Effect of osmotic shock and urea on phosphatidylserine scrambling in thrombocyte cell membranes

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Gatidis S, Borst O, Föller M, Lang F. Effect of osmotic shock and urea on phosphatidylserine scrambling in thrombocyte cell membranes. Am J Physiol Cell Physiol 299: C111–C118, 2010.—Blood passing the renal medulla enters a strongly hypertonic environment challenging functional properties and survival of blood cells. In erythrocytes, exposure to hyperosmotic shock stimulates Ca\(^{2+}\) entry and ceramide formation with subsequent cell membrane scrambling, an effect partially reversed by high concentrations of Cl\(^{-}\) or urea. Cell membrane scrambling with phosphatidylserine exposure is part of the procoagulant phenotype of platelets. Coagulation in the hypertonic renal medulla would jeopardize blood flow in the vasa recta. The present study thus explored whether hypertonic environment and urea modify phosphatidylserine exposure of human platelets. FACS analysis was employed to estimate cytosolic Ca\(^{2+}\) activity with Fluor3 fluorescence, ceramide formation, P-selectin, and glycoprotein Ib\(\alpha/I\)Ha activation with fluorescent antibodies and phosphatidylserine exposure with annexin V-binding. The spontaneous platelet aggregation was measured by impedance aggregometry. Hyperosmotic shock (addition of 500 mM sucrose or 250 mM NaCl) significantly enhanced cytosolic Ca\(^{2+}\) activity, ceramide formation, phosphatidylserine exposure, platelet degranulation, and aggregability. Addition of 500 mM urea to isotonic saline did not significantly modify cytosolic Ca\(^{2+}\) activity, ceramide abundance, or annexin V-binding but significantly blunted the respective effects of hypertonic shock following addition of 500 mM sucrose. In isotonic solutions, both ceramide (20 \(\mu\)M) and Ca\(^{2+}\) ionophore ionomycin (0.5 \(\mu\)M) increased annexin V-binding, effects again significantly blunted by 500 mM urea. Moreover, oxidative stress by addition of 0.5 mM peroxynitrite increased cytosolic Ca\(^{2+}\) activity and triggered annexin V-binding, effects again blunted in the presence of 500 mM urea. The observations reveal that hyperosmotic shock and oxidative stress trigger a procoagulant platelet phenotype, an effect blunted by the presence of high urea concentrations.

Ca\(^{2+}\); sphingomyelinase; ceramide; apoptosis; cell volume

CELL VOLUME REGULATORY MECHANISMS participate in the machinery regulating fundamental cellular functions, such as cell proliferation and suicidal cell death (7, 12, 39, 40). Specifically, suicidal cell death is typically paralleled by cell shrinkage and could be triggered by exposure to excessive osmolarity (7, 8, 39, 43, 54, 60, 67). Cellular mechanisms triggering suicidal cell death during hyperosmotic shock include opening of Ca\(^{2+}\)-permeable cation channels (44) with subsequent increase in cytosolic Ca\(^{2+}\) activity leading to Ca\(^{2+}\)-sensitive cell membrane scrambling (20, 82, 83) with subsequent exposure of phosphatidylserine exposure at the cell surface (25, 41, 42, 61, 64). Moreover, osmotic shock stimulates a sphingomyelinase with subsequent formation of ceramide (47), which potentiates the effect of cytosolic Ca\(^{2+}\) activity on cell membrane scrambling (44, 47). In erythrocytes, ceramide was proven to be a similarly important stimulator of cell membrane scrambling following osmotic shock as Ca\(^{2+}\) entry (41). Osmotic shock may further be effective through triggering of oxidative stress (65), which has been shown to similarly trigger cell membrane scrambling in several cell types (3, 16, 26, 33, 41, 42).

The effects of osmotic shock may be particularly relevant in renal medulla with the extraordinary NaCl and urea concentrations required for urinary concentration (12, 68). Hyperosmolarity due to excessive NaCl and urea concentrations perturbs cellular functions by direct denaturation of cellular macromolecules, increase in the formation of reactive oxygen species, cytoskeletal rearrangement, inhibition of DNA replication, transcription, and translation, as well as by mitochondrial depolarization (12, 22, 38, 51, 59, 73). Cells in renal medulla protect themselves, e.g., by accumulation of stabilizing organic osmolytes and increased expression of heat shock proteins (11, 12, 21, 63).

Blood cells entering kidney medulla experience an immediate exposure to the excessive NaCl and urea concentrations, which occurs too fast for adequate protection by osmolyte accumulation and heat shock protein expression. The adaptation of kidney cells depends on the rate of increase in osmolarity (14). Moreover, the urea signaling and urea protection observed in the kidney tubule cells may be specific for cells of this type and not operative in others (12). Previous observations disclosed a profound influence of osmotic shock and urea on suicidal erythrocyte death (41, 45). Nothing is known, however, on the effect of excessive osmolarity, NaCl, and urea concentration on the function of platelets.

In platelets, cell membrane phospholipid scrambling with phosphatidylserine exposure at the cell surface, a typical feature of nucleated cell apoptosis (5, 10, 19, 44), leads to a procoagulant phenotype (70). Cell membrane scrambling in platelets could be triggered by increase in Ca\(^{2+}\) activity and by oxidative stress (2, 3, 77), again well-known triggers of apoptosis of nucleated cell.

The present study was performed to test whether hyperosmolarity accomplished by addition of sucrose, NaCl, and/or urea influences cytosolic Ca\(^{2+}\) activity, ceramide formation, phosphatidylserine exposure, and aggregation of blood platelets.

MATERIALS AND METHODS

Solutions and chemicals. Platelet buffer (PB) contained (in mM) 137 NaCl, 2.7 KCl, 2 MgSO\(_4\), 10 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, and 3 CaCl\(_2\) (pH = 7.4). ACD buffer contained (in mM) 80 trisodium citrate, 52 citric acid, and 180 glucose.

Ca\(^{2+}\) ionophore ionomycin and sphingomyelinase from Staphylococcus aureus were purchased from Sigma (Taufkirchen, Germany).

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Ionomycin was used at a final concentration of 0.5 μM. Sphingomyelinase was used at a final concentration of 1 U/ml. Erythrosine-N-hexaoylphosphosine (C6-ceramide) was purchased from Biomol (Hamburg, Germany) and dissolved in ethanol to yield a 10 mM stock solution and further diluted to a final concentration of 20 μM in PB. The maximum concentration of ethanol was 0.2% in all cases, which did not significantly induce annexin V-binding (data not shown).

A monoclonal anti-ceramide antibody (clone MD 15B4; isotype IgM) was purchased from Alexis (Grüneberg, Germany).

FITC-labeled mouse anti-human P-selectin (clone AC1.2) as well as mouse anti-human glycoprotein IIb/IIIa (PAC-1) were purchased from Becton Dickinson (Heidelberg, Germany). The osmolality of the hyperosmotic solutions was measured by a freezing point osmometer and amounted to (all in mosmol/l) 767 (PB + 500 mM urea), 769 (PB + 250 mM NaCl), 777 (PB + 500 mM NaCl), 784 (PB + 500 mM sucrose), and 790 (PB + 500 mM raffinose).

Platelet isolation. Platelets were kindly provided by the blood bank of the University of Tübingen. The study was approved by the ethics committee of the University of Tübingen (184/2003V). EDTA whole blood samples were drawn from healthy volunteers (donors gave informed consent) and centrifuged at 160 g for 20 min at 20°C. Platelet-rich plasma was taken and platelets were spun down at 2,000 g for 2 min at 20°C. The pellet was washed twice in PB without CaCl2 (pH 6.8) containing 10% ACD buffer and 0.5 mg/ml bovine serum albumin (BSA) and finally resuspended in PB without CaCl2 (pH 7.4).

Experiments were performed in PB containing 3 mM CaCl2 at 37°C and a final platelet count of 2 × 10⁸/ml. Where indicated, osmolality was increased by addition of sucrose, NaCl, or urea on top of isotonic PB.

FACS analysis. For measurement of phosphatidylserine exposure, platelets were stained with Annexin-V-Fluos (Roche, Mannheim, Germany) at a 1:100 dilution in the incubation solution. In the case of hyperosmotic buffer, annexin V-binding was quantified in ohms (Ω) by comparing the deflection of the trace with the calibration mark representing 20 Ω. The data analysis was performed with APTROLOG software (Chrono-Log).

Statistical analysis. Data are expressed as arithmetic means ± SE. Statistical analysis was made by paired or unpaired ANOVA, as appropriate.

RESULTS

Effects of hyperosmotic sucrose, NaCl, and urea on thrombocyte annexin V-binding. Under control conditions, i.e., in isotonic PB, the percentage of annexin V-binding human platelets was low after 3 h (3.12 ± 0.65%, n = 6), indicating that in most cells the phospholipid asymmetry was maintained and that only in a small fraction of thrombocytes the cell membrane was scrambled with phosphatidylserine exposure at the cell surface (Fig. 1). Increase in osmolality by addition of

![Fig. 1. Effect of hypertonic sucrose, NaCl, and urea on thrombocyte annexin V-binding. A: histograms from representative FACS experiments showing the number of annexin V-binding platelets following incubation for 3 h in plain platelet buffer PB (1), in PB + 500 mM sucrose (2), and in PB + 500 mM sucrose + 500 mM urea (3). B: arithmetic means ± SE (n = 6) of the percentage of annexin V-binding platelets after 3 h of treatment with plain PB (first white bar and first black bar), with PB + 500 mM sucrose (suc), and with Ringer + 250 mM NaCl (NaCl) in the absence (white bars) or presence (black bars) of urea (500 mM). ***Significant difference from values with plain PB (ANOVA, P < 0.001); ###significant difference (ANOVA, P < 0.001) from the respective value in the absence of urea.](http://ajpcell.physiology.org/ by 10.220.33.1 on August 27, 2017)
500 mM sucrose was followed by a marked increase in annexin V-binding after 3 h, pointing to breakdown of the phosphatidylserine asymmetry (Fig. 1). The addition of 250 mM NaCl increased annexin V-binding to a much lesser extent than 500 mM sucrose. In sharp contrast, addition of 500 mM urea did not significantly enhance the percentage of annexin V-binding cells (Fig. 1). Further experiments aimed to investigate whether urea interferes with annexin V-binding. To test whether exposure of thrombocytes was preincubated for 30 min with 500 mM urea before the addition of 500 mM sucrose. The percentage of phosphatidylserine-exposing thrombocytes incubated in PB + 500 mM sucrose for 3 h, however, amounted to 55.7 ± 3.3% (n = 6) if 500 mM urea was absent during the 3-h incubation and only added to the annexin V-staining solution. Thus, urea does not interfere with annexin V-binding. To test whether exposure of hypertonicity-induced phosphatidylserine is accompanied by activation of caspase 3, thrombocytes were exposed to PB in the presence or absence of 500 mM sucrose, and a caspase 3 assay was performed. As a result, hyperosmotic shock significantly increased the percentage of caspase 3-positive cells from 1.7 ± 0.3% (n = 5) to 35.5 ± 6.4% (n = 5).

In another series of experiments, the effect of further non-ionic osmotically active substances such as mannitol and raffinose was tested. As a result a 3-h exposure of thrombocytes to PB resulted in 3.0 ± 0.4% (n = 5) annexin V-binding thrombocytes, an effect significantly enhanced by incubation in PB containing 500 mM mannitol to 52.1 ± 4.7% (n = 5) and in PB containing 500 mM raffinose to 47.4 ± 4.7% (n = 5) annexin V-binding thrombocytes.

The lack of annexin V-binding in thrombocytes exposed to 500 mM urea prompted us to explore whether urea could inhibit the triggering of cell membrane scrambling following exposure of thrombocytes to hypertonic saline. As illustrated in Fig. 1, the addition of urea significantly blunted the increase in annexin V-binding following addition of 500 mM sucrose despite the further increase in osmolarity. Another series of experiments aimed to study whether induction of caspase 3 by hyperosmotic shock can be attenuated by urea. As a result, in this series, hyperosmotic shock resulted in 41.2 ± 2.9% (n = 6) caspase 3-positive thrombocytes, whereas the presence of 500 mM urea significantly reduced the percentage of caspase 3-positive thrombocytes to 28.3 ± 2.3% (n = 6). Thus, urea also blocks hyperosmotic shock-induced activation of caspase 3.

![Fig. 2. Effect of hypertonic sucrose, NaCl, and urea on thrombocyte cytosolic Ca$^{2+}$ activity. A: histograms from representative FACS experiments showing the Fluo3 fluorescence in platelets following incubation for 3 h in plain PB (1), in PB + 500 mM sucrose (2), and in PB + 500 mM sucrose + 500 mM urea (3). B: arithmetic means ± SE (n = 6) of the percentage of Fluo3-positive platelets after 3 h of treatment with plain PB, with PB + 500 mM sucrose (suc), and with PB + 250 mM NaCl (NaCl) in the absence (white bars) or presence (black bars) of urea (500 mM). ***Significant difference from values with plain PB (ANOVA, P < 0.001); ##Significant difference (ANOVA, P < 0.01) from the respective value in the absence of urea. C: arithmetic means ± SE (n = 3) of the percentage of annexin V-binding platelets after 15 min of treatment with PB without (left bars) or with (right bars) Ca$^{2+}$ ionophore ionomycin (0.5 μM) in the absence (white bars) or presence (black bars) of 500 mM urea. ***Significant difference from values without addition of ionomycin (ANOVA, P < 0.001); #significant difference (ANOVA, P < 0.05) from the respective value in the absence of urea. D: arithmetic means ± SE (n = 6) of the percentage of annexin V-binding platelets in isotonic PB (white bars) or in PB made hypertonic by addition of 500 mM sucrose (black bars) in the presence (+Ca$^{2+}$, left bars) and absence (−Ca$^{2+}$, right bars) of Ca$^{2+}$. ***Significant difference from values in isotonic PB (ANOVA, P < 0.001); ##Significant difference from respective values in the presence of Ca$^{2+}$ (ANOVA, P < 0.001).}
Effects of hypertonic sucrose, NaCl, and urea on thrombocyte Ca\(^{2+}\) entry. Fluo3 fluorescence was utilized to determine the effect of osmotic shock and urea on the cytosolic Ca\(^{2+}\) activity in human platelets. An increase in osmolarity by addition of 500 mM sucrose and to a lesser extent of 250 mM NaCl resulted in a marked increase in Fluo3 fluorescence after 3 h, pointing to stimulation of Ca\(^{2+}\) entry by osmotic shock. In contrast, urea did not significantly enhance the Fluo3 fluorescence and thus did not stimulate Ca\(^{2+}\) entry (Fig. 2, A and B).

Similar to what was observed with annexin V-binding, the effect of 500 mM sucrose on Fluo3 fluorescence was significantly blunted by the further addition of 500 mM urea (Fig. 2, A and B).

Exposure of platelets to the Ca\(^{2+}\) ionophore ionomycin (0.5 \(\mu\)M, 15 min) was followed by a sharp increase in annexin V-binding, an effect partially inhibited when platelets had been preincubated in the presence of 500 mM urea for 15 min before the addition of ionomycin (Fig. 2C).

In the absence of extracellular Ca\(^{2+}\), the effect of hypertonic shock (+500 mM sucrose) on annexin V-binding was significantly blunted (Fig. 2D), indicating that Ca\(^{2+}\) entry participated in the stimulation of cell membrane scrambling following osmotic shock.

Effects of hypertonic sucrose, NaCl, and urea on thrombocyte ceramide formation. The effect of osmotic shock on ceramide formation was determined utilizing anti-ceramide antibodies. As illustrated in Fig. 3, A and B, an increase in osmolality by addition of 500 mM sucrose and to a lesser extent by addition of 250 mM NaCl for 3 h significantly increased binding of the anti-ceramide antibody, pointing to ceramide formation of osmotically shocked platelets. Addition of 500 mM urea tended to slightly decrease ceramide forma-
Exposure of platelets to C6-ceramide or sphingomyelinase for 4 h resulted in an increase in annexin V-binding (Fig. 3C), indicating that stimulation of ceramide formation could have participated in the stimulation of cell membrane scrambling following osmotic shock. As before, these effects were significantly blunted by the addition of 500 mM urea (Fig. 3C).

Effects of hypertonic sucrose on activation-dependent platelet surface changes and platelet aggregability. We further investigated activation-dependent platelet surface changes on platelets treated with isotonic PB or PB containing 500 mM sucrose in the absence or presence of urea. Within 3 h of incubation, hyperosmotic shock due to addition of sucrose led to a significant increase in P-selectin expression (Fig. 4A), an effect that tended to be augmented in the presence of 500 mM urea. Similarly, hyperosmotic shock due to addition of sucrose resulted in activation-induced conformational change in integrin αIIbβ3 (glycoproteins IIb/IIIa) (Fig. 4B), an effect that tended to be inhibited by 500 mM urea. Moreover, platelets exposed to hyperosmotic shock for 3 h showed spontaneous aggregation according to impedance aggregometry (Fig. 5).

Effect of oxidative stress in the presence and absence of urea on thrombocyte annexin binding. The ability of urea to reverse the stimulation of platelet cell membrane scrambling following osmotic shock raised the question of whether urea would be similarly protective during the exposure of platelets to oxidative stress. As shown in Fig. 6, the exposure of human platelets to 0.5 mM peroxynitrite led to significantly increased annexin V-binding (Fig. 6A) and Fluo3-positive platelets (Fig. 6C) compared with platelets incubated in the absence of peroxynitrite (Fig. 6B). These effects were significantly attenuated by the addition of 500 mM urea.
to the oxidant peroxynitrite (0.5 mM, 15 min) was followed by a significant increase in Fluo3 fluorescence (Fig. 6C) and annexin V-binding (Fig. 6, A and B), an effect partially reversed by preincubation of platelets in the presence of 500 mM urea before addition of peroxynitrite.

**DISCUSSION**

The present study uncovers novel stimuliators of cell membrane scrambling in human platelets. Platelet cell membrane scrambling is triggered by hyperosmotic shock. The effect is paralleled and at least partially accounted for by an increase in cytosolic Ca\(^{2+}\) activity. Hyperosmotic shock has previously been shown to open nonselective cation channels in erythrocytes (24, 31) and a variety of nucleated cells (13, 15, 27, 36, 39, 76, 79, 80). The opening of a Ca\(^{2+}\)-permeable cation channel presumably accounts for the Ca\(^{2+}\) entry into osmotically shocked platelets. In erythrocytes, the Ca\(^{2+}\) entry through the cation conductance (44) activates the Gardos channel (46), leading to hyperpolarization, loss of cellular K\(^+\), and further cell shrinkage. Accordingly, increase in extracellular K\(^+\) blunts cell shrinkage and erythrocyte annexin V-binding following increase in cytosolic Ca\(^{2+}\) (44, 69).

Hyperosmotic cell shrinkage further leads to enhanced ceramide formation in platelets, resulting presumably from stimulation of an acid sphingomyelinase. Ceramide formation following osmotic shock has previously been observed in erythrocytes (47). In those cells, the stimulation of ceramide formation during osmotic shock is secondary to release of platelet-activating factor (49), which stimulates ceramide formation in several cell types (6, 28, 52, 86).

Ceramide is well known to trigger cell membrane scrambling in erythrocytes (47) and in nucleated cells (23, 50). Ceramide is produced by a sphingomyelinase, which is similarly known to stimulate cell membrane scrambling (32, 57). Along those lines, acid sphingomyelinas and ceramide are involved in the signaling of apoptosis following CD95-triggering (29, 30) or treatment of cells with cytotoxic drugs, such as hexadecylphosphocholine (81) or daunourubicin (9).

Excessive osmolarity, as it prevails in renal medulla, has previously been shown to trigger apoptosis of a wide variety of nucleated cells (7, 8, 39, 46, 54, 60, 66, 67). Moreover, hyperosmotic shock compromises the function of lymphocytes and thus the immune defense in kidney medulla (48). In cultured renal tubular epithelial cells the apoptosis triggered by hyperosmotic saline could be reversed by urea (75, 84, 85). On the contrary, urea may sensitize cells to apoptosis (17) and trigger cell cycle delay (60). The present observations clearly demonstrate that, in platelets, urea inhibits hyperosmotic shock-induced cell membrane scrambling.

The effect of sucrose was similar to that of raffinose and mannitol but clearly more profound than the effect of NaCl. In erythrocytes, the Ca\(^{2+}\) entry is inhibited by Cl\(^-\), which thus may play a moderately protective role (45).

According to the present observations, hypertonic shock may trigger a procoagulant phenotype of platelets, which may in turn affect microcirculation in kidney medulla. Phosphatidylserine and selectin-exposing platelets may adhere to the vascular wall or foster adherence of leukocytes and thus impede blood flow (37, 62, 72). Moreover, the shedding of microparticles due to blebbing of the plasma membrane may display procoagulant and proinflammatory properties (55). Under physiological conditions, the contact time of platelets with renal medulla is presumably too short to trigger relevant cell membrane scrambling. Nevertheless, the dwelling time of platelets may be considerably enhanced in acute renal failure, which typically leads to trapping of erythrocytes (56) and presumably platelets in the tissue at the transition between renal medulla and renal cortex. Trapping of phosphatidylserine-exposing cells in renal medulla following ischemia has indeed been observed previously (45).

Urea may be partially effective through inhibition of Ca\(^{2+}\) entry. An inhibitory effect of urea on Ca\(^{2+}\) channels has previously been observed in vascular smooth muscle cells (78). In erythrocytes, urea has been shown to inhibit cell membrane scrambling mainly by interference with ceramide formation (47). In platelets, urea may exert a slight inhibitory effect on ceramide formation. Urea has further been shown to activate ras (74) and phosphatidylinositol 3-kinase (85) and to modify several further transport processes including the Na\(^{+}\)-K\(^+\)-ATPase, KCl cotransport, and Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\)cotransport (34, 35, 53, 71). The related hydroxy-urea protects erythrocytes against oxidative stress (1) and counteracts the sickling of erythrocytes (4, 18, 58). Sickle cell anemia sensitizes erythrocytes to the scrambling effect of osmotic shock, oxidative stress, and energy depletion (44). The present study reveals that urea is effective not only in erythrocytes but in platelets as well.

Urea does not only counteract cell membrane scrambling during hyperosmotic shock, but similarly blunts the cell membrane scrambling following oxidative stress, a well-known trigger of platelet phosphatidylserine exposure (2, 3, 77).

In summary, osmotic shock triggers cell membrane scrambling of platelets, inducing a procoagulant phenotype. The effect is at least partially due to increase in cytosolic Ca\(^{2+}\) activity and ceramide formation. On the contrary, urea counteracts cell membrane scrambling, an effect at least partially due to inhibition of Ca\(^{2+}\) entry. The effects are observed at NaCl and urea concentrations, as they prevail in renal medulla and may thus participate in the regulation of thrombocyte membrane stability in this tissue.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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