p38 MAPK Activation and Function following Osmotic Shock of Erythrocytes

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Key Words

p38 kinase • Eryptosis • Osmotic shock • Phosphatidylserine • Annexin • Cell volume

Abstract

p38 protein kinase is activated by hyperosmotic shock, participates in the regulation of cell volume sensitive transport and metabolism and is involved in the regulation of various physiological functions including cell proliferation and apoptosis. Similar to apoptosis of nucleated cells, erythrocytes may undergo suicidal death or eryptosis, which is paralleled by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the cell surface. Triggers of eryptosis include hyperosmotic shock, which increases cytosolic Ca^{2+} activity and ceramide formation. The present study explored whether p38 kinase is expressed in human erythrocytes, is activated by hyperosmotic shock and participates in the regulation of eryptosis. Western blotting was utilized to determine phosphorylation of p38 kinase, forward scatter to estimate cell volume, annexin V binding to depict phosphatidylserine exposure and Fluo3 fluorescence to estimate cytosolic Ca^{2+} activity. As a result, erythrocytes express p38 kinase, which

is phosphorylated upon osmotic shock (+550 mM sucrose). Osmotic shock decreased forward scatter, increased annexin V binding and increased Fluo3 fluorescence, all effects significantly blunted by the p38 kinase inhibitors SB203580 (2 μM) and p38 Inh III (1 μM). In conclusion, p38 kinase is expressed in erythrocytes and participates in the machinery triggering eryptosis following hyperosmotic shock.

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Introduction

The p38 mitogen-activated protein kinase (MAPK) is activated by hyperosmotic shock and participates in the regulation of cell volume [1-5]. The kinase is partially effective through Nuclear factor of activated T cells 5 (NFAT5) dependent regulation of osmoprotective gene products in mammalian cells [1, 6], in part by activating cell volume regulatory ion transport, such as Na^+/H^+ exchange [7] and in part by modifying cell volume sensitive metabolic pathways, such as autophagic proteolysis [8]. p38 kinase participates in the regulation of cell proliferation [9] and apoptosis [10-17].

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In analogy to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis [18]. Similar to apoptosis, eryptosis is characterized by cell shrinkage, cell membrane blebbing and cell membrane scrambling with exposure of phosphatidylserine at the erythrocyte surface [18]. Eryptosis may be triggered by hyperosmotic shock, which increases cytosolic Ca^{2+} activity and triggers the formation of ceramide, which both stimulate cell membrane scrambling [18]. Eryptosis is further triggered by a wide variety of xenobiotics and endogenous substances [19–27], and lead to anemia in several clinical disorders [18], such as iron deficiency [28], phosphate depletion [29], Hemolytic Uremic Syndrome [30], sepsis [31], malaria [18, 32, 33] or Wilson's disease [34].

The present study explored whether p38 kinase is expressed in erythrocytes and participates in the regulation of eryptosis.

Materials and Methods

Erythrocytes, solutions and chemicals

Experiments were performed in leukocyte-depleted erythrocytes from concentrates provided by the blood bank of the University of Tübingen. The volunteers providing erythrocytes gave informed consent. The study has been approved by the Ethical commission of the University of Tübingen.

The erythrocytes were incubated at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl_2 ; pH 7.4 at a hematocrit of 0.4%. Where indicated, osmolarity was increased by addition of 550 mM sucrose (Sigma, Schnellendorf, Germany), intracellular Ca^{2+} concentration was increased using the ionophore ionomycin (1 μM ; Sigma, Schnellendorf, Germany) and/or p38 kinase inhibited by addition of SB203580 (2 μM ; Tocris, Bristol, UK) or p38 Inh III (1 μM , Calbiochem, Bad soden, Germany). Where indicated erythrocytes were incubated in Ca^{2+} -free Ringer solution, where 1 mM CaCl_2 was substituted with 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). The mean corpuscular volume (MCV) of erythrocytes was measured using an electronic hematology analyzer (Sysmex, Norderstedt, Germany).

Western blotting

To examine the expression of p38 protein kinase, human erythrocytes were isolated from freshly drawn blood from 5 volunteers. Isolated erythrocytes were lysed in lysis buffer containing (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM Na_2VO_4 (sodium vanadate), 0.4% β -mercaptoethanol, protease inhibitor cocktail (Sigma, Taufkirchen, Germany). Proteins (120 μg) were solubilized in Laemmli sample buffer at 95°C for 5 min and resolved by 10%

SDS-PAGE. For immunoblotting proteins were electro-transferred onto a nitrocellulose membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 (TBST) at room temperature for 1 h. Then, the membrane was incubated with affinity purified rabbit anti-p38 kinase antibody (1:1000; Cell Signaling, Freiburg, Germany) and/or anti-p-p38 kinase antibody (1:1000, Cell Signaling, Freiburg, Germany) at 4°C overnight. After washing 3 times with TBST (10 min each) the blots were incubated with horseradish peroxidase conjugated secondary anti-rabbit antibody (1:2000; Cell Signaling, Freiburg, Germany) for 1 h at room temperature. After washing antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany). Antibody-binding was quantified densitometrically with Quantity One Software (Biorad, München, Germany). Rabbit anti-GAPDH antibody (1:1000; Cell Signaling, Freiburg, Germany) was used for detecting a loading control.

Phosphatidylserine exposure and forward scatter

For FACS analysis erythrocytes were washed after incubation once in Ringer solution containing 5 mM CaCl_2 . The cells were then stained with FITC-conjugated Annexin V (ImmunoTools, Friesoythe, Germany) at a 1:200 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

After incubation, 50 μl suspension of erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 30 min and then resuspended in 150 μl Ringer. The Ca^{2+} -dependent fluorescence intensity was then measured in fluorescence channel FL-1 in FACS analysis.

Confocal microscopy and immunofluorescence

For the visualization of eryptotic erythrocytes, 4 μl of erythrocytes, incubated in respective experimental conditions, were stained with FITC-conjugated annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 μl Ringer solution containing 5 mM CaCl_2 . Then the erythrocytes were washed twice and finally resuspended in 50 μl of Ringer solution containing 5 mM CaCl_2 . 20 μl were smeared onto a glass slide, covered with a coverslip and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Statistics

Data are expressed as arithmetic means $\pm$ SEM. n is the number of batches used. As different erythrocyte batches used in distinct experiments are differently susceptible to eryptotic effects, comparisons were always made between erythrocytes from the same batch. Paired ANOVA with Tukey's test as post-

Fig. 1. Expression and phosphorylation of p38 kinase in erythrocytes. A. Original Western blots demonstrating the expression of phosphorylated p38 kinase protein (p-p38, 1st line) and unphosphorylated p38 kinase protein (p38, 2nd line) and of the respective GAPDH protein abundance (3rd line) in isolated human erythrocytes of two representative volunteers out of five volunteers. Proteins were isolated from erythrocytes without prior incubation (0hi), following a 2 h incubation in isotonic (300 mosm) extracellular fluid (2hi) or following 2 h of incubation in hypertonic (+550 mM sucrose) extracellular fluid (2hh). B. Arithmetic means ± SEM (n = 5) of the densitometric western blot analysis showing the P-p38 kinase protein abundance over the p38 kinase protein abundance prior to incubation (white bar) as well as following a 2 h incubation in isotonic (grey bar) or hypertonic (black bar) extracellular fluid. *** indicates significant (p < 0.001) difference from incubation in isotonic extracellular fluid.

test or un paired *t* test was used for statistical analysis as indicated in the Figure legends. Where indicated in some cases, a normalization of values to control values was appropriate and is denoted in the respective graphs. p < 0.05 was considered statistically significant.

Results

Western blotting was employed to determine, whether erythrocytes express the p38 protein kinase. As illustrated in Fig. 1, human erythrocytes expressed the p38 kinase protein. In isotonic extracellular fluid, only a very small portion of the protein was phosphorylated. Exposure of erythrocytes for 2 h to hyperosmotic shock

Fig. 2. Effect of osmotic shock on erythrocyte cell volume. A. Arithmetic means ± SEM (n = 6) of the mean corpuscular volume (MCV, left bars) and the erythrocyte forward scatter (FSC, right bars) of erythrocytes following a 5 h incubation in isotonic (white bars) or hypertonic (black bars) Ringer solution. *** indicates significant (p < 0.001) difference from isotonic Ringer solution (paired *t* test). B. Time dependence of the arithmetic means ± SEM (n = 6) of the forward scatter of erythrocytes following incubation in isotonic (black squares) and in hypertonic (white squares) Ringer solution. **, *** indicates significant (p < 0.01, p < 0.001) difference from isotonic Ringer solution at the same time point (ANOVA). C. Arithmetic means ± SEM (n = 6) of the forward scatter of erythrocytes following a 5 h incubation in isotonic (left bars) or hypertonic (right bars) Ringer solution in the presence (white bars) or absence (black bars) of extracellular Ca²⁺ (1 mM). *** (p < 0.001) indicates significant difference from isotonic Ringer solution, ### (p < 0.001) indicates significant difference from the respective values in the presence of Ca²⁺ (ANOVA).

(+550 mM sucrose), however, was followed by a marked increase of p38 kinase phosphorylation. Subsequent experiments were performed to explore whether p38

Fig. 3. Effect of osmotic shock on erythrocyte forward scatter in the presence or absence of p38 kinase inhibitors. A. Histograms of forward scatter in representative experiments of erythrocytes exposed for 5 h to hypertonic Ringer solution (addition of 550 mM sucrose) in the absence (black line) or presence (red line) of p38 kinase inhibitor SB203580 (2 μ M). B. Arithmetic means $\pm$ SEM (n = 6) of the forward scatter of erythrocytes following a 5 h incubation in isotonic (white bars) or hypertonic (black bars) Ringer solution in the absence (control) or presence of 2 μ M SB203580 (SB203580) or 1 μ M p38 Inh III (p38 Inh III). *** indicates significant (p < 0.001) difference from isotonic Ringer solution. ### indicates significant (p < 0.001) difference from the absence of p38 kinase inhibitors (ANOVA).

Fig. 4. Effect of osmotic shock on Ca^{2+} concentration on erythrocytes in the presence or absence of p38 kinase inhibitors. A. Histograms of the percentage of Fluo3 positive cells reflecting cytosolic Ca^{2+} concentration in representative experiments of erythrocytes exposed for 5 h to hypertonic Ringer solution (addition of 550 mM sucrose) in the absence (black line) or presence (red line) of p38 kinase inhibitor SB203580 (2 μ M). B. Arithmetic means $\pm$ SEM (n = 6) of the percentage of Fluo3 positive erythrocytes following a 5 h incubation in isotonic (white bars) or hypertonic (black bars) Ringer solution in the absence (control) or presence of 2 μ M SB203580 (SB203580) or 1 μ M p38 Inh III (p38 Inh III). *** indicates significant (p < 0.001) difference from isotonic Ringer solution, ### indicates significant (p < 0.001) difference from the absence of p38 kinase inhibitors (ANOVA).

kinase is involved in the regulation of suicidal erythrocyte death or eryptosis following hyperosmotic shock.

In a first series of experiments, the regulation of cell volume by hyperosmotic shock was investigated. To this end, changes in erythrocyte forward scatter were compared to the mean corpuscular volume. Hyperosmotic shock indeed triggered a decrease in both forward scatter and the mean corpuscular volume pointing to cell shrinkage (Fig. 2A). Exposure of erythrocytes to hyperosmotic Ringer solution triggered a significant time-dependent decrease in erythrocyte forward scatter (Fig. 2B). Furthermore, removal of Ca^{2+} from the extracellular medium significantly blunted the hyperosmotic shock-induced erythrocyte cell shrinkage (Fig. 2C).

According to forward scatter, exposure of erythrocytes to hyperosmotic extracellular fluid was followed by a significant decrease of cell volume (Fig. 3). The addition of the specific p38 kinase inhibitor SB203580 (2 μ M) did not significantly modify forward scatter in isotonic extracellular fluid but slightly and significantly blunted the decrease of forward scatter following hyperosmotic shock. Similarly, the specific p38 kinase inhibitor p38 Inh III (1 μ M) did not significantly modify forward scatter in isotonic extracellular fluid but slightly and significantly blunted the decrease of forward scatter following hyperosmotic shock.

Exposure of erythrocytes to hyperosmotic extracellular fluid was further followed by increase of

Fig. 5. Effect of osmotic shock on erythrocyte cell membrane scrambling in the presence or absence of p38 kinase inhibitors. A. Histograms of annexin V binding reflecting phosphatidylserine exposure in representative experiments of erythrocytes exposed for 5 h to hypertonic Ringer solution (addition of 550 mM sucrose) in the absence (black line) or presence (red line) of p38 kinase inhibitor SB203580 (2 μ M). B. Arithmetic means $\pm$ SEM (n = 6) of the annexin V binding of erythrocytes following a 5 h incubation in isotonic (white bars) or hypertonic (black bars) Ringer solution in the absence (control) or presence of 2 μ M SB203580 (SB203580) or 1 μ M p38 Inh III (p38 Inh III). *** indicates significant (p < 0.001) difference from isotonic Ringer solution, ### indicates significant (< 0.001) difference from the absence of p38 kinase inhibitors (ANOVA).

Fig. 6. Confocal images of phosphatidylserine-exposing erythrocytes treated with p38 kinase inhibitors. Confocal microscopy of FITC-dependent fluorescence (lower panels) and light microscopy (upper panels) of human erythrocytes stained with FITC-conjugated annexin following 5 h incubation in (left to right) isotonic Ringer solution (Isotonic), hypertonic Ringer solution (addition of 550 mM sucrose; Hypertonic), hypertonic Ringer solution with 2 μ M SB203580 (SB203580) and hypertonic Ringer solution with 1 μ M p38 Inh III (p38 Inh III).

Fluo3 fluorescence, pointing to increase of cytosolic Ca^{2+} activity (Fig. 4). The addition of the specific p38 kinase inhibitor SB203580 (2 μ M) did not significantly modify Fluo3 fluorescence in isotonic extracellular fluid but slightly and significantly blunted the increase of Fluo3 fluorescence following hyperosmotic shock. Similarly, the specific p38 kinase inhibitor p38 Inh III (1 μ M) did not significantly modify Fluo3 fluorescence in isotonic extracellular fluid but slightly and significantly blunted the decrease of forward scatter following hyperosmotic shock.

Exposure of erythrocytes to hypertonic extracellular fluid further triggered cell membrane scrambling with exposure of phosphatidylserine at the erythrocyte surface thereby resulting in a significant increase in annexin V-binding quantified by FACS analysis (Fig. 5) and visualized by confocal microscopy (Fig. 6). Neither SB203580 (2 μ M) nor p38 Inh III (1 μ M) modified significantly annexin V binding in the isotonic extracellular fluid but both blunted significantly its increased binding after hypertonic shock (Fig. 5 and Fig. 6).

Fig. 7. Effect of Ca^{2+} ionophore ionomycin on erythrocyte cell membrane scrambling in the presence or absence of p38 kinase inhibitors. A. Histograms of annexin V binding reflecting phosphatidylserine exposure in representative experiments of erythrocytes exposed for 30 min with 1 μM ionomycin in the absence (black line) or presence (red line) of p38 kinase inhibitor SB203580 (2 μM). B. Arithmetic means $\pm$ SEM ($n = 6$) of the annexin V binding of erythrocytes following a 30 min exposure without (white bars) or with 1 μM ionomycin (black bars) in the absence (control) or presence of 2 μM SB203580 (SB203580). *** indicates significant ($p < 0.001$) difference from the absence of ionomycin, ### indicates significant ($p < 0.001$) difference from the absence of 2 μM SB203580 (ANOVA).

Additional experiments were performed to investigate whether the p38 kinase inhibitors blunt the enhanced annexin V-binding following addition of 1 μM ionomycin for 30 min to the extracellular fluid. As a result, the specific p38 kinase inhibitor SB203580 (2 μM) did not significantly modify annexin V binding in isotonic extracellular fluid but significantly blunted the increase of annexin V binding following addition of ionomycin (1 μM) (Fig. 7). However, the specific p38 kinase inhibitor p38 Inh III did not significantly inhibit the increase of annexin V binding following exposure to 1 μM ionomycin (data not shown).

Discussion

The present study uncovers a novel player in the regulation of erythrocyte survival following hyperosmotic shock. The p38 kinase is expressed in erythrocytes and, similar to what has been observed in nucleated cells [1–5], activated by hyperosmotic shock. Moreover, the present observations reveal that p38 kinase participates in the cellular machinery triggering suicidal erythrocyte death following osmotic shock. p38 kinase has similarly been implicated in the signaling of nucleated cell apoptosis [10–17]. The Fluo3 measurements suggest that p38 kinase is upstream of Ca^{2+} entry and thus participates in the regulation of Ca^{2+} entry. The increase of cytosolic Ca^{2+} is followed by Ca^{2+} dependent stimulation of cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface as well as stimulation of Ca^{2+} activated K^{+} channels with subsequent hyperpolarization, exit of KCl with osmotically obliged water and thus cell shrinkage [18]. The Ca^{2+} induced water exit and cell shrinkage adds to the water exit and cell shrinkage following increase of extracellular osmolarity during hyperosmotic shock. The former but not the latter is expected to be sensitive to K^{+} channel blockers such as clotrimazole, which has previously been shown to prevent the cell shrinkage following treatment of the erythrocytes with the Ca^{2+} ionophore ionomycin [35].

The regulation of eryptosis by p38 kinase may impact on erythrocyte survival and thus on the life span of circulating erythrocytes. Phosphatidylserine-exposing cells are bound to [36] and are engulfed by [37] phagocytosing cells. The eryptotic cells are thus rapidly cleared from circulating blood [28]. Does enhanced eryptosis occur to the extent that the so accelerated loss of erythrocytes is not compensated by similarly accelerated formation of new erythrocytes, enhanced eryptosis leads to anemia [18]. Eryptosis is further likely to compromise microcirculation. Phosphatidylserine-exposing erythrocytes adhere to endothelial cells of the vascular wall [38–42] and foster the assembly of prothrombinase and tenase as well as the generation of thrombin and thus blood clotting [38, 43, 44]. For instance, enhanced trapping of eryptotic erythrocytes has previously been observed in renal medulla following ischemia [45]. Along those lines suicidal erythrocytes may contribute to the vascular injury in patients suffering from metabolic syndrome [46].

In conclusion, the present observations disclose that p38 kinase contributes to the stimulation of eryptosis following hyperosmotic shock by participating in the triggering of Ca^{2+} entry into shrunken erythrocytes.

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