Stimulation of Suicidal Erythrocyte Death by Methylglyoxal

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Key Words
Cell volume • Phosphatidylserine • Cation channels • Calcium • Apoptosis • Glycolysis

Abstract
Diabetes increases the percentage of circulating erythrocytes exposing phosphatidylserine (PS) at the cell surface. PS-exposing erythrocytes are recognized, bound, engulfed and degraded by macrophages. Thus, PS exposure, a feature of suicidal erythrocyte death or eryptosis, accelerates clearance of affected erythrocytes from circulating blood. Moreover, PS-exposing erythrocytes bind to the vascular wall thus interfering with microcirculation. The present study explored mechanisms involved in the triggering of phosphatidylserine exposure by methylglyoxal, an extra- and intracellular metabolite which is enhanced in diabetes. PS exposure, cell size and cytosolic Ca²⁺-activity after methylglyoxal treatment were measured by FACS analysis of annexin V binding, forward scatter and Fluo-3-fluorescence, respectively, and it was shown that the treatment significantly enhanced the percentage of PS-exposing erythrocytes at concentrations (0.3 µM) encountered in diabetic patients. Surprisingly, methylglyoxal did not significantly increase cytosolic Ca²⁺ concentration, and at concentrations up to 3 µM, did not decrease the forward scatter. Instead, exposure to methylglyoxal inhibited glycolysis thus decreasing ATP and GSH concentrations. In conclusion, methylglyoxal impairs energy production and anti-oxidative defense, effects contributing to the enhanced PS exposure of circulating erythrocytes and eventually resulting in anemia and deranged microcirculation.

Introduction
Diabetes is known to decrease the life span of erythrocytes [1]. The shortened survival is presumably due to triggering of phosphatidylserine exposure [2-5]. As macrophages are equipped with receptors specific for phosphatidylserine [6-8], erythrocytes exposing phosphatidylserine at their surface will be rapidly recognized, engulfed and degraded [9, 10].

Recent experiments allowed some insight into the mechanisms involved in the stimulation of phosphatidylserine exposure. Treatment of erythrocytes with the Ca²⁺-ionophore ionomycin leads to Ca²⁺-mediated eryth-
rocyte shrinkage, membrane blebbing and breakdown of cell membrane phosphatidylserine asymmetry, all features typical for programmed cell death or apoptosis of nucleated cells [11-14]. In contrast to nucleated cells, however, Ca2+-triggered programmed cell death of erythrocytes occurs in the absence of caspase activation, DNA fragmentation and mitochondrial depolarisation [11-14]. To describe this particular programmed death of erythrocytes, the term eryptosis was coined recently [15]. Eryptosis is induced by different well-known proapoptotic stressors, namely hyperosmotic shock, oxidative stress and energy depletion, which all activate Ca2+-permeable unselective cation channels allowing Ca2+ entry [14, 16, 17]. The resulting increase of cytosolic Ca2+ activity triggers erythrocyte cell membrane scrambling with subsequent breakdown of the phosphatidylserine asymmetry [18, 19]. Erythrocyte Ca2+ entry [20] and phosphatidylserine exposure [21] have further been observed following activation of protein kinase C. Eryptosis shares some mechanisms of but may be distinct from other types of suicidal erythrocyte death, such as erythrocYTE senescence [15, 22-24] or neocytolysis [25].

Metabolites known to be increased during hyperglycemia of diabetes include methylglyoxal [26-30], which has further been shown to be enhanced in chronic renal failure [31, 32]. The link between hyperglycemia and enhanced methylglyoxal formation has been clearly demonstrated by incubation of erythrocytes to elevated glucose concentrations resulting in an increased triosephosphate pool of glycolytic intermediates and a subsequent increase in the methylglyoxal concentration [33-35]. Furthermore, methylglyoxal has been implicated in the pathophysiology of vascular and renal disease [36-42] and shown to induce neuronal injury [43].

The present study has been performed to elucidate the putative role of methylglyoxal in the stimulation of phosphatidylserine exposure of erythrocytes.

**Materials and Methods**

*Assay of methylglyoxal in human blood plasma and red blood cells*

Type 1 diabetic patients and age-matched normal healthy human subjects were recruited from patients and staff of the George Haik Eye Clinic (New Orleans, LA, USA) with their informed consent. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and was approved by the local Ethics Committee. Twenty-one normal subjects (10 male, 11 female) of age 60 ± 17 years had a mean plasma glucose concentration of 5.9 ± 0.7 mM and a mean HbA1c of 5.8 ± 0.5 %. Thirty-one type 1 diabetic patients were recruited (13 male, 18 female) of age 64 ± 14 years, duration of diabetes 20 ± 9 years, plasma glucose concentration 9.3 ± 5.0 mM and HbA1c 8.3 ± 1.6 %. Blood was collected from subjects with informed consent by intravenous puncture with heparin as anticoagulant. The plasma was separated by centrifugation immediately (2,000 g, 5 min), plasma and red blood cells mixed with one volume of 100 mM and 500 mM acetic acid, respectively (to prevent analyte formation during storage and processing) and shipped and stored in liquid nitrogen until processed further for analysis. Methylglyoxal was determined by derivatisation with 1,2-diamino-4,5-dimethoxybenzene and quantitation of the quinoxaline derivative by reversed phase HPLC with fluorimetric detection as described earlier [44].

**Cells and solutions**

Human erythrocytes were drawn from healthy volunteers and used either without purification or after separation by centrifugation for 25 min; 2000 g over Ficoll (Biochrom KG, Berlin, Germany). Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO4, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH, 5 glucose, 1 CaCl2, pH 7.4.

Where indicated, methylglyoxal (Sigma, Taufkirchen, Germany) has been added at the final concentrations as indicated. Since methylglyoxal is rapidly degraded [45], the indicated amount of methylglyoxal has been added consecutively every 60 min for up to 8 hours. In some experiments, the Ca2+ ionophore ionomycin (Sigma) was diluted into Ringer solution from a 1 mM dimethylsulfoxide (DMSO) stock solution. Appropriate amounts of solvent were added to control erythrocyte suspensions.

**FACS analysis**

FACS analysis was performed as described previously [14]. After incubation of erythrocytes (0.3 % or 6 % hematocrit) for 24 or 48 h with the respective solutions, cells were washed in annexin V-binding buffer containing 125 mM NaCl, 10 mM HEPES (pH 7.4), and 5 mM CaCl2. Erythrocytes were stained with Annexin-Fluos (Roche Diagnostics, Mannheim, Germany) at a 1:50 dilution. After 15 min, samples were diluted 1:5 and measured by flow cytometric analysis using a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Cells were analyzed by forward and side scatter and annexin V-fluorescence intensity was measured in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Intracellular Ca2+ measurements were performed as described [46]. Briefly, erythrocytes from healthy volunteers were loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) by addition of 10 µl of a Fluo-3/AM stock solution (2.0 mM in DMSO) to 10 ml erythrocyte suspension (0.16 % hematocrit in Ringer). The cells were incubated at 37°C for 15 min under vigorous shaking and light protection. An additional 10 µl of Fluo-3/AM was added and incubation continued for another 25 min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1000 g for 3 min. at 22°C and washed twice with Ringer solution containing 0.5 % bovine serum albumin.

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Fig. 1. Effect of methylglyoxal on phosphatidylserine exposure of human erythrocytes. A. Original histograms of annexin-V-binding of erythrocytes incubated for 24 h in the absence (left) or presence (right) of 10 µM methylglyoxal. B. Means ± SEM (n = 7) of annexin-V-binding of erythrocytes after 24 h in the absence (control, open bar) or presence (closed bars) of 0.1 µM, 0.3 µM, 1.0 µM, 3.0 µM and 10 µM methylglyoxal. *different from control (ANOVA; P<0.05). C. Means±SEM (n = 7) of forward scatter of erythrocytes incubated for 24 h in the absence (control, open bar) or presence (closed bars) of 0.1 µM, 0.3 µM, 1.0 µM, 3.0 µM and 10 µM methylglyoxal. *different from control (ANOVA; P<0.05).

Table 1. Enhanced plasma levels and intraerythrocytic concentrations of methylglyoxal in diabetic patients. Data are means ± SD.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Subject group</th>
<th>Means ± SD</th>
<th>Minimum - maximum</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Methylglyoxal]Plasma</td>
<td>(21)</td>
<td>Control</td>
<td>139 ± 98</td>
<td>67 - 180</td>
<td></td>
</tr>
<tr>
<td>(nmol/g Hb)</td>
<td></td>
<td>Diabetic</td>
<td>483 ± 181</td>
<td>103 - 575</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>[Methylglyoxal]RBC</td>
<td>(21)</td>
<td>Control</td>
<td>1.37 ± 0.51</td>
<td>0.68 - 2.23</td>
<td></td>
</tr>
<tr>
<td>(nmol/g Hb)</td>
<td></td>
<td>Diabetic</td>
<td>2.49 ± 1.94</td>
<td>0.37 - 6.00</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Determination of intracellular ATP concentration
Erythrocytes were washed (3 x 5 min) in PBS, centrifuged and 100 µl of the blood cell pellet were incubated for 48 h at 37°C with standard Ringer solution with or without 30 µM, 100 µM or 300 µM methylglyoxal (final hematocrit ~ 5-7 %). After incubation, cells were lysed in distilled water and proteins were precipitated by HClO4 (5 %). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated KHCO3 solution. All manipulations were performed at 4°C to avoid ATP degradation. After dilution of the supernatant, the ATP concentration of the aliquots was determined utilizing the luciferin-luciferase assay kit (Roche Diagnostics, Mannheim, Germany) and a luminometer (Biolumat LB9500; Berthold, Bad Wildbad, Germany). ATP concentrations are expressed as mmol/l packed erythrocyte volume.

Determination of reduced glutathione (GSH)
Freshly drawn human erythrocytes (6 % hematocrit) were washed twice in PBS and incubated for 48 h at 37°C in Ringer solution or glucose-free Ringer solution, in the presence or absence of methylglyoxal (3 µM). After incubation, cells were again washed twice in PBS. Then, 50 µl of the erythrocyte pellet was lysed in 200 µl distilled water and centrifuged at 14,000 g. 150 µl of the supernatant was deproteinized by adding 150 µl metaphosphoric acid (10 %). All manipulations after the 24 or 48 h incubation period were performed at 4°C. Glutathione (GSSG and GSH) was measured with the Glutathione Assay Kit from Cayman Chemicals (purchased from IBL-Hamburg, Hamburg, Germany) according to the manufacturer’s protocol. GSH concentrations are expressed in µmol/l packed erythrocyte volume.

Determination of glucose utilization
Freshly drawn human erythrocytes (6 % hematocrit) were washed twice in PBS and incubated for 48 h at 37°C in Ringer solution (7.2 mM glucose and 0 mM lactate) in the presence or absence of methylglyoxal (300 µM). Following incubation, glucose (electrochemical measurement by the glucose dehydrogenase method) and lactate (lactate oxidase method and electrochemical determination of H2O2) concentrations were determined as described.
Statistics

Data are expressed as arithmetic means ± SEM and statistical analysis was made by paired or unpaired student’s t-test, or by ANOVA using Dunnett’s or Tukey’s test as post hoc test, where appropriate. P < 0.05 was considered statistically significant.

Results

Metabolites known to be increased during hyperglycemia of diabetes include methylglyoxal. To estimate the impact of diabetes on methylglyoxal concentration, a group of 31 type 1 diabetic patients was recruited, and the plasma levels as well as the intraerythrocytic concentrations of methylglyoxal were determined. As shown in Table 1, the methylglyoxal plasma levels of diabetic patients were significantly enhanced by approx. 3.5-fold as compared with the plasma levels of age-matched normal individuals (n=21). Interestingly, the diabetic condition not only enhanced the patients’ methylglyoxal plasma levels but also led to a significant elevation of intraerythrocytic methylglyoxal by about 82 % (Table 1).

Thus, the effect of methylglyoxal was tested on erythrocytes from healthy individuals. As shown in Fig. 1A,B, methylglyoxal enhanced the phosphatidylserine exposure, an effect statistically significant at concentrations of 0.3 µM methylglyoxal. As outlined in the methods section, addition of methylglyoxal has been repeated up to 8 times to compensate for methylglyoxal degradation. At concentrations below 10 µM, methylglyoxal did not significantly affect the forward scatter of the erythrocytes. The exposure to 10 µM methylglyoxal was, however, followed by a slight, but significant decrease of forward scatter (Fig. 1C).

Both, enhanced phosphatidylserine exposure and decreased forward scatter are typical consequences of increased cytosolic Ca²⁺ activity. Thus, the effect of methylglyoxal on cytosolic Ca²⁺ activity has been tested using Fluo3 fluorescence. As illustrated in Fig. 2, 3 µM methylglyoxal did not significantly increase cytosolic Ca²⁺ activity up to 6 hours incubation. At concentrations of 10 µM, methylglyoxal treatment was even followed by a slight but significant decrease of cytosolic Ca²⁺ activity.

Oxidative stress is also known to stimulate phosphatidylserine exposure. To explore whether methylglyoxal influences anti-oxidative defense, reduced (GSH) and oxidized (GSSG) glutathione were determined in erythrocytes from healthy volunteers incubated for 48 hours in Ringer solution without or with 3 µM methylglyoxal. As illustrated in Fig. 3, methylglyoxal treatment led to a marked decline of GSH levels without significantly affecting GSSG levels. Glucose depletion, which was used as a positive control for the dissipation of the anti-oxidative defense, similarly decreased the GSH levels.

The decline of GSH levels following methylglyoxal treatment could have resulted from energy depletion. Thus, in the next series of experiments, ATP concentrations were determined in erythrocytes from healthy volunteers incubated for 48 hours in Ringer solution without or with 30 µM, 100 µM or 300 µM methylglyoxal.

![Fig. 2. Cytosolic Ca²⁺ activity in the presence of methylglyoxal. A. Original histograms of Fluo3-fluorescence in erythrocytes incubated for 6 h in the absence (upper left) or presence of 3 µM (upper right) and 30 µM (lower left) methylglyoxal or 0.1 µM ionomycin, as a positive control (lower right). B. Means ± SEM (n = 4) of Fluo3-fluorescence in erythrocytes incubated for 1, 3 and 6 h in the absence (control, open bars) or presence of 0.3 µM, 3 µM, 30 µM methylglyoxal (MG), or for 1 h with 1 µM ionomycin (black bar). *different from control (ANOVA;P<0.05).]
methylglyoxal. As shown in Fig. 4, the treatment with methylglyoxal indeed led to a significant decrease of erythrocyte ATP concentration, an effect statistically significant at 100 µM and 300 µM methylglyoxal. Glucose depletion, which was again used as a positive control, similarly decreased the ATP concentration (Fig.4).

The decline of the ATP concentration could have resulted from impairment of the flux through glycolysis or pentose phosphate pathway. To test for the effect of methylglyoxal treatment on glucose metabolism, the consumption of glucose and formation of lactate were determined in erythrocytes from healthy volunteers incubated for 48 hours in Ringer solution without or with 300 µM methylglyoxal. As shown in Fig. 5, methylglyoxal treatment indeed significantly decreased lactate production.

If decreased flux through the glycolytic pathway were sufficient to trigger phosphatidylserine exposure, glucose depletion should be similarly effective as methylglyoxal. As shown in Fig. 6, this was indeed the case. In these experiments, glucose depletion led within 48 hours to marked phosphatidylserine exposure.

**Discussion**

The present results confirm that diabetes leads to enhanced phosphatidylserine exposure of erythrocytes, a key event in suicidal erythrocyte death or eryptosis. More importantly, the experiments reveal that eryptosis is triggered by methylglyoxal, a metabolite accumulated in sustained hyperglycemia. The stimulation of eryptosis
by methylglyoxal could contribute to or even account for the triggering of cryptosis in diabetic individuals. The mean plasma concentration of methylglyoxal, which was measured in diabetic patients, amounted to 0.5 µM, a value which is in the same range as the lowest, effective methylglyoxal concentration (0.3 µM) significantly inducing phosphatidylserine exposure in our experimental system. However, it should be kept in mind that the in vitro measurements were performed in the absence of plasma. Since methylglyoxal reversibly binds to thiol groups of albumin [47], this will decrease the potency of methylglyoxal and thereby inhibit its proeryptotic effect. Apparently, methylglyoxal is not primarily effective through increase of cytosolic Ca²⁺ activity. An increase of cytosolic Ca²⁺ activity was expected to activate the Ca²⁺ sensitive K⁺ channel (Gardos channel) [48, 49]. The subsequent hyperpolarisation of the cell membrane would drive Cl⁻ out of the cell and the cellular loss of KCl with osmotically obliged water thus lead to cell shrinkage [50, 51]. Loss of cellular K⁺ is considered a requirement of apoptosis in several cell types [52-58] and activation of Ca²⁺ sensitive K⁺ channels participates in the cell shrinkage and deformation of desoxygenized sickle cells [48, 49, 59-61] which have most recently been shown to be highly sensitive to cryptosis following osmotic shock or oxidative stress [62]. However, exposure for 6 hours to 3 µM methylglyoxal had little effect on forward scatter, suggesting that at those concentrations and exposure times methylglyoxal did not substantially increase cytosolic Ca²⁺ activity. At higher concentrations methylglyoxal even decreased cytosolic Ca²⁺ activity. On the other hand, millimolar concentrations of methylglyoxal have been shown to increase Ca²⁺ activity in MDCK cells [63].

Methylglyoxal is at least partially effective by an effect on glucose metabolism. Methylglyoxal has previously been shown to interfere with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [64]. The energy depletion compromises the formation of GSH thus interfering with anti-oxidative defense [65]. Besides its effect on cellular GSH levels, methylglyoxal may impede anti-oxidative defense by inactivating glutathione peroxidase [66]. GSH depletion has been observed in...
diabetes mellitus and GSH levels have been shown to negatively correlate with diabetic complications, highlighting the pathophysiological significance of weakened anti-oxidative defense [67]. Energy depletion may further impede the function of ATP consuming pumps. Decreased Na+/K+ ATPase activity has indeed been observed in erythrocytes from diabetic patients [68].

Methylglyoxal similarly stimulates apoptosis of nucleated cells [69-77], an effect involving oxidative stress and activation of p38 kinase [72, 78, 79]. On the other hand, methylglyoxal could interact with the permeability transition pore of mitochondria [80, 81] and may exert anti-apoptotic effects in nucleated cells [82]. Whether or not impaired flux through the glycolytic pathway contributes to the influence of methylglyoxal on apoptosis of nucleated cells has not yet been explored. In the present study, we show that methylglyoxal impedes the anti-oxidative defense of erythrocytes thereby inducing phosphatidylserine exposure. As shown previously, oxidative stress by treating erythrocytes with tert-butylhydroperoxide leads to programmed erythrocyte death [14].

Phosphatidylserine exposing erythrocytes bind to phosphatidylserine receptors at macrophages [6-8], leading to their engulfment and subsequent degradation. Erythrocytic vesicles may further be removed from circulation via scavenger receptors by hepatic Kupffer cells [24, 83-85]. Thus, phosphatidylserine exposure leads to premature clearance of affected erythrocytes from circulating blood. Moreover, enhanced phosphatidylserine exposure may allow adhesion of erythrocytes to the vascular wall [10, 86] leading to impairment of microcirculation [87-89]. On the other hand, phosphatidylserine exposure has been reported to impair adhesion to bovine pulmonary artery endothelial cells [90]. Enhanced adhesiveness of erythrocytes could support hemostasis [20, 91-94] and enhance the risk to develop thrombosis [95]. Along those lines trapping of cryptic erythrocytes has been observed in the renal medulla following renal ischemia [96]. Enhanced adherence to endothelial cells has indeed been observed in erythrocytes from diabetic patients [97] and methylglyoxal has indeed been implicated in the pathophysiology of vascular and renal disease [36-42, 98-100]. Thus, methylglyoxal induced phosphatidylserine exposure may contribute to the development of diabetic microangiopathy. However, methylglyoxal may be effective by further mechanisms, such as crosslinking of matrix proteins [101] or glycosylation of plasma proteins [102].

Premature death of phosphatidylserine exposing erythrocytes may affect the interpretation of glycated hemoglobin (HbA1c) concentration, which is widely used to monitor metabolic control in diabetic patients. The degree of glycation of hemoglobin is a function of blood glucose concentration and the time of exposure, i.e. the life span of the erythrocyte. Our data, together with previous results show that hyperglycemia via methylglyoxal increases the percentage of phosphatidylserine exposing erythrocytes thus decreasing the erythrocyte life span. Accordingly the resulting HbA1c value is lower in patients with reduced erythrocyte life span and does not fully reflect the poor metabolic control. Thus, decreased erythrocyte life span should be considered at the interpretation of HbA1c concentrations, particularly in patients with anemia and high reticulocyte numbers. A recent study demonstrated that the life span of erythrocytes is negatively correlated with glycemia [103]. The average erythrocyte life span averages ~80 days in patients with poor metabolic control as compared to the normal range of 123 days [103].

In conclusion, methylglyoxal stimulates phosphatidylserine exposure of erythrocytes at least partially by interfering with glucose utilization, ATP production, GSH formation and anti-oxidative defense. The effect of methylglyoxal contributes to or even accounts for the stimulation of erythrocyte phosphatidylserine exposure in diabetic patients and subsequent anemia and/or diabetic microangiopathy.

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