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Hydroxyurea stimulates the release of ATP from rabbit erythrocytes through an increase in calcium and nitric oxide production

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Abstract

Hydroxyurea, a proven therapy for sickle cell disease, is known to improve blood flow and reduce vaso-occlusive crises, although its exact mechanism of action is not clear. The objective of this study was to determine if hydroxyurea results in an increase of ATP release from the red blood cell (RBC) via the drug's ability to stimulate nitric oxide (NO) production in these cells. A system enabling the flow of RBCs through microbore tubing was used to investigate ATP release from the RBC. Incubation of rabbit RBCs (7% hct) with 50 μ M hydroxyurea resulted in a significant increase in the release of ATP from these cells. This level of ATP release was not detected in the absence of flow. Studies also showed that increments in hydroxyurea and NO (from spermineNONOate) resulted in an initial increase in ATP release, followed by a decrease in this release at higher concentrations of hydroxyurea and the NO donor. Incubation with L-NAME abolished the effect of the hydroxyurea, suggesting that NO production by the RBC was involved. Indeed, in the presence of 50 μ M hydroxyurea, the amount of total Ca^{2+} measured (by atomic absorption spectroscopy) in a 7% solution of RBCs increased from 363 ± 47 ng/ml and 530 ± 52 ng/ml. Finally, EPR studies suggest that an increase in nitrosylated Hb in the RBC is only measured for those studies involving hydroxyurea and a Ca^{2+} -containing buffer.

Keywords

nitric oxide; hydroxyurea; ATP; eNOS; red cells; sickle cell

1. Introduction

Although one of the primary tasks of the red blood cell (RBC) is to supply oxygen to organs and tissues, it has now become increasingly clear that the RBC itself is a determinant of overall blood flow to these organs and tissues. Specifically, it has been shown that, when exposed to certain stimuli (both physical and pharmacological), the RBC releases ATP that is not due to hemolysis. Upon binding to purinergic-type receptors, this ATP has the ability

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to stimulate nitric oxide synthase (NOS) activity in the endothelium, thereby increasing the levels of nitric oxide (NO) produced by these cells. In this construct, via its ability to release ATP, the RBC thus becomes a determinant in blood flow.

As mentioned above, there exist both physical and pharmacological methods to stimulate ATP release from the RBC. For example, it has been shown that mechanical deformation through micron-scale pores and flow-induced deformation through tubing having micron-scale inside diameters will induce ATP release from the RBC. Furthermore, reagents such as mastoporan, iloprost, pentoxifylline, and C-peptide (a substance co-released with insulin) will also induce increased levels of ATP release from the RBC. Interestingly, pentoxifylline and C-peptide have also been reported to increase the overall deformability of the RBC, a characteristic that has been previously reported to improve the ability of these cells to release ATP.

Hydroxyurea is another substance that has been reported to affect RBC deformability, although its effects are not completely understood. In fact, it has been reported that hydroxyurea both increases and decreases RBC deformability. Since 1995 hydroxyurea has been regarded as a proven treatment for sickle cell disease. Improvement of rheological properties including increased whole cell deformability, reduced red cell density and improved erythrocyte hydration status have been observed with patients receiving hydroxyurea. However, the mechanism of action of hydroxyurea in relation to improved rheological properties still remains to be elucidated, especially regarding the improvements of vascular properties, although various mechanisms have been proposed in the recent literature.

A widely accepted mechanism of action of hydroxyurea is its ability to induce fetal hemoglobin (HbF) production in RBCs. Interestingly, detectable improvements in the levels of HbF may not appear for a week or two, (Rodgers et al., 2003) if at all. Collectively, the lack of an exact mechanism of action and the timing of the rheological improvements prior to HbF formation suggests the existence of other possible mechanisms or effects from hydroxyurea.

Here, we present results that may provide information concerning the immediate rheological improvements from hydroxyurea therapy. Specifically, we have found that incubation of rabbit RBCs with hydroxyurea improves these cells' ability to release flow-induced ATP. Moreover, the results presented here imply that NO produced by the RBC itself is a major determinant in the ability of these cells to release ATP.

2. Materials and Methods

2.1. Animals

Rabbits (male New Zealand whites, 2.0-2.5 kg) were anaesthetised with ketamine (8 ml/kg, i.m.) and xylazine (1 mg/kg, i.m.) followed by pentobarbital sodium (15 mg/kg i.v.). A cannula was placed in the trachea and the animals were ventilated with room air. A catheter was placed into a carotid artery for administration of heparin and for phlebotomy. After

heparin (500 units, i.v.), the animals were exsanguinated. All procedures were approved by the Animal Investigation Committee at Michigan State University.

2.2. RBC Preparation

After exsanguination, blood was immediately centrifuged for 10 min at 500 *g* and 4°C. The plasma and buffy coat were collected for other studies within the laboratory. The RBCs were resuspended and washed three times in a physiological salt solution (PSS) (in mmol/l: 4.7 KCl, 2.0 CaCl₂, 140.5 NaCl, 12 MgSO₄, 21.0 tris[hydroxymethyl]aminomethane, 11.1 dextrose with 5% w/v bovine serum albumin [final pH 7.4]). Cells were prepared on the day of use and experiments were finished within 6 h of removal from the animal.

2.3. Preparation of RBCs for Measurements Involving Effects of hydroxyurea

For studies involving hydroxyurea, a 2 mM hydroxyurea solution was prepared by dissolving 4 mg of hydroxyurea (Sigma Aldrich, St. Louis, MO) in 25 ml of PSS. The range of hydroxyurea concentrations (0 – 200 µM) was made by diluting appropriate volumes (0 – 500 µl) of the 2 mM solution in PSS followed by the addition of RBCs to create a 7% hematocrit (hct) solution of RBCs in buffered hydroxyurea. Specifically, when preparing the hydroxyurea treated RBCs, hydroxyurea was first added to PSS before adding RBCs to create a homogeneously treated RBC solution. RBC samples were then incubated for 20 minutes at room temperature followed immediately by the ATP measurement portion of the analysis.

2.4. Preparation of NO Standards

Spermine NONOate solutions were prepared by dissolving 1.0 mg of spermine NONOate (Cayman Chemicals, Ann Arbor, MI) in 1 ml of 0.20 M NaOH, resulting in a spermine NONOate solution with a concentration of 38 mM. Working solutions were prepared by adding different volumes of the stock solution in deoxygenated phosphate buffered saline (PBS) at a pH of 7.4. These solutions were then pipetted into the PSS immediately preceding the addition of RBCs for those studies involving ATP release as a function of added NO. The ATP measurements were performed following 30 minute incubation in the spermine NONOate-containing samples.

2.5. Preparation of RBCs with L-NAME

A 1 mM stock solution of L-NAME was prepared by dissolving 3 mg of L-NAME in 10 ml of phosphate buffered saline (pH 7.4). An appropriate amount of this 1 mM stock was added to the PSS to create a 100 µM solution of buffered L-NAME, which was immediately followed by the addition of RBCs. The RBCs were allowed to incubate in the buffered L-NAME solution for 30 minutes prior to performing measurements.

2.6. Preparation of RBCs with Glibenclamide

A solution of 0.01 mol/l glibenclamide was prepared by adding 49 mg of glibenclamide (Sigma Chemical, St. Louis, MO) to 2 ml of a 0.1 mol/l solution of sodium hydroxide. To this, 7.94 ml of a dextrose solution (1 g dextrose in 20 ml of distilled and deionized water (DDW)) was added. The mixture was heated to 52 °C until all of the glibenclamide was

dissolved. Once dissolved, 1 ml of this solution was added to 9 ml of PSS, resulting in a solution with a concentration 1 mmol/l. This solution was then used to create buffered solutions containing glibenclamide at 100 μ M for inhibiting ATP release from the RBCs. Briefly, RBCs were incubated in the glibenclamide-containing PSS, centrifuged, and then resuspended in PSS containing the appropriate reagents (e.g., hydroxyurea).

2.7. Non-Flow ATP Assay

RBC-derived ATP was measured by the luciferin-luciferase assay where the amount of light generated by the reaction of ATP with firefly tail extract (Sigma Chemical Co., St. Louis, MO, USA) is dependent on the ATP concentration. The luciferin-luciferase solution was prepared according to manufacturer's instructions (i.e., 5 ml of DDW were added to a vial containing the firefly tail extract). Sensitivity was augmented by the addition of 2 mg of synthetic D-luciferin (Sigma Chemical) to the crude firefly tail extract. Measurement of the ATP release was performed by mixing a 100 μ l aliquot of RBCs (typically prepared as a 7% hct) pipetted into a standard cuvette immediately followed by 200 μ l of the luciferin-luciferase solution. A photomultiplier tube housed in a light-excluding box was used to detect the emitted light. An ATP standard curve was prepared on the day of each experiment by determining the light efflux in response to the addition of known amounts of authentic ATP (Sigma Chemical) to the luciferin/luciferase reaction mixture. Total intracellular ATP was measured in a similar manner; however, the RBCs were lysed in DDW and diluted 1:1000 in DDW prior to measurement.

2.8. Flow-Based ATP Assay

All RBC solutions were diluted to a 7% hct in PSS. To measure the RBC derived ATP release, the luciferin-luciferase reaction mixture was placed in a 500 μ l syringe (Hamilton, Fischer Scientific). The RBC solution was placed in another 500 μ l syringe and both were loaded on to a syringe pump (Harvard Apparatus, Boston, MA) operating at 6.8 μ l min⁻¹ and connected to two sections of silica microbore tubing having an internal diameter of 50 μ m and an outer diameter of 362 μ m (Polymicro Technologies, Phoenix, AZ). The pumping rate was chosen to approximate linear rates estimated to exist in arterioles. The microbore tubes (each about 30 cm in length) were then connected to a T-junction (Upchurch Scientific, Oak Harbor, WA) having a volume of 256 nl. The combined contents were passed through a 6 cm section of microbore tubing having an internal diameter of 75 μ m. The resultant chemiluminescence produced by the RBC derived ATP and the luciferin-luciferase reaction was detected by the photomultiplier tube described above. Prior to placing the tubing over the PMT for measurement, the polyimide coating was removed from the tubing to enhance light transmission. This assay has been used by our group for other studies involving ATP release from the RBC.

2.9. Determination of erythrocyte Ca²⁺ increase using Atomic Absorption Spectroscopy (AAS)

Ca²⁺ standards ranging in concentrations from 2.50 to 25000 μ g/l, or ppb) were prepared by a serial dilution of a 10 mg/l stock solution of standard CaCO₃ in 25 ml volumetric flasks. The CaCO₃ stock solution was prepared by dissolving 4 mg of CaCO₃ in 1000 ml of DDW (the resistivity of the DDW was 18.2 M Ω , thereby ensuring the absence of any Ca²⁺ from

the solvent). RBCs in a 7% hct solution were incubated with 50 μM hydroxyurea for 20 minutes in PSS (which, as described above, contains Ca^{2+}). Next, the RBC sample was centrifuged at $500 \times g$ at 4°C for 10 min to remove the Ca^{2+} -containing supernatant. The packed RBCs were resuspended and washed 3 times in Ca^{2+} -free PSS. The Ca^{2+} -free PSS was prepared by using Ca^{2+} -free Ringers buffer; however, the ionic strength was balanced by substituting an equivalent amount of MgSO_4 . Finally the washed RBCs were lysed in DDW, and the membrane ghosts were removed by centrifuging at $3000 \times g$ at 4°C for 10 min. The supernatant was then analyzed using the atomic absorption spectrophotometer fitted with a hollow cathode lamp specific for exciting Ca^{2+} .

2.10. Electron Paramagnetic Resonance Studies

RBC solutions were diluted to a 7% hematocrit and incubated in 50 μM hydroxyurea for 20 minutes in normal PSS and Ca^{2+} -free PSS, as needed. The incubated samples were transferred to 3 mm quartz tubes and frozen immediately in liquid nitrogen. The NO standards using spermine NONOate were prepared as followed: a 38 μM spermine NONOate solution was prepared by diluting 10 μl of 38 mM stock spermine NONOate solution in 10 ml of deoxygenated phosphate buffered saline (PBS). Next, a series of standard solutions (20, 40, 100, 200, 1000 μM) were prepared by using varying volumes of 38 μM spermine NONOate solution in a total of 5000 ml deoxygenated PBS. The PBS was deoxygenated in separate vials by the effervescence of nitrogen gas for 4 minutes.

For some experiments, NO standards were prepared by dissolving gaseous NO in deoxygenated PBS. A Schlink line vacuum system was employed in deoxygenating the PBS. NO gas was bubbled into the airtight flasks having deoxygenated PBS to form the stock solution of NO (1.9 mM). The series of dilutions were made by diluting the appropriate volumes of the NO stock solution in deoxygenated PBS buffer in air tight flasks.

All EPR measurements were carried out in a Bruker ESP-300E spectrometer operating at 9.46 GHz using a Bruker ST4102 (TE102) microwave cavity. An Oxford Instruments ESR-900 liquid helium cryostat together with a model ITC-502 temperature controller was used to maintain the sample temperature at 4.2 K. The typical EPR settings were as follows; power 5 mW, field modulation frequency 100 kHz, field modulation amplitude 2.8 G, conversion time 327 ms, scan average 16. Each spectrum of either spermine NONOate treated or hydroxyurea treated RBCs were obtained by subtracting the EPR spectrum from untreated RBCs.

2.11. Statistical Analysis

Either a student t-test or ANOVA was performed to determine statistical significance. Values of $P < 0.05$ were considered to be significant and are indicated in the figures.

3. Results

3.1. Determination of RBC-derived ATP upon stimulation with hydroxyurea

Upon mechanical deformation through microporous filters, microbore tubing, or channels in a microfluidic device, RBCs are capable of releasing ATP, a recognized stimulus of NO

production in various cell types. Here, RBCs were incubated with 50 μ M hydroxyurea for 20 minutes and assayed for ATP release through the flow system described above. As shown in figure 1a, RBCs (obtained from rabbits) that were incubated in hydroxyurea exhibited an $\sim$ 83% increase in the release of ATP in comparison to those cells incubated in buffer alone with no hydroxyurea. Also shown in figure 1a is data showing the release of ATP from RBCs that have been incubated with both hydroxyurea and glibenclamide, an agent known to inhibit the release of ATP from RBCs. The decrease in ATP release in the presence of both hydroxyurea and glibenclamide is evidence that the increase in ATP release in the presence of hydroxyurea is not due to cell lysis. Moreover, our studies suggest there was no change in the intracellular levels of ATP upon incubation with hydroxyurea (1.14 ± 0.09 mM/RBC for control cells vs. 1.20 ± 0.03 mM/RBC for cells incubated with hydroxyurea, $n=3$). Collectively, these data suggest that the measured ATP release is through a secretory mechanism, as opposed to lysis or intracellular ATP depletion.

3.2. Release of ATP Requires a Secondary Stimulus

Results in figure 1 also indicate that the ATP release from the RBCs in the presence of hydroxyurea requires a stimulus, in this case a flow component. Specifically, the results in figure 1b, performed on 4 separate rabbits from those used to obtain the data in figure 1a, show the ATP release from RBCs incubated with hydroxyurea and subjected to flow increased by $61.0 \pm 2.7\%$ in comparison to a $12.9 \pm 2.9\%$ increase for those same cells not subjected to flow. While the ATP increased significantly for both flow and non-flow measurements, these results indicate that hydroxyurea has a much more pronounced effect on the RBC's ability to release ATP when the cells are subjected to flow after hydroxyurea treatment.

3.3. Biphasic Effect of hydroxyurea and Nitric Oxide on RBC-derived ATP Release

An examination of the data in figure 2 shows that increasing concentrations of hydroxyurea in RBCs result in an increase in ATP release up to an hydroxyurea concentration of 100 μ M (in a 7% hematocrit); beyond this value, the ATP release begins to decrease. Figure 3 contains data showing a similar trend for RBCs incubated with increasing concentrations of an NO donor. These two sets of data are intriguing because, as mentioned above, there are many reports suggesting that the effect of hydroxyurea may be NO-mediated. The strikingly similar trends in ATP release found in figures 2 and 3 suggest that this relationship between hydroxyurea and NO may be true. Therefore, subsequent experiments were performed to examine if the hydroxyurea was somehow stimulating NOS in the RBC and producing NO in concentrations that could behave as an agent to improve ATP release.

3.4. Possible Role for Nitric Oxide Synthase in hydroxyurea-Mediated ATP Release

It has been reported that RBCs do contain small amounts of endothelial nitric oxide synthase (eNOS). In addition, numerous reports suggest that the beneficial effect of hydroxyurea may be related to NO. The data in figure 4 shows an increase in ATP release from those RBCs incubated in hydroxyurea and subjected to flow ($38.6\% \pm 9.2\%$ increase in comparison to RBCs alone). However, another aliquot of these same cells were first incubated in L-NAME, a competitive inhibitor of eNOS activity, for 1 hour prior to the addition of hydroxyurea. As shown, the ATP release decreased to a value that was not significantly

different from RBCs alone. Moreover, similar results were obtained for those cells incubated in L-NAME first followed by the addition of hydroxyurea and for those cells where the reagents were added simultaneously. L-NAME by itself (third bar) had no effect on the RBCs ability to release additional ATP, suggesting that the effect of the hydroxyurea is related to NO production by the cell and that L-NAME itself does not appear to affect ATP release on RBCs alone that are subjected to flow.

3.5. Calcium Requirement for hydroxyurea-induced Release of ATP

In order to investigate the NO synthesis in RBCs in more detail, an experiment was performed to examine whether there is an effect from an increase of RBC calcium on the RBCs ability to release ATP. Figure 5 shows the measured ATP release from RBCs in response to hydroxyurea in PSS and Ca^{2+} -free PSS using the flow-based system described above. Similar to studies shown in previous figures, incubation of the RBCs in a buffer containing hydroxyurea increased the ATP release from $0.26 \mu\text{M} \pm 0.02 \mu\text{M}$ (in the absence of hydroxyurea) to $0.48 \pm 0.01 \mu\text{M}$. However, when this same study was repeated (i.e., using the flow-based system) using Ca^{2+} -free PSS, there was no significant change in ATP release from the RBCs. Of course, one possible reason for the decreased ATP release from the RBC may be due to the lack of Ca^{2+} alone, as opposed to the lack of Ca^{2+} affecting hydroxyurea activity on the RBC. Therefore, we also measured ATP release in the presence of C-peptide in Ca^{2+} -free PSS. C-peptide has been shown to increase the ATP release from RBC via a glucose uptake pathway that is independent of any type of secondary stimulus (e.g., deformation or hypoxia). As expected, C-peptide treated RBCs released higher amounts of ATP regardless of the Ca^{2+} content in the PSS, compared to RBCs without C-peptide, confirming that the observed increase in ATP release from RBCs with hydroxyurea in Ca^{2+} -free PSS is not due to an impairment of all the physiological processes in the absence of Ca^{2+} (data not shown).

3.6. Validation Study

To validate that an increase of total Ca^{2+} could be measured in the RBC after incubation with hydroxyurea, another experiment was designed to measure the intracellular Ca^{2+} concentrations in these cells. The RBCs were first incubated with $50 \mu\text{M}$ hydroxyurea for 20 minutes. Lysed RBC samples were then prepared as described above and determined by AAS. The total Ca^{2+} for the RBCs in Ca^{2+} -free PSS and normal PSS was $363 \pm 47 \text{ ng/ml}$ and $530 \pm 52 \text{ ng/ml}$, respectively, indicating an increase in Ca^{2+} into the RBC upon incubation with hydroxyurea in the presence of Ca^{2+} .

3.7. EPR Determination of hydroxyurea-stimulated Nitrosylated Hb

An EPR experiment was carried out at 4 K to detect the HbNO signal in RBC samples treated with hydroxyurea. NO reacts rapidly with deoxygenated hemoglobin to give HbNO. However, in this case, the RBCs were not subjected to deoxygenation in order to preserve the physiological conditions of the circulation.

The spectrum obtained for hydroxyurea treated RBCs was subtracted from the spectrum obtained from untreated RBCs to obtain the difference spectrum, which is in fact showing the HbNO signal due to hydroxyurea. The subtraction method was applied to eliminate the

EPR signal for any copper containing protein, ceruloplasmin ($g = 2.06$ where g is the spectroscopic splitting factor), in RBCs as it overlaps almost the same field where HbNO occurs. The difference spectrum was compared with the difference spectrum obtained from RBCs incubated with a known concentration of spermine NONOate. The comparison of the two subtracted spectra obtained from hydroxyurea and spermine NONOate is shown in figure 6a for comparison. The triplet hyperfine coupling and the g values at 2.01 are the characteristic features of HbNO formation. The data in figure 6a suggest that hydroxyurea is not leading to any significant increase in HbNO formation. However, the experiments performed to generate the data in figure 6a were repeated as the initial EPR studies were carried out in Ca^{2+} -free PBS. RBCs were incubated with $50 \mu\text{M}$ hydroxyurea for 20 minutes in normal PSS and Ca^{2+} -free PSS. Interestingly, a higher HbNO signal was observed for RBCs in the normal PSS, where Ca^{2+} was present, compared to the RBCs that were in Ca^{2+} -free PSS. The signal subtraction method was employed here to eliminate the interference from the ceruloplasmin protein. A comparison of the difference spectra obtained for hydroxyurea treated RBCs in Ca^{2+} -free and normal PSS is shown in figure 6b. The triplet structure obtained at $g = 2.0$ confirmed the presence of HbNO signal for calcium containing buffer, suggesting the involvement of Ca^{2+} in the NO formation due to hydroxyurea.

4. Discussion

Sickle cell disease is one of the most common genetic diseases in the United States, affecting more than 85,000 people. Formation and the presence of hemoglobin S (HbS) in the red blood cell (RBC) results in the majority of pathophysiological conditions associated with the disease. Although sickle cell disease was originally considered a disease of the RBC, its major pathophysiological complications are also associated with the alteration of vascular properties. People with sickle cell are known to have stiffened RBCs, low nitric oxide (NO) bioavailability, and increased RBC- endothelial interactions.

Even though hydroxyurea is approved and has proven beneficial in the treatment of sickle cell disease, there remain some interesting features involved with the therapy that, if better understood, would lead to improved therapeutic intervention. Specifically, based on clinical results, hydroxyurea may be beneficial by means other than induction of HbF levels. For example, in some instances, improvements in blood flow are seen in patients hours after taking hydroxyurea, although increased HbF levels may not be detectable for weeks or more. Such data suggests that the beneficial rheological/vasculature effects may involve something other than increased levels of HbF.

It is now well-established that RBCs will release ATP in response to a number of stimuli including deformation, hypoxia, acidosis, as well as a number of pharmacological/molecular stimuli including iloprost and C-peptide. There are also a number of agents that will inhibit this release such as glibenclamide, niflumic acid, and, as reported by Olearczyk et al, NO.

Interestingly, NO has been reported to increase and decrease RBC deformability. As shown here, lower concentrations of NO enhance deformation-induced ATP release, but that higher concentrations decrease the ATP release. A comparison between Olearczyk's results and those reported here reveals similar trends; for example, Olearczyk reported a decrease in

ATP release (per 2×10^4 cells) from those RBCs incubated with an NO donor (100 nM in the incubation solution). The data reported here used similar levels of NO, but per 3×10^6 RBCs; in other words, the experiments performed here employed approximately 100 moles less of NO per cell in those studies where an increase of ATP release was determined. In those studies using similar levels of NO as those reported by Olearczyk, the results are the same (a decrease in ATP release). It should also be noted that two recent reports have also reported that addition of NO or NO metabolites to aliquots of RBCs resulted in an increased ability of these cells to release ATP.

Similar to the addition of NO to RBCs, there are also conflicting reports that hydroxyurea results in an increase in deformability, while others suggest a decrease in RBC deformability upon incubation with hydroxyurea. Many groups have demonstrated a relationship between NO and the addition of hydroxyurea. Collectively, the data presented here, in combination with previous reports, suggest that hydroxyurea may be stimulating increases in NO which, in turn, is enabling the RBC to release increased levels of ATP under certain conditions.

Intracellular calcium is important in the NO synthesis in all the cell types containing the constitutive form of NOS and RBCs are not an exception. Therefore, an influx of calcium can increase the formation of calcium-calmodulin complex, thereby increasing the NO synthesis within the cell. An earlier study has shown the ability of hydroxyurea to increase the intracellular calcium concentration in endothelial cells by immobilizing the calcium pools in the endoplasmic reticulum. However, the work here shows for the first time that an increase in calcium influx occurs upon addition of hydroxyurea to RBCs.

In contrast to the data presented here, which requires eNOS activity in the RBC, there are other reports that hydroxyurea stimulates NO production in the RBC independently of NOS activity. Specifically, Nahavandi reported that hydroxyurea does increase NO production (similar to that reported here), although this NO production was independent of NOS activity as inhibition with L-NMMA had no effect on the NO production. One interesting feature of this previous work, however, is that the authors added 1 mM arginine to the samples in addition to the NOS inhibitor. L-NMMA is a competitive inhibitor of NOS; therefore, with this amount of L-arginine added (which were not added in the studies reported here), it may be possible that the L-NMMA would have minimal effect due to the high levels of exogenous substrate added to the samples.

Although to date we have not performed any measurements involving deformability of the RBC in the presence of hydroxyurea, it is evident that the increase in ATP release only occurs in the presence of flow. In fact, recent work has shown that nitrite-enhanced ATP release involved an increased binding of nitrite-modified hemoglobin to the RBC membrane that displaces glycolytic enzymes from the membrane, resulting in the formation of a pool of ATP that is released from the RBC.

In conclusion, the results obtained provide further information regarding a relationship between hydroxyurea and NO in RBCs. Strong evidence of increased NO production in the presence of hydroxyurea has been previously reported by other groups. However, the mechanism by which that NO was produced was not totally clear. Here, data suggests that

the increase in NO production by the RBC in the presence of hydroxyurea is due to a calcium influx into the cell. Importantly, the resultant increase in ATP from hydroxyurea-incubated RBCs (upon being subjected to flow) may provide some insight into the immediate improvements in overall blood flow when hydroxyurea is administered to people with sickle cell. Admittedly, the exact relationship between the improvements in ATP release from the RBCs and the NO production by these cells is not fully clear, although due to the necessity of “flow”, the improvement seems to be related to an enhancement in RBC deformability.

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Figure 1.

In (A) RBC-derived ATP release in the absence (7% RBC) and presence (7% RBC + hydroxyurea) of 50 μ M hydroxyurea is shown. An aliquot of RBCs was also incubated with glibenclamide (7% RBC + Gli + hydroxyurea) to verify that the ATP release was not due to cell lysis. In (B), the ATP release was determined in the presence (first set of bars) and absence (second set of bars) of flow for RBCs in the presence and absence of hydroxyurea. In (A) the error bars are reported as S.E.M. for $n = 5$ rabbits. In (B), the bars corresponding to flow conditions were reported as S.E.M. for $n = 4$ rabbits. In (A), the asterisk (*) represents a statistically significant difference from both RBCs without hydroxyurea and with hydroxyurea in the presence of glibenclamide; in (B), * represents significance when comparing the RBCs with hydroxyurea to RBCs without hydroxyurea.

Figure 2.

RBC-derived ATP release under conditions of flow measured as a function of hydroxyurea added to a 7% Hct of RBCs. The highest ATP release was observed for RBCs incubated with 100 μ M hydroxyurea for 20 minutes. Beyond this point, the ATP release decreased, reaching a value that was not significantly different from untreated RBCs at 200 μ M hydroxyurea. All values of ATP release were significantly different from basal levels except for the 200 μ M incubation. The error bars are reported as S.E.M. for n =7 rabbits.

Figure 3.

Effect of spermine NONOate on deformation-induced ATP release from RBCs (in the presence of flow). The ATP release was initially increased gradually with increasing concentration of NO donor and the highest release was observed for 7% RBCs incubated with 250 nM Spermine NONOate. Beyond this level, the ATP release gradually decreased to a point at 1000 nM where the ATP release was not significantly different from the initial untreated 7% RBCs. The asterisk (*) represents statistical significance between samples in comparison to RBCs in the absence of any NO donor.

Figure 4.

Measurement of ATP release from RBCs in the presence of L-NAME and hydroxyurea under conditions of flow. While a significant increase in ATP release was measured for RBCs in the presence of hydroxyurea (7% + hydroxyurea) in comparison to RBCs alone (7%), the addition of L-NAME to the RBCs prior to hydroxyurea (7% + L-NAME + hydroxyurea) or after hydroxyurea (7% + hydroxyurea + L-NAME) resulted in ATP release values that were not increased from basal levels. Importantly, the addition of L-NAME alone to the RBCs did not affect flow-induced ATP release from these cells, suggesting that the effect of the L-NAME is related to changes due to the presence of hydroxyurea. The values reported are averages from $n = 4$ rabbits and the error bars are represented as S.E.M. The asterisk (*) represents a statistically significant difference between the RBCs with hydroxyurea and those without (first and third bars) or those with hydroxyurea and L-NAME.

Figure 5.

Measurement of RBC-derived release of ATP (in the presence of flow) in the presence and absence of calcium ions in the medium. The black bars represent the ATP release of untreated RBCs and gray bars represents the ATP release from RBCs treated with 50 μ M hydroxyurea. Increased ATP release is observed for hydroxyurea treated RBCs in PSS (a calcium-containing buffer) while no significant increase of ATP release from RBCs is observed for RBCs treated with hydroxyurea in PSS without the addition of calcium. The data represented are reported for $n = 5$ rabbits and the error bars are reported as S.E.M. The asterisk (*) represents a statistically significant difference between those cells with hydroxyurea in the presence of Ca^{2+} and those with either Ca^{2+} alone (first bar) or no Ca^{2+} at all (third and fourth bars). This data suggests that the presence of Ca^{2+} is requisite for measured effects of hydroxyurea on the RBC.

Figure 6.

The difference spectra obtained for spermine NONOate treated RBCs and hydroxyurea treated RBCs in the absence of calcium is shown in (a). The triplet hyperfine structure at $g = 2.01$ is the characteristic signal of HbNO in RBCs. The triplet hyperfine structure corresponding to the g values of HbNO in NO treated RBCs is not significant in hydroxyurea treated RBCs. The EPR settings were as follows; power 5 mW, modulation frequency 9.46 GHz, field modulation amplitude 2.8 G, conversion time 327 ms, temperature 4.2 K, scan average 4. In (b), the difference EPR spectra obtained from RBCs incubated with 50 μM hydroxyurea in calcium free PSS and in calcium containing PSS are shown. The triplet hyperfine structure with g values at 2.02 indicates the presence of HbNO in the spectrum.