor inhibited platelet aggregation to ADP, a finding with potential implications for hypoxic patients taking platelet inhibitors.

Authors’ Contributions

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Conflict of Interest
The authors declare no competing financial interests. A.A. R.T. reports non-financial support from Actelion UK Ltd, outside the submitted work.

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