ADP Acting on P2Y\textsubscript{13} Receptors Is a Negative Feedback Pathway for ATP Release From Human Red Blood Cells

Lingwei Wang, Göran Olivecrona, Matthias Götberg, Martin L. Olsson, Maria Sörhede Winzell, David Erlinge

Abstract—Red blood cells may regulate tissue circulation and O\textsubscript{2} delivery by releasing the vasodilator ATP in response to hypoxia. When released extracellularly, ATP is rapidly degraded to ADP in the circulation by ectonucleotidases. In this study, we show that ADP acting on P2Y\textsubscript{13} receptors on red blood cells serves as a negative feedback pathway for the inhibition of ATP release. mRNA of the ADP receptor P2Y\textsubscript{13} was highly expressed in human red blood cells and reticulocytes. The stable ADP analogue 2-MeSADP decreased ATP release from red blood cells by inhibition of cAMP. The P2Y\textsubscript{12} and P2Y\textsubscript{13} receptor antagonist AR-C67085 (30 \textmu mol/L), but not the P2Y\textsubscript{1} blocker MRS2179, inhibited the effects of 2-MeSADP. At doses where AR-C67085 only blocks P2Y\textsubscript{12} (100 \textmu mol/L), it had no effect. AR-C67085 and the nucleotidase apyrase increased cAMP per se, indicating a constant cAMP inhibitory effect of endogenous extracellular ADP. 2-MeSADP reduced plasma ATP concentrations in an in vivo pig model. Our results indicate that the ATP degradation product ADP inhibits ATP release by acting on the red blood cell P2Y\textsubscript{13} receptor. This negative feedback system could be important in the control of plasma ATP levels and tissue circulation. (Circ Res. 2005;96:189-196.)

Key Words: ATP release ■ cAMP ■ P2 receptors ■ microdialysis ■ red blood cells

It has become increasingly clear that, in addition to functioning as an intracellular energy source, ATP and ADP can serve as important extracellular signaling molecules.\textsuperscript{1,2} Extracellular ATP in the circulation is rapidly degraded into ADP, AMP, and adenosine by ectonucleotidases.\textsuperscript{3} ATP and ADP activate P2 receptors on endothelium, platelets,\textsuperscript{4,5} and other blood cells,\textsuperscript{6} regulating several physiological responses including vascular tone,\textsuperscript{7} platelet aggregation, and the release of endothelial factors. At least 15 nucleotide-activated cell surface receptors (7 of P2X and 8 of P2Y) have been found in man, with remarkably broad and varied physiological responses.

The matching of oxygen supply with demand requires a mechanism that increases blood flow in response to decreased tissue oxygen levels. Several reports suggest that the red blood cell (RBC) acts as a sensor for hypoxia, and different mechanisms have been suggested by which the deoxygenated RBC stimulates vasodilatation.\textsuperscript{7-10} RBCs contain millimolar amounts of ATP and possess the membrane-bound glycolytic enzymes necessary for its production.\textsuperscript{11-13} ATP is released in response to reductions in oxygen tension and pH.\textsuperscript{7,9} It has been shown in vitro that the vessels dilate in response to low O\textsubscript{2} levels only when blood vessels are perfused with RBCs.\textsuperscript{14} Recently, in vivo studies in man demonstrated that ATP is released in working skeletal muscle circulation depending on the number of unoccupied hemoglobin O\textsubscript{2} binding sites.\textsuperscript{7,9} The released ATP then binds to P2Y receptors on the endothelium and stimulates vasodilatation by the release of nitric oxide (NO), prostaglandins,\textsuperscript{2} and endothelium-derived hyperpolarizing factor (EDHF).\textsuperscript{15,16} Thus, the RBC functions as an O\textsubscript{2} sensor, contributing to the regulation of blood flow and O\textsubscript{2} delivery by releasing ATP depending on the oxygenation state of hemoglobin.

Physiologically important signaling systems are usually regulated by negative feedback systems, for example, noradrenaline and ATP release from sympathetic nerves is inhibited by presynaptic \alpha\textsubscript{2} and P\textsubscript{1} (A1 subtype) receptors.\textsuperscript{17} We hypothesized that ATP release from RBCs is regulated by a P2 receptor-mediated negative feedback pathway.

Materials and Methods

The studies were approved by the local Ethics Committee of the Lund University and were conducted according to the principles of the Declaration of Helsinki. All participants gave written consent for the study.

Preparation of RBCs and Reticulocytes

Human RBCs were collected as described.\textsuperscript{12} The packed RBCs were resuspended and washed three times in a physiological salt solution (PSS; in mmol/L: 4.7 KCl, 2.0 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 140.5 NaCl, 21.0 Tris, and 11.1 dextrose; pH 7.4). The oxygen tension was 85 to 90 mm Hg. RBCs were prepared on the day of use. Previous...
experiments using real-time PCR have demonstrated high levels of P2Y12 in platelets,4 P2Y1 in buffy coat,3 and P2X4 receptors in monocytes and lymphocytes.18 Very low levels of mRNA for these receptors (Figure 1) rule out contamination of other circulating cells.

Human reticulocytes were prepared from peripheral blood samples. A dry reticulocyte-enriched cell pellet was obtained by separation of the sample on a Lymphoprep gradient. The cells present in the liquid phase below the lymphocytic layer were harvested, washed repeatedly in PBS, and frozen at −80°C.19

Incubating RBCs and RBC Ghosts In Vitro With Microdialysis Equipment

Washed RBCs were brought to a 20% hematocrit in PSS buffer. RBC ghosts were prepared as described20 from washed RBCs with 20% hematocrit, resuspended into the original volume in PSS buffer. The RBCs or RBC ghosts were kept in an incubation chamber (2.5 mL, 37°C). Microdialysis probes (CMA20; Microdialysis AB, Sweden) were inserted into the solution and perfused at a rate of 0.5 μL/min with PSS. Samples were balanced for 30 minutes before pharmacological agents were added. RBCs and RBC ghosts were incubated with different pharmacological agents for 10 minutes, if not otherwise indicated, and samples were collected during the final 5 minutes for ATP quantification.

Our microdialysis method eliminates the need for centrifugation and pipetting of RBCs that is known to cause nonspecific release of ATP. Furthermore, it enables the investigation of real-time changes of ATP release during sequential addition of drugs. When defined concentrations of ATP (10−5 to 10−3 mol/L) were added in the incubation chamber without RBCs, the dialysate ATP concentrations had a linear relationship compared with the added ATP concentrations (r2=1.0, P<0.0001). Relative recovery (RR) was 7.6±0.5%, determined as follows: RR=cd/cs, with cd being the concentration of ATP in a dialysate fraction and cs its known concentration of ATP within a sample solution.21 To further verify the specificity of ATP assays, defined concentrations of ATP were incubated with 2-MeSADP (1 μmol/L), MRS2179 (10 μmol/L), adrenaline (1 μmol/L), and AR-C67085 (30 μmol/L), which did not interfere with the results of ATP measurements (data not shown).

In Vivo Pig Model With Microdialysis for Determining ATP Levels in Blood

Healthy domestic pigs (Larsson, Lund, Sweden) of both sexes weighing 25 kg were premedicated with azapenone (Stresnil Vet), 2 mg/kg. After induction of anesthesia with thiopental 5 to 25 mg/kg, the animals were orally intubated and ventilated. A slow infusion of 1.25 μL/mL Fentanyl (Pharmalink AB) in Ringer-acetate was started and adjusted as needed. 10 000 IU Heparin were given. An 8F introducer (Onset, Cordis Co) was inserted into the right internal jugular vein, and an 8F AL 1.0 was inserted into the coronary sinus. A venography was obtained using contrast medium Omnipaque 300 mg I/mL (Nycomed) to ensure positioning of the catheter. Through a catheter, the microdialysis probe (CMA70) was inserted and the microdialysis tip was placed in the coronary sinus. A 6F introducer sheath was inserted into the surgically exposed left carotid artery. Through the left carotid artery, a catheter was placed in the left anterior descending artery (LAD). Finally, 2 mL of 2-MeSADP (10 μmol/L) was infused in the LAD for 1 minute. Samples were collected by microdialysis (5 μL/min) from the coronary sinus every 5 minutes.

Measurement of ATP

ATP was measured by ATP Bioluminescent assay kit (Sigma) according to the supplier’s instructions.

Measurement of cAMP

Washed RBCs were brought to a 20% hematocrit in PSS buffer and incubated with different pharmacological agents for 10 minutes if not otherwise indicated. After the incubation, the samples were treated as described.12 The concentration of cAMP was determined in duplicate using the cAMP EIA System (Amersham Biosciences)

Quantitative TaqMan Real-Time Reverse Transcription Polymerase Chain Reaction Assay

Total cellular RNAs were extracted as described.4,5,18,22,23 The TaqMan Reverse Transcription (RT) Reagents Kit was used to transcribe mRNA into cDNA. Real-time polymerase chain reaction (PCR) was performed by means of a PRISM 7700 Sequence Detector as described.4,23 Oligonucleotide primers and TaqMan probes were designed by using Primer Express, based on sequences from the GenBank database.22 Constitutively expressed glyceraldehyde-phosphodehydrogenase (GAPDH) was selected as external endogenous control genes to correct for any potential variation in RNA loading, cDNA synthesis, or efficiency of the amplification reaction. The threshold cycle (Ct) is defined as the fractional cycle number at which the reporter fluorescence reaches a certain level (set to 10 times the SD of the baseline). The target gene normalized to GAPDH was expressed as ΔCt (Ct of target gene minus Ct of GAPDH). The normalized value is given by the equation 2−ΔΔCt. To further verify the specificity of the PCR assays, the PCR was performed with non-reverse-transcribed total cellular RNA and samples lacking the DNA template. No significant amplifications were obtained in any of these samples (data not shown).

SDS-PAGE and Western Blot

RBC membranes24 and platelets4,5 were isolated. The resultant pellets were dissolved in RIPA buffer and frozen at −20°C. SDS-PAGE and Western blot were performed as described.4,23 Membranes were incubated with anti-P2Y1 (Alomone) and anti-P2Y12 (AstraZeneca) antibodies. P2Y1 and P2Y12 antibodies were preabsorbed with specific antigen peptides to examine specificity.
The anti-P2Y1 antibody was also tested in transfected astrocytoma cells (hP2Y1-1321N1), indicating specificity of the antibody. Our previous studies of tissues known to express P2Y1 receptors (platelets and endothelium) have also shown 180-kDa bands. The anti-P2Y12 antibodies were also tested in P2Y12 transfected cells and shown to represent specific binding compared with nontransfected cells by AstraZeneca, Sweden. Then membranes were reprobed with the anti-GAPDH antibody (Chemicon) as control.

Statistical Methods
Data are expressed as mean±SEM. n indicates the number of subjects tested. Statistical analysis of the normalized Ct values (ΔCt) was performed with a one-way ANOVA, followed by a multiple comparison post test (Tukey’s test). For other comparisons, the Student t test was used. Differences were considered significant at *P<0.05, **P<0.01, and ***P<0.001 (two-tailed test), compared with control if not indicated.

mRNA Quantification in RBCs and Reticulocytes
In RBCs, P2Y13 was the most abundantly expressed mRNA (P>0.0001, compared with other P2 receptors), whereas P2Y1, P2Y5, P2Y6, P2Y4, P2Y11, P2Y12, P2X6, and P2X7 had extremely low expression levels (Figure 1A). The expression pattern was strikingly different compared with our previous mRNA quantifications of P2 receptors in vascular smooth muscle cells, endothelial cells, monocytes, lymphocytes, and CD34+ stem and progenitor cells. To illustrate expression of the P2 receptors relative to each other, the GAPDH was used as endogenous control gene, ie, the other receptors were expressed as a ratio of the GAPDH. For verifying the mRNA expressions, ADP receptors in reticulocytes were also detected and the P2Y13 receptor was expressed at a strikingly higher level than the other known ADP receptors (P2Y1 and P2Y12) (Figure 1B). A preparation of reticulocytes (purity >95%) was also isolated using FACS (cells from a peripheral blood sample were labeled with FITC- and PE-conjugated monoclonal antibodies against surface markers CD71 and glycophorin A, and double positive cells subsequently sorted directly to PCR tubes for real-time quantitative PCR). In this sample, P2Y13 had markedly higher levels than P2Y1 (2.1%) and P2Y12 (2.8% in % of P2Y13 100%).

Western Blotting for ADP Receptors in RBCs
P2Y13 is one of three ADP receptors in man; the others are P2Y1 and P2Y12. To confirm expression on the protein level, Western blotting analysis was performed. Unfortunately, no antibodies against the P2Y13 receptor are available. However, the other ADP receptor subtypes P2Y1 and P2Y12 were not expressed in erythrocytes, yet expressed in platelets as expected. GAPDH was expressed at similar levels in erythrocytes and platelets (Figure 2A and 2B). This indicates that the effects of ADP on erythrocytes are mediated by P2Y13 receptors.

Regulation of ATP Release From RBCs
In the present assay, baseline ATP levels outside erythrocytes were 0.76±0.12 μmol/L, n=12. Incubation of RBCs with the more stable ADP analogue 2-MeSADP, dose-dependently reduced extracellular ATP concentrations with a maximum reduction of 48% after 10 minutes (Figure 3A).

During the incubation of intact RBCs, the ATP levels were unaltered over time, indicating that a constant release of ATP from RBCs is necessary for the maintenance of extracellular ATP levels (Figure 3D). This balance was affected by 2-MeSADP (1 μmol/L), causing a reduction in extracellular ATP concentrations, which were reduced to 83% after 5 minutes and to 75% after 10 minutes (Figure 3D).

There is no selective P2Y13 antagonist, but AR-C67085, an antagonist of P2Y12 receptors in nanomolar concentrations, is also an antagonist of P2Y13 receptors in micromolar concentrations. In the presence of 30 μmol/L of AR-C67085 to block both P2Y13 and P2Y12 receptors, the ATP reducing effect of 2-MeSADP was abolished (Figure 3C). In contrast, no effect of AR-C67085 was seen in concentrations where it only blocks P2Y12 receptors (0.1 μmol/L). MRS2179, is an antagonist of the P2Y1 receptor, another ADP receptor known to be present on the endothelium and on platelets. MRS2179 did not have any effect on the RBCs and did not block the inhibition of ATP release by 2-MeSADP. Furthermore, 30 μmol/L of AR-C67085 raised extracellular ATP levels (Figure 3B) and the effect was additive to adrenaline (6B).

After the decrease of extracellular ATP levels with 2-MeSADP, adrenaline caused a stepwise increase of ATP levels (Figure 4A). Adrenaline increases ATP release and this effect was inhibited by 2-MeSADP (Figure 4B). The β-adrenergic antagonist propranolol (1 μmol/L) but not the α-adrenergic antagonist phenolamine (100 μmol/L), blocked the effect of adrenaline (Figure 6B).

Regulation of Plasma ATP Concentrations in an In Vivo Pig Model
After intracoronary injection of 2-MeSADP, samples were obtained from the venous effluent of the heart in the coronary sinus with a microdialysis probe. Analysis showed decreased concentrations of plasma ATP after intracoronary injection of 2-MeSADP (Figure 4C).
Effect of Ectonucleotidases in RBCs
When a certain amount of ATP (1 μmol/L) was added to the RBC ghost solution, the ATP level was reduced to 22% after 5 minutes and to 9% after 10 minutes (Figure 3E). No changes were found without RBC ghosts. The rate of ATP degradation was not changed by coincubation with 2-MeSADP or AR-C67085 (Figure 3E).

Participation of cAMP-Regulating ATP Release
Incubation of erythrocytes with 2-MeSADP decreased intracellular cAMP levels (Figure 5A) and this change was dose-dependent (Figure 5C). Adrenaline caused an increase of cAMP levels both alone and after a decrease of cAMP levels by 2-MeSADP (Figure 5B). The β-adrenergic antagonist propranolol (1 μmol/L) but not the α-adrenergic antag-
onist phentolamine (100 μmol/L) blocked the effect of adrenaline on cAMP (Figure 6A). Incubation of erythrocytes with 30 μmol/L of AR-C67085 increased intracellular cAMP levels (Figure 5A) and the effect was additive to adrenaline (Figure 6A). The ATP degrading enzyme apyrase was used to eliminate ATP and ADP from the media, which lead to markedly increased cAMP levels (Figure 5D).

**Discussion**

The main finding of the study is that ADP activates a negative feedback pathway for ATP release from human RBCs via P2Y13 receptors. Because blood consists of approximately 40% RBCs, containing a 1000-fold higher ATP concentration than plasma (mmol/L versus μmol/L), even a minor release of ATP from the high intracellular concentrations could have major circulatory effects. A negative system may therefore be of great physiological importance to mitigate ATP release. Furthermore, the previous view of the RBC as a “passive bag that transports oxygen” is challenged. It now turns out that it releases ATP in response to stimuli and as with most important signaling systems, it has a negative feedback system to terminate its release.

**Selective Expression of the P2Y13 Receptor in RBCs**

We have recently developed a sensitive quantitative mRNA assay for most of the P2 receptors and demonstrated that it was possible to quantify mRNA levels in platelets even though they only contain minute levels of mRNA.4,22,23 The RBC is also an anucleated cell with very low mRNA levels. However, it was possible to extract and quantify mRNA for P2 receptors. Surprisingly, the P2Y13 receptor was found to be by far the highest expressed P2 receptor in RBCs. To verify the mRNA expressions, ADP receptors in reticulocytes were detected. It was found that the P2Y13 receptor had markedly higher expression than P2Y1 and P2Y12 receptors. A role for the P2Y13 receptor has not been clearly shown in any cell type and its distribution has been assumed to mainly be in cells of the immune system based on the observation of high mRNA levels in the spleen and bone marrow.27,28 In light of our findings, this mRNA is more likely to represent RBC mRNA. It is unlikely that our finding should represent contamination from other circulating cells because platelets do not express P2Y13, and analysis of buffy coat mRNA revealed a completely different P2 receptor expression profile.4

In Western blotting, the other ADP receptor subtypes P2Y1 and P2Y12 were not expressed in RBCs, but in platelets as expected. This gives support on the protein level that the effects of ADP on RBCs are mediated by P2Y13 receptors. The P2Y13 receptor is coupled to Gi proteins mediating inhibition of intracellular cAMP levels.27 Previous reports have shown that increased cAMP levels in RBCs lead to release of ATP.12 It is therefore possible that ADP acting on P2Y13 receptors could represent a negative feedback mechanism for ATP release by inhibition of intracellular cAMP levels.

**ATP Release From RBCs Affected by P2Y13 Receptor Activation**

To study ATP release from RBCs, we first tried to separate the extracellular buffer solution from RBCs by centrifugation or dilution as suggested in previous studies.12,13 The problem is that large amounts of ATP are released on centrifugation and handling of samples and this caused a large variation of the results. We therefore used a small microdialysis probe with an external inlet and outlet, placed in the RBC solution. This resulted in stable values with low variability. Furthermore, it made it possible to perform continuous registration of extracellular ATP levels during subsequent in vitro additions of different compounds.
Incubation of RBCs with the stable ADP analogue 2-MeSADP, dose-dependently reduced extracellular ATP concentrations. We interpret this as a reduced ATP release from RBCs. A constant release of ATP is necessary to maintain extracellular levels because of the rapid degradation of extracellular ATP by ectonucleotidases on the RBC membrane.29 The RBC ghosts are deprived of their intracellular ATP thus lacking the possibility to release ATP and can therefore only alter the extracellular ATP levels by degradation via membrane-bound ectonucleotidases. When a certain amount of ATP was added to the RBC ghost solution, the ATP level was reduced (Figure 3D and 3E). No changes were found without RBC ghosts, demonstrating rapid ATP degradation by RBC ectonucleotidases. The rate of ATP degradation was not changed by coincubation with 2-MeSADP or AR-C67085, which indicates that the changes in extracellular ATP levels were not caused by a direct effect of 2-MeSADP or AR-C67085 on ectonucleotidases. During the incubation of intact RBCs, the ATP levels were unaltered over time, indicating that a constant release of ATP from RBCs is necessary for the maintenance of extracellular ATP levels. This balance is affected by 2-MeSADP, causing a reduction in extracellular ATP concentrations. Thus, the degradation of ATP by ectonucleotidases and the release of ATP from RBCs appear to be key points in the regulation of the extracellular ATP levels surrounding RBCs.

There is no selective P2Y13 antagonist, but AR-C67085 (an antagonist of P2Y1 receptors in nanomolar concentrations) is also an antagonist of P2Y13 receptors in micromolar concentrations.25 In the presence of 30 μmol/L of AR-C67085 to block both P2Y11 and P2Y12 receptors, the ATP reducing effect of 2-MeSADP was abolished. In contrast, no effect of AR-C67085 was seen in concentrations where it only blocks P2Y12 receptors (0.1 μmol/L). MRS2179, an antagonist of the P2Y1 receptor,26 did not have any effect on the RBCs and did not block the inhibition of ATP release by 2-MeSADP. This indicates that among the ADP receptors, P2Y1, P2Y12, and P2Y13, only the P2Y13 receptor contributes to the regulation of ATP release in RBCs.

Interestingly, 30 μmol/L of AR-C67085 raised extracellular ATP levels per se, indicating antagonism of endogenous extracellular ADP. Thus, the extracellular ATP level is constantly regulated by ADP. A rapid loop must exist in which ATP is secreted, subsequently degraded to ADP, AMP, and adenosine followed by intracellular reuptake and phosphorylation back to ATP. The extracellular concentration of ATP is dependent on its release from RBCs. The release of ATP may be controlled by negative feedback inhibition mediated by ADP acting on the P2Y13 receptor. Although less potent as an agonist of P2Y13 compared with ADP, ATP may have an effect in high concentrations and inhibit its own release.27,28

Adrenaline increases ATP release and 2-MeSADP inhibits this effect. Adrenaline stimulates β-adrenergic receptors on the RBC membrane that increase intracellular cAMP by coupling to Gs.24 After decrease of extracellular ATP levels with 2-MeSADP, adrenaline increased ATP levels. This indicates that the external ATP levels could be both raised or lowered by receptor agonists.

In an in vivo pig model, plasma ATP concentrations were decreased after injecting 2-MeSADP, indicating that 2-MeSADP could regulate plasma ATP concentrations by stimulation of P2Y13 receptors on RBCs in vivo. These findings may be affected by other mechanisms. Vasodilatation by 2-MeSADP may increase the oxygenation rate of hemoglobin and thereby reduce ATP release from RBCs. On the other hand, platelet activation by 2-MeSADP may cause ATP release.

### Participation of cAMP in the P2Y13-Activated Signal Transduction Pathway That Regulates ATP Release
The P2Y11 receptor is coupled to Gs proteins mediating inhibition of intracellular cAMP levels.27 Incubation of RBCs...
with 2-MeSADP decreased intracellular cAMP levels. The inhibition of cAMP by 2-MeSADP could be reversed by incubation of RBCs with adrenaline. Incubation of RBCs with 30 \( \mu \)mol/L of AR-C67085 not only resulted in ATP release, but also increased intracellular cAMP levels per se. This indicates that AR-C67085 can block the P2Y13 activation by endogenous ADP. To further test if endogenous ADP mediates a constant inhibitory effect on cAMP levels, the ATP degrading enzyme apyrase was used to eliminate ATP and ADP from the media. This led to a marked increase in cAMP levels. Thus, endogenous ADP may control ATP levels outside the RBC by inhibition of intracellular cAMP levels via activation of P2Y13 receptors (Figure 7). It is an interesting parallel that the \( \beta \)-receptor mediated negative feedback pathway of noradrenaline release in nerves also acts via G\(_i\) and cAMP inhibition. However, a recent article\(^{30}\) demonstrated that direct G\(_i\)-stimulation with mastoparan increased ATP release. In contrast to stimulation of G\(_i\), via the P2Y13 receptor (and in contrast to the usual cAMP inhibition of G\(_i\) proteins), they found increased cAMP levels in response to mastoparan. Thus, the link between cAMP levels and ATP release is similar, but differences depending on G\(_i\) protein activation or subtypes may exist.

Several mechanisms of ATP release outside the cell have been suggested, including ATP binding cassette (ABC) transporters, connexin hemichannels, mitochondrial porins, and stretch activated channels (See Lazarowski et al\(^{31}\) for review). In RBCs, evidence suggests ATP release through an anion channel, possibly the cystic fibrosis transmembrane conductance regulator (CFTR), which is known to be regulated by cAMP.\(^{32}\)

**A Negative Feedback Pathway for ATP Release From Human RBCs by P2Y\(_{13}\) Receptors**

Our finding of a negative feedback system for ADP release fits well with previous evidence of ATP as an extracellular transmitter, as first proposed by Burnstock,\(^{1}\) and it is possible that this negative feedback system plays an important role in the control of peripheral circulation. In underperfused organs, hypoxia stimulates an increase in intracellular cAMP, which leads to the release of ATP to the blood plasma. This increase of cAMP could be enhanced by adrenaline, that is in itself elevated during hypoxia.\(^{33}\) Extracellular ATP stimulates endothelial cells to release dilatory factors such as NO, mediating vasodilatation and improved oxygen delivery to the organ. To shut down the ATP release on the venous side (where the blood is still deoxygenated), and to avoid unnecessary ATP release, the degradation product ADP acting on P2Y\(_{13}\) receptors could inhibit cAMP and decrease ATP release from RBCs. ATP and ADP are then degraded by ectonucleotidases to adenosine, which is quickly taken up in the RBCs where it can be recycled to ATP by the glycolytic pathway. The described negative feedback pathway may be important to avoid high extracellular concentrations of ATP. At levels above 100 \( \mu \)mol/L, ATP concentrations may exceed the catalytic capacity of ectonucleotidases and, could in fact, stimulate ATP release by increasing permeability of the RBC.\(^{34}\) At high concentrations of ATP, a self sustaining process may thus be instigated, which may contribute to the irreversible stage of circulatory shock that can develop rapidly in severely ill patients.

According to one study, raising the plasma ATP levels by expanding the intracellular ATP concentrations of the RBC pool results in anticancer activities.\(^{35}\) It could be therefore be speculated that a P2Y\(_{13}\) receptor antagonist, in addition to infusions of ATP, could be of value in the treatment of cancer by increasing ATP delivery to the tissues. It is possible that in extreme conditions such as circulatory or septic shock with acidosis and hypoxia, high ATP levels could be deleterious, leading to a drop in blood pressure. However, in most situations increased ATP levels ought to
have positive effects by increasing tissue perfusion. ATP stimulates NO release from the endothelium and might be beneficial in situations of endothelium dysfunction, such as atherosclerosis, congestive heart failure, and diabetes. ATP, in contrast to adenosine, has recently been demonstrated to be sympatholytic, ie, counteracts sympathetic vasoconstriction.6 Increased ATP levels may therefore reduce hypertension and decrease peripheral vascular resistance in, for example, congestive heart failure. Patients with primary pulmonary hypertension have an impaired ATP release from RBCs that may be a pathogenic factor for the etiology of their pulmonary hypertension.13 Thus, a P2Y13 receptor antagonist that increases ATP levels extracellularly by inhibiting the negative feedback pathway may be valuable in congestive heart failure, hypertension, pulmonary hypertension, atherosclerosis, and diabetes.

In conclusion, we have shown that the ATP degradation product ADP inhibits ATP release by acting on the RBC P2Y13 receptor. This negative feedback system could be important in the control of plasma ATP levels and tissue circulation.

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