Nitric Oxide Inhibits ATP Release from Erythrocytes

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Abstract

Erythrocytes have been reported to release adenosine triphosphate (ATP) from intracellular stores into the surrounding environment in response to decreased oxygen tension and mechanical deformation. This erythrocyte-derived ATP can then act on purinergic receptors present on vascular endothelial cells resulting in the synthesis and bi-directional release of nitric oxide (NO). NO released abluminally, produces relaxation of vascular smooth muscle, thereby increasing vascular caliber, leading to a decrease in deformation-induced ATP release from erythrocytes. In contrast, NO released into the vascular lumen could interact directly with formed elements in the blood, including the erythrocyte. Here we investigate the hypothesis that NO functions in a negative-feedback manner to inhibit ATP release from the erythrocyte. The NO donor, N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate), decreased total pulmonary resistance in a dose-dependent fashion when administered to isolated perfused rabbit lungs. ATP release from rabbit erythrocytes in response to decreased oxygen tension or mechanical deformation was inhibited by pre-incubated with spermine NONOate (100 nM, 20min). Importantly, incubating rabbit erythrocytes with spermine (100 nM, 20 min), the polyamine remaining after the liberation of NO from spermine NONOate, did not affect decreased oxygen tension-induced ATP release. Mechanical deformation-induced ATP release was also inhibited when erythrocytes were pre-incubated with spermine NONOate. However, NO-depleted spermine NONOate had no effect on mechanical deformation-induced ATP release from rabbit erythrocytes. These data provide support for the hypothesis that NO inhibits ATP release from erythrocytes, thereby identifying an additional role of NO in the regulation of vascular resistance.

The erythrocyte, via its ability to release adenosine triphosphate (ATP), has been identified as a potential regulator of vascular resistance (Ellsworth et al., 1995; Sprague et al., 1996). The release of ATP from intracellular stores into the surrounding environment is reported to occur in response to distinct physiological stimuli. One such stimulus, mechanical deformation, can occur as the erythrocyte passes through small resistance vessels and capillaries of the microcirculation or in response to increases in linear flow velocity. Sprague *et. al.* reported that both rabbit and human erythrocytes release ATP in response to mechanical deformation produced by passage of erythrocytes through filters with an average pore size of 5 μm (Sprague et al., 1996). Human and hamster erythrocytes have also been reported to release ATP in response to decreased oxygen tension and/or reduced pH (Bergfeld and Forrester, 1992; Ellsworth et al., 1995), environments the erythrocyte would encounter as it traverses skeletal muscle and other oxygen consuming organs.

Once released from the erythrocyte into the circulation, ATP can activate purinergic receptors, specifically those of the P_{2y} subfamily, present on the vascular endothelium resulting in the synthesis and release of NO. NO released abluminally interacts with vascular smooth muscle resulting in its relaxation. The resultant increase in vascular caliber would decrease the stimulus for deformation-induced ATP release. An increase in vascular caliber would also decrease ATP release in response to decreased oxygen tension by increasing oxygen delivery to the tissue, resulting in improved matching of oxygen supply with metabolic demand (Ellsworth, 2000).

In addition to being released abluminally, NO is released luminally where it can interact directly with formed elements in the blood, including the erythrocyte. NO is a highly reactive free radical that has been identified as a regulatory molecule in a number of systems including,

but not limited to, the immune, nervous and cardiovascular systems (Garthwaite, 1991; Bredt and Snyder, 1994; Garthwaite and Boulton, 1995). NO regulates these systems by stimulating or inhibiting the activity of several proteins (Garthwaite, 1991; Bredt and Snyder, 1994; Garthwaite and Boulton, 1995). Therefore, it is possible that luminal release of NO could interact with circulating erythrocytes modulating their release of ATP.

In the present studies, we investigate the hypothesis that NO functions in a negative-feedback manner to inhibit ATP from circulating erythrocytes. We determined the effect of increasing concentrations of N-(2-aminoethyl-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate), a NO donor, on total pulmonary vascular resistance in the isolated rabbit lung. In addition, we investigated the effect of spermine NONOate on ATP release in response to decreased oxygen tension and mechanical deformation. Finally, we examined the effect of spermine, the polyamine remaining after the liberation of NO from spermine NONOate, on ATP release in response to decreased oxygen tension, as well as the effect of NO-depleted spermine NONOate on mechanical deformation-induced ATP release from erythrocytes.

Materials and Methods

Isolated Perfused Rabbit Lungs. New Zealand white rabbits (random sex, 2 to 3 kg body weight) were anesthetized with ketamine (12.5 ml/kg im) and xylazine (1 mg/kg im) followed by pentobarbital sodium (10 mg/kg iv). Following tracheal intubation, the animals were mechanically ventilated with room air (tidal volume 10 ml/kg, rate 20-25 breaths/min). A catheter was placed into the carotid artery for administration of heparin (500 U) and for phlebotomy. Ten minutes after the administration of heparin, the animals were exanguinated. The heart and lungs were removed en block during continuous ventilation. Catheters were placed in the main pulmonary artery and the left atrium. The heart and lungs were suspended in a humidified chamber maintained at 37°C and ventilated with 15%O_2-6% CO_2, balance N_2. Blood was removed from the lungs by perfusion with 200 mL of physiological salt solution (PSS, 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 0.026 mM Na-EDTA, 11.1 mM glucose and 5% hetastarch (Baxter Healthcare, Deerfield, IL) without recirculation. The lungs were then perfused with PSS at 80 ml/min with a recirculating volume of 100 mL. The pulmonary arterial pressure (P_{pa}), left atrial pressure (P_{la}), and airway pressure (P_{aw}) were recorded continuously. P_{la} was set to a pressure of 2-3 mmHg with a screw clamp. Lungs were perfused under zone III conditions ($P_{pa}>P_{la}>P_{aw}$). Samples of perfusate were analyzed for pH, O_2 and CO_2 tensions under control and experimental conditions (pHOx blood gas analyzer, Nova Biomedical; Waltham, MA). After hemodynamic and blood gas stability was achieved under baseline conditions (flow rate of 80 ml/min), P_{pa} was increased by infusion of 5-hydroxytryptamine. When hemodymamic stability was achieved at the increased P_{pa}, spermine NONOate was added to the reservoir and the pressures recorded after 3 min.

Isolation of rabbit and human erythrocytes. Rabbit were anesthetized, ventilated, and exsanguinated as described above. Human erythrocytes were obtained by venipuncture performed in an antecubital vein without the use of a tourniquet. Sixty milliliters of blood were collected into a syringe containing 50 U of heparin. Protocols used to obtain rabbit and human blood were approved by the Institutional Animal Care and Use Committee and the Institutional Review Board of Saint Louis University, respectively.

Rabbit or human blood, was centrifuged at 500 x g for 10 min at 4 °C. The plasma, buffy coat, and uppermost erythrocytes were removed by aspiration and discarded. The remaining erythrocytes were washed three times in buffer solution containing 140.5 mM NaCl, 21.0 mM Tris, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.1% dextrose, and 0.5% bovine albumin, fraction V (ICN Biomedicals, Inc., Aurora, OH), final pH 7.4. The hematocrit of the washed erythrocytes was determined using an Autocrit Ultra 3 centrifuge (Becton Dickinson, Bedford, MA).

Decreased oxygen tension-induced ATP release. Washed rabbit or human erythrocytes were brought to a 10% hematocrit with a buffered solution containing 140.5 mM NaCl, 21.0 mM Tris, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.5% dextrose, pH 7.4. The resuspended erythrocytes were then diluted to a 0.1% hematocrit with buffered solution in a tissue chamber set at 37 °C. The buffered solution in each tissue chamber was equilibrated with either 15% O_2, 6% CO_2, 79% N_2 (P_{O2} = 100-110 mmHg, P_{CO2} = 30-32 mmHg, pH = 7.38 ± 0.01) or 0% O_2, 6% CO_2, 94% N_2 (P_{O2} = 35-40 mmHg, P_{CO2} = 30-32 mmHg, pH = 7.38 ± 0.01). Immediately following the addition of the erythrocytes to the tissue chamber, the chamber was covered and the gas turned off. The ATP released into the buffered solution was measured one minute after the addition of the erythrocytes to the chamber. The pH and gas tensions were determined

immediately following the measurement of ATP, using a blood-gas analyzer (Radiometer; Bronshoj, Denmark).

Mechanical Deformation of Washed Erythrocytes. Washed rabbit erythrocytes were brought to 10% hematocrit with a physiological salt solution containing 140.5 mM NaCl, 21.0 mM Tris, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.1% dextrose, and 0.5% bovine albumin (pH 7.4) and then subjected to mechanical deformation using the St. George's Blood Filtrometer (Carri-Med Ltd., Dorking, U.K.). As described previously, this device creates a calibrated pressure gradient across a vertically mounted filter with pores having an average diameter of 5 μm (Sprague et al., 1996). Deformation was achieved by passing the suspended erythrocytes through the filter. The red cell transit time (RCTT), a measure of erythrocyte deformability, as well as the concentration of ATP in the filter effluent were determined.

Measurement of ATP. ATP was measured using the luciferin-luciferase technique (Strehler, 1968). A 200 μl sample of an erythrocyte suspension was injected into a cuvette containing 100 μl of a 1 mg/ml crude firefly-tail extract (Sigma, St. Louis, MO) and 100 μl of a 0.5 mg/ml solution of D-luciferin (Sigma). The addition of D-luciferin to the reaction mixture increases the sensitivity of the assay (Bergfeld and Forrester, 1992). The light emitted from the reaction of ATP with the crude firefly extract/D-luciferin was measured using a luminometer set at a wavelength of 565 nm (Turner Designs, Sunnyvale, CA, model TD-20/20). The emission peak was compared to an ATP standard curve generated on the day of the experiment. The concentration of ATP was determined by comparing the sample signal with that of the standard curve. We have established that standard curves run in the absence of erythrocytes are not different from those run in the presence of non-stimulated erythrocytes. Spermine NONOate, at the concentrations used in these experiments, did not alter the ATP signal.

Measurement of erythrocyte lysis. To exclude the possibility that erythrocyte lysis contributes to the measurement of ATP release, after measuring ATP in the erythrocyte suspension, erythrocytes were sedimented by centrifugation at 500 x g for 10 min. The presence of hemoglobin in the supernatant was determined by light absorption at 405nm (van Kampen and Zijlstra, 1983).

To ensure that the method used to detect erythrocyte lysis is as sensitive as the assay used to measure ATP release, washed rabbit erythrocytes were lysed and serial dilutions made. The concentration of ATP and the absorbance at 405nm was measured for each dilution. In dilutions where ATP was measured, an absorbance at 405nm was also detected; verifying that the assay used to measure erythrocyte lysis is as sensitive as the assay used to measure ATP release.

Statistical Methods. Statistical significance between experimental periods was determined with an ANOVA, or where appropriate a student's t-test. When using an ANOVA, a least significant difference test was used to identify individual differences, in the event that the F ratio indicated that changes had occurred. *P* values of 0.05 or less were considered statistically significant. Results are reported as means ± SEM.

Results

Effect of spermine NONOate administration on vascular resistance in isolated rabbit lungs perfused with physiological salt solution.   To determine the effect of NO liberated from an NO donor on pulmonary vascular resistance, spermine NONOate was added to the perfusate of isolated rabbit lungs perfused with physiological salt solution.   Following hemodynamic, pH, and blood gas stability at a flow rate of 80 ml/min (minimum of 15 min), pulmonary artery pressure (P_{pa}) was increased from 9.5 ± 0.6 to 24.0 ± 0.7 mmHg by the infusion of 5-hydroxytryptamine (5-HT), while keeping the left atrial pressure constant at 2.3 ± 0.1 mmHg.   When hemodynamic stability was attained at the increased P_{pa}, spermine NONOate was added to the reservoir to achieve circulating concentrations of 1, 10, 100 and 1,000 nM.   As depicted in figure 1, the addition of spermine NONOate to the perfusate resulted in dose-dependent decreases in P_{pa}.   In separate experiments, administration of spermine (1,000 nM), the polyamine resulting from the liberation of NO from spermine NONOate, had no effect on P_{pa} in lungs constricted with 5-HT.   In the latter studies, a decrease in the P_{pa} from 19.8 ± 2.2 to 13.6 ± 1.9 mmHg while keeping the left atrial pressure constant at 2.5 ± 0.1 mmHg following the addition of nitroglycerine (1 µM) demonstrated that the lungs were capable of relaxation.

Effect of spermine NONOate on ATP release from erythrocytes in response to decreased oxygen tension.   To demonstrate that NO inhibits ATP release in response to decreased oxygen tension, washed rabbit erythrocytes were incubated with the NO donor spermine NONOate (100 nM, 20 min) and then exposed to either a normal or a decreased oxygen tension.   As illustrated in figure 2a, preincubation of rabbit erythrocytes with spermine NONOate inhibited ATP release in response to decreased oxygen tension.   In separate experiments, spermine NONOate also inhibited ATP release from human erythrocytes in

response to decreased oxygen tension, figure 2b. In experiments using rabbit or human erythrocytes, there was no measurable difference in the total intracellular concentration of ATP between erythrocytes treated with spermine NONOate and those that were untreated (rabbit: 1.2 x 10^{-3} M and 1.2 x 10^{-3} M ATP; human: 1.6 x 10^{-3} M and 1.9 x 10^{-3} M ATP for untreated and spermine NONOate treated erythrocytes, respectively). Importantly, increases in ATP release measured from both rabbit and human erythrocytes were not the result of erythrocyte lysis, as free hemoglobin was not detected in any of the samples.

Basal ATP release is measured when erythrocytes are exposed to a normal oxygen tension (figure 2). To determine whether spermine NONOate could inhibit the basal release of ATP, washed rabbit erythrocytes were incubated with spermine NONOate (100 nM, 20 min) and then exposed to a normal oxygen tension. Spermine NONOate had no effect on basal release of ATP (15.9 $\pm$ 2.7 vs. 17.4 $\pm$ 4.1 nM ATP per 2×10^4 erythrocytes/mm^3 for untreated and spermine NONOate treated erythrocytes, respectively, n = 4). It is possible that the ATP measured under a normal oxygen tension is the result of the method used to obtain erythrocytes.

Effect of spermine on ATP release from erythrocytes in response to decreased oxygen tension. Spermine NONOate is synthesized by exposing a nucleophilic species, spermine, to NO in the absence of oxygen. This process yields the stable NO donor in powdered form. When spermine NONOate is dissolved it dissociates to regenerate NO and the nucleophilic species, spermine, as a by-product. Although the by-products of most NO donors are inactive in most biological systems, it is not known whether the by-products of spermine NONOate, spermine, can influence ATP release from erythrocytes. To ensure that the inhibition in ATP release in response to decreased oxygen tension resulted from the action of NO and not spermine, washed rabbit erythrocytes were incubated with spermine, and then exposed to either a

normal or decreased oxygen tension. As described in figure 3, spermine had no effect on ATP

release from rabbit erythrocytes in response to decreased oxygen tension. Incubating rabbit

erythrocytes with spermine did not alter intracellular concentration of ATP ($1.6 \times 10^{-3} \pm 2.4 \times 10^{-4}$ M ATP for untreated erythrocytes versus $1.7 \times 10^{-3} \pm 3.1 \times 10^{-4}$ M ATP for treated

erythrocytes) or induce erythrocyte lysis.

Effect of spermine NONOate on ATP release from erythrocytes in response to

mechanical deformation. To provide support for the hypothesis that NO inhibits ATP release

from erythrocytes, and that this inhibition is not unique to decreased oxygen tension, washed

rabbit erythrocytes were incubated with spermine NONOate (100 nM, 20 min) and then

mechanically deformed by passage through filters with a pore size of 5 μm using the St. Georges

Blood Filtrometer. As depicted in figure 4a, spermine NONOate inhibited mechanical

deformation-induced ATP release from rabbit erythrocytes. In these experiments incubating

rabbit erythrocytes with spermine NONOate did not alter erythrocyte deformability or

intracellular concentrations of ATP. In addition, increases in ATP were not the result of

erythrocyte lysis as increases in free hemoglobin were not detected in any of the samples.

Effect of NO-depleted spermine NONOate on mechanical deformation-induced ATP

release from erythrocytes. To ensure that the inhibition of mechanical deformation-induced

ATP release was not the result of the by-product formed from the liberation of NO, washed

rabbit erythrocytes were incubated with NO-depleted spermine NONOate. In these experiments,

spermine NONOate was dissolved in aqueous solution 12 hours prior to its use. This time

interval allows for the complete liberation of NO leaving only the degradation product. Washed

rabbit erythrocytes were incubated with the NO-depleted spermine NONOate and then

mechanically deformed. The NO-depleted donor had no effect on mechanical deformation-

induced ATP release, figure 4b. In addition, incubation of erythrocytes with spermine NONOate did not alter the red cell transit time, indicating that incubating with NO-depleted spermine NONOate did not alter erythrocyte deformability. This provides support for the hypothesis that NO itself, not the by-product resulting from the liberation of NO, inhibits ATP release from erythrocytes in response to mechanical deformation.

Discussion

Erythrocytes have been reported to release ATP in response to either decreased oxygen tension or mechanical deformation (Bergfeld and Forrester, 1992; Ellsworth et al., 1995; Sprague et al., 1996). Previously, we reported that ATP released from erythrocytes in the pulmonary circulation produced decreases in vascular resistance via stimulation of endogenous NO synthesis (Sprague et al., 1996). NO that is released abluminally from the endothelium interacts with vascular smooth muscle resulting in vasorelaxation. However, NO is also released into the vascular lumen where it can interact directly with formed elements in the blood, including the erythrocyte. In the present study, we hypothesize that NO released luminally from the vascular endothelium in response to erythrocyte-derived ATP serves as a signal to inhibit additional ATP release from circulating erythrocytes.

Addition of the NO donor, spermine NONOate, to the perfusate of isolated rabbit lungs in which the pulmonary vascular resistance is increased with 5-HT resulted in a dose-dependent decrease in total pulmonary vascular resistance (see figure 1). In identical experiments, spermine, the polyamine remaining from the liberation of NO from spermine NONOate, had no effect on total pulmonary vascular resistance. These experiments demonstrate that NO liberated from spermine NONOate can influence vascular reactivity and serve to identify a physiologically relevant dose of spermine NONOate (100 nM) used in subsequent experiments. Concentrations of NO in the nanomolar range are consistent with previously reported measurements from endothelial cells *in vitro* and *in vivo* (Kalinowski et al., 2001; Balbatun et al., 2003; Kalinowski et al., 2003) Spermine NONOate, but not spermine, the polyamine remaining from the liberation of NO from spermine NONOate, inhibited ATP release from rabbit and human erythrocytes in response to decreased oxygen tension (see figures 2 and 3, respectively). In separate

experiments, spermine NONOate, but not NO-depleted spermine NONOate, inhibited ATP release in response to mechanical deformation (see figure 4). Taken together, these data provide support for the hypothesis that NO inhibits ATP release from erythrocytes.

The ability of NO to serve as a regulatory molecule is not limited to the inhibition of ATP release from erythrocytes. NO has been identified as an important regulatory molecule in other systems including the immune, nervous and cardiovascular systems (Garthwaite, 1991; Bredt and Snyder, 1994; Garthwaite and Boulton, 1995). NO can regulate these systems by stimulating or inhibiting the activity of several proteins (Garthwaite, 1991; Bredt and Snyder, 1994; Garthwaite and Boulton, 1995). Among these proteins are adenylyl cyclase and the heterotrimeric G protein Gi, both of which are components of a proposed signal transduction pathway for ATP release from erythrocytes (Sprague et al., 2001; Olearczyk et al., 2003). NO gas as well as the NO donors 3-morpholinosydnonimine and S-nitroso-N-acetlypenicillamine were reported to directly inhibit the catalytic activity of adenylyl cyclase types V and VI, but not other types of adenylyl cyclase (Tao et al., 1992; Duhe et al., 1994; McVey et al., 1999; Hill et al., 2000). Inhibition of adenylyl cyclase activity by NO was proposed to result from S-nitrosylation. S-nitrosylation is the non-enzymatic addition of NO to a protein thiol, a cysteine residue in the case of adenylyl cyclase (Stamler, 1994). Therefore, if the adenylyl cyclase in the proposed signal transduction pathway for ATP release from erythrocytes is type V or type VI, then NO could inhibit ATP release via S-nitrosylation.

In addition to inhibiting adenylyl cyclase activity, NO has also been reported to indirectly inhibit the activity of the heterotrimeric G protein Gi/o (Brune and Lapetina, 1989; Dimmeler and Brune, 1991; Pozdnyakov et al., 1997). Incubating the cytosolic or membrane fractions from a variety of cell types with NO gas or various NO donors in the presence of $[^{32}P]NAD$ increased

the [^{32}P]-labeling of several proteins, including the heterotrimeric G protein Gi/o (Pozdnyakov et al., 1993; Sheffler et al., 1995; Zoche and Koch, 1995; Sullivan et al., 1997). This action of NO resulted from the stimulation of an endogenous mono-ADP-ribosyltransferase, an enzyme that catalyzes the addition of the ADP-ribose moiety from nicotinamide adenine dinucleotide to protein (Brune and Lapetina, 1989; Dimmeler and Brune, 1991; Schuman et al., 1994). An endogenous mono-ADP-ribosyltransferase has been identified and purified from the cytosol of human erythrocytes (Tanuma et al., 1988). This mono-ADP-ribosyltransferase mono-ADP-ribosylates the α-subunit of Gi/o. We have identified the heterotrimeric G proteins Gαi1, Gαi2 and Gαi3 but not Gαo in rabbit and human erythrocyte membranes (Olearczyk et al., 2003). The identification of an endogenous mono-ADP-ribosyltransferase in erythrocytes that ADP-ribosylates Gi and that may be activated by NO suggests a second possible mechanism for the regulation of ATP release from erythrocytes.

The ability of NO to directly inhibit adenylyl cyclase activity and/or indirectly inhibit the activity of the heterotrimeric G protein Gi/o, via stimulation of an endogenous mono-ADP-ribosyltransferase, is consistent with the hypothesis that NO inhibits a signal transducation pathway for ATP release from erythrocytes. Under this regulatory mechanism, ATP released from the erythrocyte, in response to mechanical deformation or decreased oxygen tension, would activate P$_{2y}$ receptors located on the vascular endothelium, stimulating the synthesis and bi-directional release of NO. NO released abluminally would result in relaxation of vascular smooth muscle, thereby increasing vascular caliber and decreasing mechanical deformation-induced ATP release. In addition, an increase in vascular caliber would decrease ATP release in response to decreased oxygen tension by increasing oxygen delivery to the tissue, thereby matching oxygen supply with metabolic demand. The NO released luminally would interact the

erythrocyte where it could directly inhibit adenylyl cyclase activity and/or indirectly inhibit the activity of Gi/o via stimulation of an endogenous mono-ADP-ribosyltransferase. Either action of NO would be expected to inhibit a signal transduction pathway for ATP release from erythrocytes and thereby identifies an additional role of NO in the regulation of vascular resistance.

Acknowledgements

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Footnotes

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Figure 1. Effect of spermine NONOate administration on vascular resistance in the isolated rabbit lung perfused with physiological salt solution. Rabbit lung was isolated and perfused with physiological salt solution (80 ml/min) until hemodynamic and blood gas stability was achieved. The P_{pa} was increased from 9.5 ± 0.6 to 24.0 ± 0.7 mmHg by infusion of 5-hydroxytryptamine. Spermine NONOate was then added to the circulating volume (100 ml) to achieve a final concentration of 1, 10, 100 or 1,000 nM (n = 5). Values are means $\pm$ SEM and are expressed as the percent change from post-5-HT measurements. **$P < 0.01$, different from baseline, †$P < 0.01$, different from 1 and 10 nM spermine NONOate, ††$P < 0.01$, different from 100 nM spermine NONOate, †††$P < 0.01$, different from 1,000 nM spermine NONOate.

Figure 2. A. Effect of spermine NONOate on ATP release from rabbit erythrocytes in response to decreased oxygen tension. Washed rabbit erythrocytes were incubated in the presence or in the absence of spermine NONOate (spNO, 100 nM, 20 min) and then exposed to either a normal oxygen tension ($P_{O2} = 111.8 \pm 0.7$ mmHg, $P_{CO2} = 29.9 \pm 0.6$ mmHg, pH $= 7.378 \pm 0.002$) or a reduced oxygen tension ($P_{O2} = 36.2 \pm 0.2$ mmHg, $P_{CO2} = 29.3 \pm 0.3$ mmHg, pH $= 7.377 \pm 0.002$), n = 5. The amount of ATP released is reported as the mean $\pm$ SEM. **$P < 0.01$, different from erythrocytes exposed to normal oxygen tension and erythrocytes exposed to decreased oxygen tension after pretreatment with spermine NONOate. B. Effect of spermine NONOate on ATP release from human erythrocytes in response to decreased oxygen tension. Washed human erythrocytes were incubated in the presence or in the absence of spermine NONOate (spNO, 100 nM, 20 min) and then exposed to either a normal oxygen tension ($P_{O2} = 113.1 \pm 0.6$ mmHg, $P_{CO2} = 31.4 \pm 0.1$ mmHg, pH $= 7.381 \pm 0.002$) or a reduced oxygen tension ($P_{O2} = 37.3 \pm 0.8$ mmHg, $P_{CO2} = 31.6 \pm 0.1$ mmHg, pH $= 7.382 \pm 0.002$), n = 5. The amount of ATP released reported as

the mean $\pm$ SEM. **$P < 0.01$, different from erythrocytes exposed to normal oxygen tension and erythrocytes exposed to decreased oxygen tension after pretreatment with spNO.

Figure 3. Effect of spermine on ATP release from rabbit erythrocytes in response to decreased oxygen tension. Washed rabbit erythrocytes were incubated in the presence or in the absence of spermine (100 nM, 20 min) and then exposed to either a normal oxygen tension ($P_{O2} = 113.1 \pm 0.6$ mmHg, $P_{CO2} = 31.4 \pm 0.1$ mmHg, pH $= 7.381 \pm 0.002$) or a reduced oxygen tension ($P_{O2} = 37.3 \pm 0.8$ mmHg, $P_{CO2} = 31.6 \pm 0.1$ mmHg, pH $= 7.382 \pm 0.002$), n = 5. The amount of ATP released is reported as the mean $\pm$ SEM. **$P < 0.01$, different from erythrocytes exposed to normal oxygen tension.

Figure 4. A. Effect of spermine NONOate on mechanical deformation-induced ATP release from rabbit erythrocytes. Washed rabbit erythrocytes were incubated in the presence or in the absence of spermine NONOate (spNO, 100 nM, 20 min) and then mechanically deformed by passage through filters with a pore size of 5 µm using the St. Georges Blood Filtrometer, n = 7. The amount of ATP released is reported as the mean $\pm$ SEM. **$P < 0.01$, different from baseline and deformation + spNO. B. Effect of NO-depleted spermine NONate on mechanical deformation-induced ATP release from rabbit erythrocytes. Spermine NONOate (spNO, 100 nM) was prepared 12 hours prior to use in the experiment. This ensures that the NO is completely liberated from the donor leaving only the degradation product, spermine. Washed rabbit erythrocytes were incubated in the presence and in the absence of the NO-depleted NO donor and then mechanically deformed by passage through a filter with an average pore size of 5

μm using the St. Georges Blood Filtrometer, n = 7. The amount of ATP released is reported as

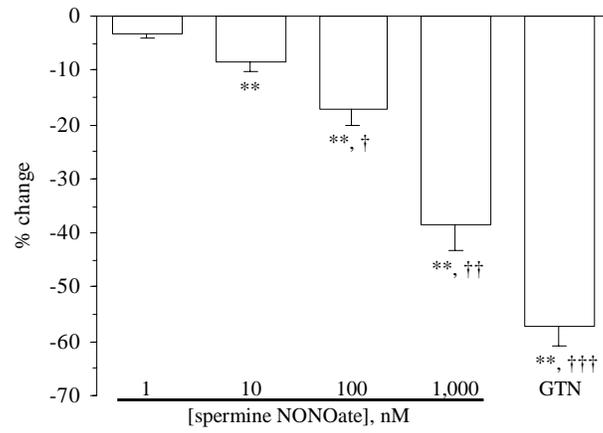the mean ± SEM. **$P < 0.01$, different from baseline.
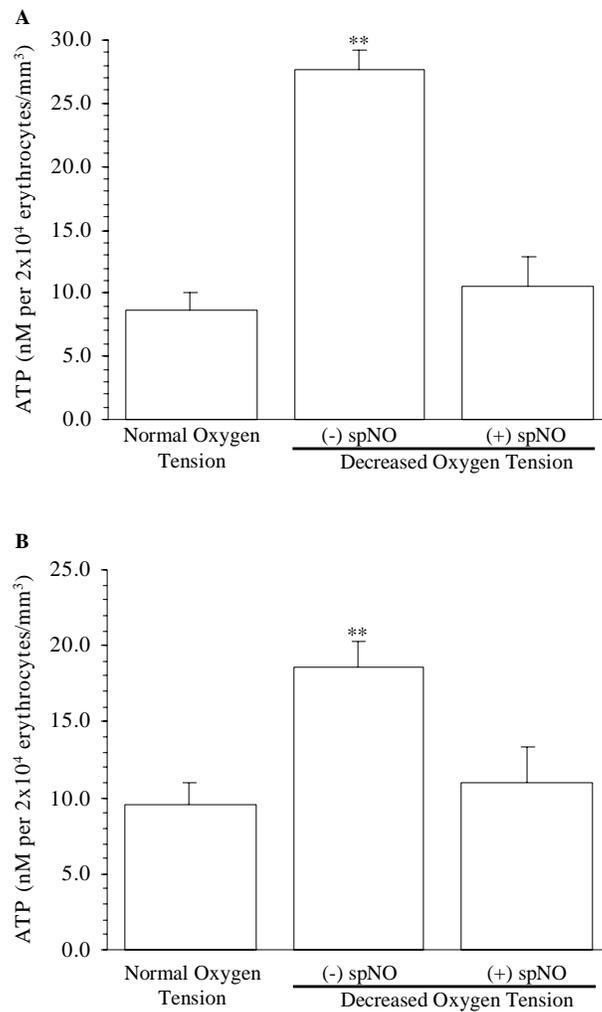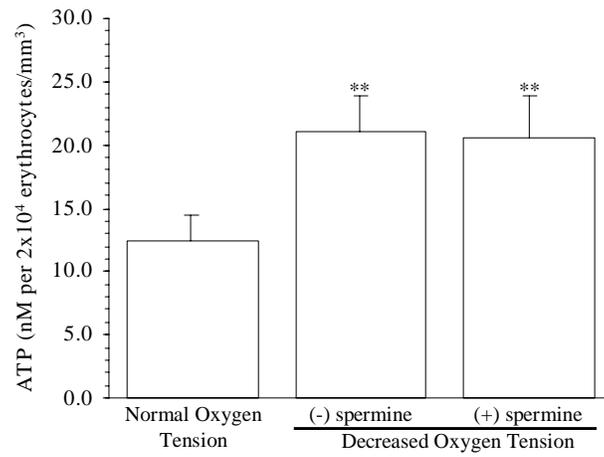
Figure 1

Figure 2

Figure 3

Figure 4