To the editor:

Liberation of ATP secondary to hemolysis is not mutually exclusive of regulated export

In their recent report published in *Blood*,[1] Sikora and colleagues correlate cell-free hemoglobin (Hb) and extracellular adenosine triphosphate (ATP_{ec}), and conclude that cell rupture from hypotonic shock accounts for ATP_{ec} increases in isolated red blood cell (RBC) suspensions. Although this conclusion may apply to the authors' experimental conditions (>25% reduction in tonicity with RBCs from hemochromatosis patients, stored 0-14 days), it cannot be generalized to studies in which (patho)physiologically relevant conditions (eg, hypoxia, acidosis, adrenergic stimulation, mechanical stress) were used to promote ATP release from RBCs freshly isolated from normal humans independently of hemolysis.[1-11] We raise the following concerns.

(1) The selected stimuli directly and independently evoke hemolysis

Cell lysis can result directly from stimuli such as hypotonic shock (a nonphysiological stimulus), toxins (eg, dimethyl sulfoxide [DMSO]), and mechanical or hypoxic stress. RBCs, particularly, are readily ruptured, with expulsion of intracellular contents, including the classical hemolysis indicators, cell-free Hb, potassium, and lactate dehydrogenase. But liberation of ATP secondary to hemolysis is not mutually exclusive of regulated export. In some cases, the authors used banked RBCs, which are particularly vulnerable to osmotic lysis, in contrast to fresh human RBCs, which do not lyse in response to a 25% reduction in tonicity.[12] Moreover, Sikora and colleagues did not perform osmotic fragility curves for the hemochromatosis RBCs studied, or compare their susceptibility to lysis to that of freshly isolated normal human RBCs.[1]

(2) Prior reports of extracellular ATP

Contrary to the authors' statements, reports (some uncited) investigating RBC ATP release do address and preempt concerns over increased $[ATP]_{ec}$ via hemolysis, with rigorous intrasample comparison of cell-free [Hb] and [ATP] used to distinguish genuine ATP export from breakage of RBCs.[2-4,6-8,10,11,13] Even the earliest investigations, from Bergfeld and Forrester,[9] parsed authentic ATP release from that due to cell damage, and numerous publications document regulated ATP export from RBCs while controlling for hemolysis. If increases in $[ATP]_{ec}$ are solely from hemolysis, then $[ATP]_{ec}$ should be unaffected by antagonizing ATP-export signaling. In fact, ATP export from mouse RBCs lacking pannexin 1 (Px1; an ATP conduit) or pretreated with Px1 inhibitors is low, or even absent, without altered hemolysis[3-5]; Sikora and colleagues did not address this important point.

(3) Experiment-specific results may preclude definitive conclusions

The authors intended to test the hypothesis that hemolysis is *the primary cause* for $[ATP]_{ec}$ elevations in response to selected stimuli. However, multiple experimental design flaws exist, limiting the conclusions.

First, the lysis-induced ATP liberation was not discriminated from genuine ATP release. Lysis can mask regulated ATP release because the expulsion of intracellular nucleotidases leads to rapid ATP hydrolysis (as in Figure 2 of their article), so that the measure of $[ATP]_{ec}$ would underestimate actual ATP release, and because intracellular [ATP] far exceeds exported [ATP].[2,3,11] Masking may also arise from: centrifugation (mechanical stress) used to separate the ATP_{ec} and free Hb from RBCs, the vulnerability of the banked RBCs studied (especially given their deficient ATP-release capacity[8,10]), DMSO solvent (other vehicles can dissolve cyclic AMP [cAMP] analogs[3,11]), RBC temperature shift (promotes vulnerability), and poly-L-lysine (higher concentrations perturb distribution of band 3,[3] a determinant of controlled ATP export[7,9]). In the case of the ATP-release stimulus hypoxia, special caution and controls using normoxic gas flow are lacking in Sikora's study but necessary because the force of gas flow itself can lyse cells. Therefore, the signal from genuine ATP release was likely overwhelmed here by the noise of hemolysis.

Second, Sikora and colleagues observed no ATP release in response to cAMP-stimulating reagents (forskolin + papaverine + isoproterenol, hereafter referred to as "3V"). In contrast, adding 100% DMSO lysed RBCs, but 10% DMSO was innocuous. Consequently, they propose that hemolysis rather than cAMP could have caused ATP release in Montalbetti et al.[11] This argument is unsustainable because in Montalbetti[11]: (1) identical volumes of 3V (as 10 times stock) and vehicle (phosphate-buffered saline [PBS]) were added to cells in 1% DMSO; (2) cAMP-elevating isoproterenol (in PBS) markedly potentiated forskolin-evoked ATP release; (3) dibutyryl-cAMP (aqueous solution) promoted ATP release; and (4) inhibitors of Px1 abolished ATP release in the absence of measurable hemolysis. Lastly, Sikora and colleagues ignored the observation that 3V promoted ATP release in RBCs from wild-type but not $Px1^{-/-}$ mice.[3] The fact that the authors detected no cAMP-evoked ATP release in 0- to 14-day-old RBCs from hemochromatosis patients cannot invalidate studies using fresh, normal human and mouse RBCs.[3,11] Notably, ATP export has been shown to decline with RBC storage time,[10] and the report by Sikora and colleagues might have been strengthened by showing the data in support of their assertion that hemolysis and ATP_{ec} did not differ as a function of RBC storage for 0 to 2 days vs up to 14 days.

Finally, the authors leave unresolved the intriguing but contradictory observation that in their experiments, the increases in ATP_{ec} precede the hemolysis.

(4) In vivo evidence on ATP and hemolysis

One can definitively determine RBC hemolysis in vitro, but direct study of intravascular hemolysis in vivo is more challenging. Nevertheless, plasma ATP rises during physiological stress in vivo and cannot be attributed to hemolysis. These data are consistent with regulated ATP export.[2]

We agree that RBC hemolysis elevates $[ATP]_{ec}$ as demonstrated by Sikora and colleagues. However, in our opinion, their findings are correlational and not mechanism-probing, and contrast with well-controlled experiments showing RBC ATP export independent of hemolysis and inhibited by agents that antagonize authentic ATP release. The evidence does not support hemolysis as a primary ATP-release mechanism in human RBCs.

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Response

Hemolysis is a primary and physiologically relevant ATP release mechanism in human erythrocytes

We appreciate the willingness of Kirby and colleagues to discuss methodological concerns in red blood cell (RBC) adenosine triphosphate (ATP) release studies. In our investigations, before moving to mechanism-probing experiments, we spent most of the time evaluating the different methods that have been used in the field to assess RBC ATP release and the contribution of hemolysis to that release. Although we began with the hypothesis that regulated ATP release mechanisms do operate in RBCs, after extensively verifying each experimental approach, we came to the conclusion expressed in the title: ie, that "Hemolysis is a primary ATP release mechanism in human erythrocytes."[1] In the course of the study, while attempting to reproduce previously published findings, we identified a number of methodological pitfalls (the major ones are discussed in the supplemental Methods available on *Blood* Web site), which likely precluded a proper evaluation of ATP release and also the contribution of hemolysis in the earlier studies[2-10] cited by Kirby et al.

We agree that "liberation of ATP secondary to hemolysis is not mutually exclusive of regulated export."[11] Our point is that without the controls described in our paper, a confounding effect of hemolysis cannot be excluded.

We determined that the most reliable and direct evaluation of a hemolysis contribution to RBC ATP release requires the following. (1) The use of RBC supernatants for extracellular ATP (ATP_{ec}) evaluation rather than RBC suspensions is necessary. We verified that centrifugation (500*g*, 10 minutes, 4°C), even when repeated 5 times, does not elevate ATP_{ec} above the low basal level seen after the first or second centrifugation. (2) The measurement of free hemoglobin (Hb) in an aliquot of the same supernatant used for ATP_{ec} determination is necessary. To the best of our knowledge, such paired measurements have been performed only for plasma ATP-Hb determinations by Gorman et al[12] and Kirby et al[2] or toxin-induced ATP release.[13] When ATP_{ec}-Hb values of all samples were plotted to evaluate the correlation between these variables, they showed a linear relationship and exactly matched those obtained with freshly prepared RBC lysates (see Figure 1D in Sikora et al[1]). Any "upward" deflection in the linear ATP-Hb relationship would indicate a contribution of nonlytic ATP release.

With these restrictions, we found that ATP and Hb release were tightly correlated for all stimuli tested and exactly matched expectations based on the number of lysed cells and independently determined intracellular ATP concentration. Our conclusions were fully confirmed by directly visualizing single-cell ATP release and cell lysis using luminescence ATP imaging and simultaneous infrared cell imaging, respectively. We encourage other investigators to use these criteria and to examine the correlation between ATP and Hb release in individual experiments, so that conclusions can be made concerning regulated, hemolysis-independent mechanisms.

Specific responses

Gas flow was used in both normoxia- and hypoxia-treated samples, although indeed this was not stated clearly in the paper. Fresh and stored RBCs gave qualitatively similar results, despite somewhat elevated hemolysis after prolonged storage. The measured