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Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes

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Abstract

Erythrocytes lack nuclei and mitochondria, the organelles important for apoptosis of nucleated cells. However, following increase of cytosolic Ca^{2+} activity, erythrocytes undergo cell shrinkage, cell membrane blebbing and breakdown of phosphatidylserine asymmetry, all features typical for apoptosis in nucleated cells. The same events are observed following osmotic shock, an effect mediated in part by activation of Ca^{2+}-permeable cation channels. However, erythrocyte death following osmotic shock is blunted but not prevented in the absence of extracellular Ca^{2+} pointing to additional mechanisms. As shown in this study, osmotic shock (950 mOsm) triggers sphingomyelin breakdown and formation of ceramide. The stimulation of annexin binding following osmotic shock is mimicked by addition of ceramide or purified sphingomyelinase and significantly blunted by genetic (aSM-deficient mice) or pharmacologic (50 μM 3,4-dichloroisocoumarin) knockout of sphingomyelinase. The effect of ceramide is blunted but not abolished in the absence of Ca^{2+}. Conversely, osmotic shock-induced annexin binding is potentiated in the presence of sublethal concentrations of ceramide. In conclusion, ceramide and Ca^{2+} entry through cation channels concert to trigger erythrocyte death during osmotic shock.

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Abbreviations: C_6-ceramide, D-erythro-*N*-hexanoylsphingosine; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; PBS, phosphate-buffered saline; SM, sphingomyelin; SMase, sphingomyelinase

Introduction

Apoptosis is a physiological form of death in which cells turn on a program eventually leading to self-destruction. It has been shown that the loss of cell volume is an early and fundamental feature of this form of cell death (for reviews, see Gomez-Angelats *et al.*[1] and Gomez-Angelats and Cidlowski[2]). Among the well-known triggers of apoptotic cell death is osmotic shock, that is, exposure to hypertonic extracellular fluid.[3–5] The cellular mechanisms invoked in the triggering of apoptosis following osmotic shock have been intensively studied in nucleated cells. These mechanisms include ligand-independent clustering of multiple growth factor and cytokine receptors, such as tumor necrosis factor receptor, by physical stress,[6] upregulation of tumor necrosis factor-alpha expression[3] and triggering of death receptor membrane trafficking.[7] These plasma membrane-located signals are then transduced by a network of different pathways, for example, activation of the phosphatidylinositol 3-kinase/Akt pathway,[8] induction of transcriptionally active p53[4] and activation of the heat-shock transcription factor 1.[9] Further studies pointed to a significant role of mitochondria during osmotic stress, either as a stress-sensing module[10] or as part of the apoptotic pathway itself.[11] This plasticity of effects involved in apoptotic pathways stimulated the search for less complex systems that are able to operate in the absence of intracellular organelles or gene transcription.

Erythrocytes are devoid of nuclei and mitochondria, intracellular organelles involved in the induction of apoptosis of nucleated cells.[12,13] Nevertheless, elevation of intracellular Ca^{2+} activity by treatment of erythrocytes with the Ca^{2+} ionophore ionomycin induces erythrocyte shrinkage, membrane blebbing and breakdown of cell membrane phosphatidylserine asymmetry,[14–16] all typical features of apoptosis in nucleated cells. Thus, the postulate has been made that those events reflect erythrocyte apoptosis.[14–16] The exposure of phosphatidylserine at the surface is evidenced from binding of annexin.[14,15] Most recently, erythrocytes have similarly been shown to bind annexin following osmotic shock,[17] which activates a Ca^{2+}-permeable cation channel, thus leading to increase of cytosolic Ca^{2+} activity.[17] Intracellular Ca^{2+} then activates a scramblase that translocates phosphatidylserine to the outer leaflet of the cell membrane.[18,19] However, erythrocyte annexin binding following osmotic shock is only partially inhibited in the nominal absence of Ca^{2+} or in the presence of a cation channel inhibitor.[17] Thus, osmotic shock is likely to trigger additional mechanisms eventually leading to breakdown of phosphatidylserine asymmetry. The present study aimed to disclose those additional mechanisms. As activation of acidic sphingomyelinase (aSMase) with subsequent formation of ceramide participates in the signaling of CD95-induced apoptosis of nucleated cells[20,21] and as CD95 has also been implicated in the osmotic stress response,[7] we specifically explored the influence of ceramide on erythrocyte apoptosis.

Results

Ca^{2+} ionophore ionomycin and hyperosmolarity trigger erythrocyte annexin binding

Treatment of erythrocytes with the Ca^{2+} ionophore ionomycin (1 μM) increases, within 1 h, the percentage of annexin binding cells from 1.4 $\pm$ 0.4 to 73.4 $\pm$ 7.7% (n=4). Thus, increase of intracellular calcium leads to breakdown of phosphatidylserine asymmetry. As illustrated in Figure 1a, annexin binding of erythrocytes is further increased following an 8-h exposure to hypertonic extracellular fluid (600 and 950 mOsm). In the following experiments, we routinely used 950 mOsm to induce annexin binding. In contrast to Jurkat T-cells, phosphatidylserine exposure in erythrocytes is not caspase-dependent. The pan-caspase inhibitor zVAD-fmk reduces annexin binding after osmotic shock in Jurkat cells (Figure 1c) but not in erythrocytes (Figure 1b). Furthermore, although erythrocytes contain significant amounts of procaspase-3, limited proteolysis of the enzyme to the active p17 subunit of caspase-3 did not occur after osmotic shock (Figure 2b, c). Again, Jurkat T-cells served as a positive control and these cells clearly displayed osmotic shock-induced formation of the active p17 subunit (Figure 2a). The morphology of erythrocytes exposed to osmotic shock is shown in Figure 3a. The cells are shrunken, annexin-positive and show invaginations of the plasma membrane.

If erythrocyte membrane integrity is disrupted, then annexin could bind to phosphatidylserine facing the cytosol. Disruption of the erythrocyte cell membrane should lead to release of cellular proteins including hemoglobin. To test for this possibility, we determined hemoglobin release. An 8-h exposure to 950 mOsm led to the release of hemoglobin from only 3.8 $\pm$ 0.6% (n=4) of the erythrocytes. Thus, the erythrocyte cell membrane is not disrupted and annexin would not have access to the intracellular leaflet of the cell membrane. This was further confirmed in erythrocytes infected with *Plasmodium falciparum* and counterstained with propidium iodide: after an 8-h treatment with 950 mOsm 78.2 $\pm$ 2.3% (n=3) of the infected cells were annexin-positive and 82.2 $\pm$ 7.3% (n=3) were propidium iodide-negative.

We then investigated the Ca^{2+} dependence of erythrocyte death. In the nominal absence of extracellular Ca^{2+} annexin binding following osmotic shock has been significantly decreased but not abolished. The respective values were 3.1 $\pm$ 0.4% (n=4) for controls, 58.5 $\pm$ 4.0% (n=4) for cells treated with 950 mOsm in the presence of Ca^{2+} and 33.8 $\pm$ 6.1% (n=4) for cells treated with 950 mOsm in the absence of Ca^{2+} (Figure 3b). As illustrated in Figure 3b, the annexin binding triggered by osmotic shock in the presence of

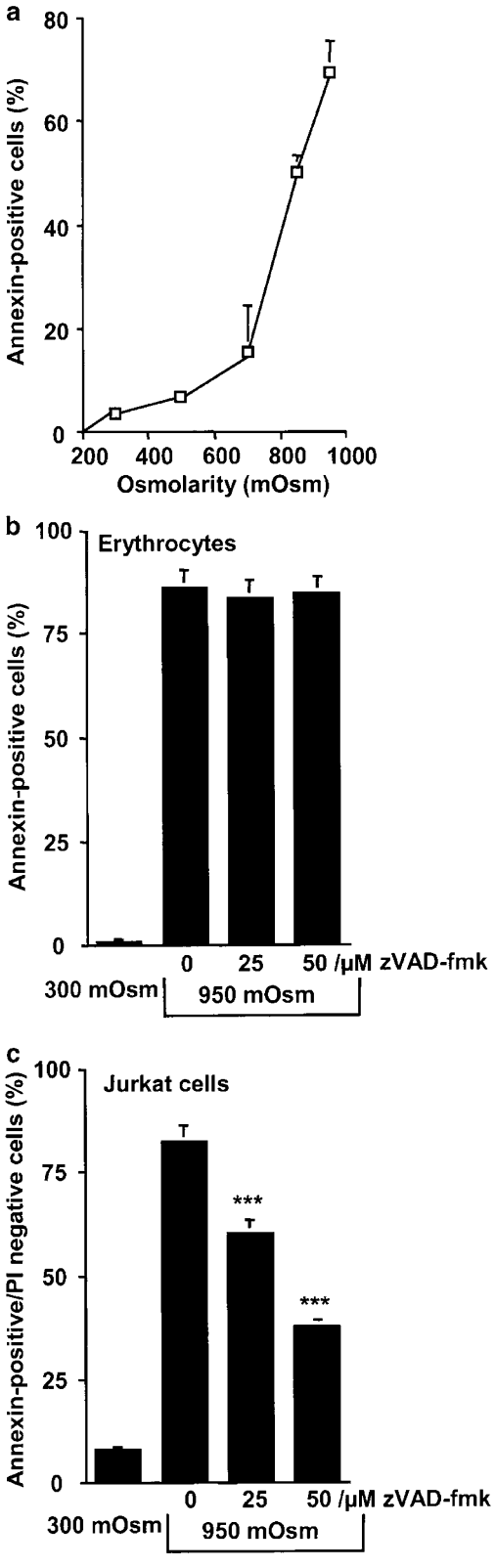


Figure 1 Hyperosmotic shock induces caspase-independent annexin binding of erythrocytes. (**a**) Annexin binding of erythrocytes at different osmolarities after 8 h of incubation. Arithmetic means $\pm$ S.E.M. (n=3) of annexin-positive cells are given in % of the total population. (**b**) Annexin binding of erythrocytes after 8 h of incubation with 950 mOsm in the absence or presence of 25 or 50 μM zVAD-fmk. Control refers to erythrocytes exposed to an isotonic buffer solution (300 mOsm) for 8 h. Arithmetic means $\pm$ S.E.M. (n=3) of annexin-positive cells are given in % of the total population. (**c**) Annexin binding of Jurkat T-cells after 8 h of incubation with 950 mOsm in the absence or presence of 25 or 50 μM zVAD-fmk. Control refers to Jurkat T-cells exposed to isotonic medium (300 mOsm) for 8 h. Arithmetic means $\pm$ S.E.M. (n=3) of annexin-positive/propidium iodide-negative cells are given in % of the total population. ***Significantly different from cells treated with 950 mOsm in the absence of zVAD-fmk ($P<0.01$)

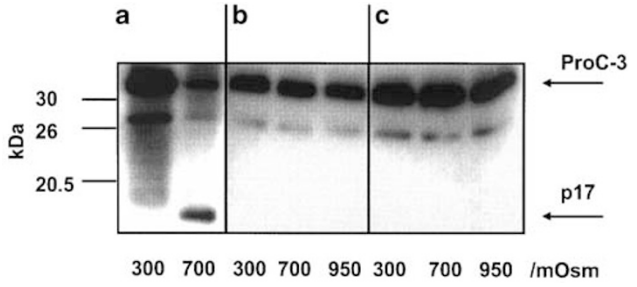


Figure 2 Hyperosmotic shock does not induce caspase-3 activation in erythrocytes. (**a**) Jurkat T-cells were treated either with isotonic medium (300 mOsm) or with medium adjusted to 700 mOsm. Then, 25 μg of cellular extract was loaded per lane and activation of caspase-3 was analyzed by Western blot as described in Materials and Methods. (**b**) Erythrocytes were treated either with isotonic medium (300 mOsm) or with medium adjusted to 700 or 950 mOsm. Then, 300 μg of cellular extract was loaded per lane and activation of caspase-3 was analyzed by Western blot. (**c**) Erythrocytes were treated either with isotonic medium (300 mOsm) or with medium adjusted to 700 or 950 mOsm. Then, 500 μg of cellular extract was loaded per lane and activation of caspase-3 was analyzed by Western blot. Positions of molecular mass markers are indicated at the left. Arrows indicate the positions of procaspase-3 (ProC-3) and the 17 kDa active subunit of caspase-3 (p17)

extracellular Ca^{2+} was only partially inhibited by the cation channel blocker amiloride. In cells depleted from Ca^{2+} by a 1-h exposure to Ca^{2+} chelators (EGTA), the application of ionomycin for another 1 h remained without any increase of annexin binding ($6.5 \pm 1.1\%$, $n=5$), but the subsequent exposure to osmotic shock for 8 h still led to significant triggering of annexin binding ($80.5 \pm 10.1\%$, $n=3$). These data indicate that osmotic shock-induced erythrocyte death is not exclusively mediated by a Ca^{2+} increase originating from intra- or extracellular sources.

Ceramide induces annexin binding of erythrocytes and cell shrinkage but unlike osmotic shock does not increase Ca^{2+} uptake into erythrocytes

Treatment of erythrocytes with 50 μM C_6-ceramide or addition of 0.01 U/ml SMase to the culture medium for 4 h strongly enhanced the number of annexin binding cells (Figure 4a–c). The effect of both C_6-ceramide and SMase was blunted in the nominal absence of extracellular Ca^{2+} by 66 and 77%, respectively (Figure 4d). Thus, the presence of Ca^{2+} sensitizes the erythrocytes against ceramide, but is not required for the cell death-inducing effect of ceramide. We also used long-chain natural ceramide to induce annexin binding of erythrocytes. In these experiments, annexin binding of erythrocytes after treatment with 0.1 and 0.25 μM D-erythro-*N*-palmitoylsphingosine (C_{16}-ceramide) approached $56.7 \pm 3.8\%$ ($n=3$) and $53.9 \pm 10.2\%$ ($n=3$), respectively, as compared with $7.0 \pm 2.6\%$ ($n=3$) in Ringer's solution-treated controls. However, the ethanol/dodecane vehicle also had a slight effect and annexin binding was increased to $25.4 \pm 10.5\%$ ($n=3$).

Ceramide-induced cell death was investigated in more detail and forward scatter analysis revealed that all maneuvers (osmotic shock, C_6-ceramide and SMase treatment) led

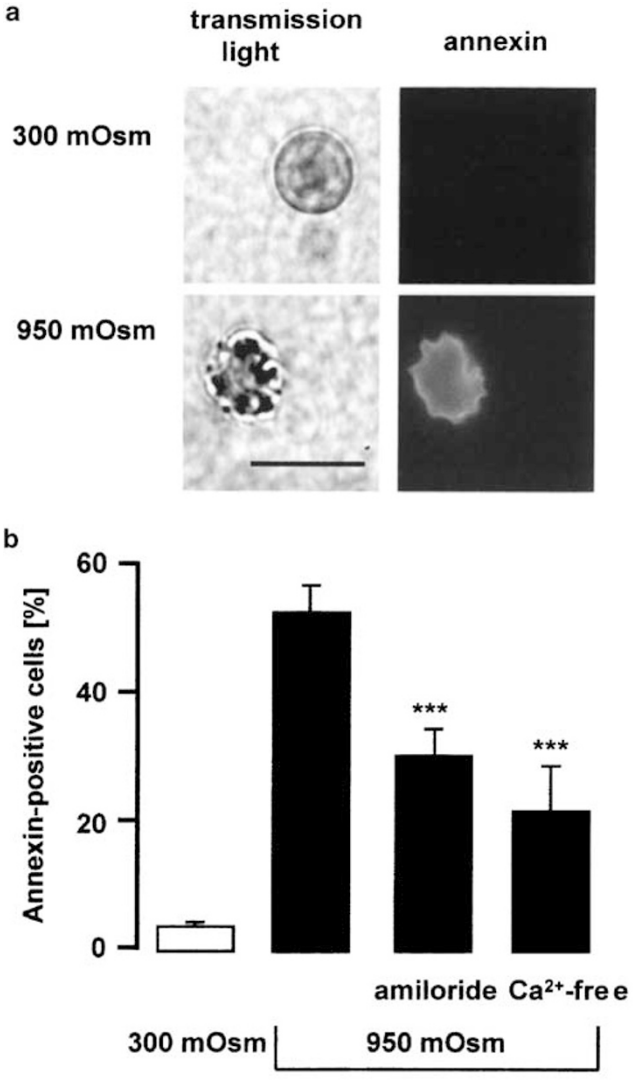


Figure 3 Hyperosmotic shock induces morphological changes and Ca^{2+}-dependent and Ca^{2+}-independent annexin-binding of erythrocytes. (**a**) Transmission light illustrations of erythrocytes treated with 300 mOsm for 8 h (upper left) or 950 mOsm for 8 h (lower left). The corresponding fluorescence illustrations for 300 mOsm are shown on the upper right and for 950 mOsm on the lower right. Bar=10 μm. (**b**) Annexin binding of erythrocytes after 8 h of incubation with 950 mOsm in the absence or presence of 1 mM amiloride, or in the absence of extracellular Ca^{2+} (Ca^{2+}-free). Control refers to erythrocytes exposed to an isotonic buffer solution (300 mOsm) for 8 h. Arithmetic means $\pm$ S.E.M. ($n=3$) of annexin-positive cells are given in % of the total population. ***Significantly different from cells treated with 950 mOsm in the absence of amiloride ($P<0.01$)

to a significant decrease of the forward scatter indicating that the cells were shrunken (Figure 5a, b). Consistent with the absence of hemolysis, the cell number was not reduced after osmotic shock, C_6-ceramide nor SMase treatment within the 8-h time period (Figure 6a). However, at later time points (24 h of incubation), all maneuvers led to a significant reduction of the cell number (Figure 6b) indicating that the erythrocytes are terminally damaged.

In the next series of experiments, we measured intracellular Ca^{2+} during ceramide-mediated erythrocyte death. Neither

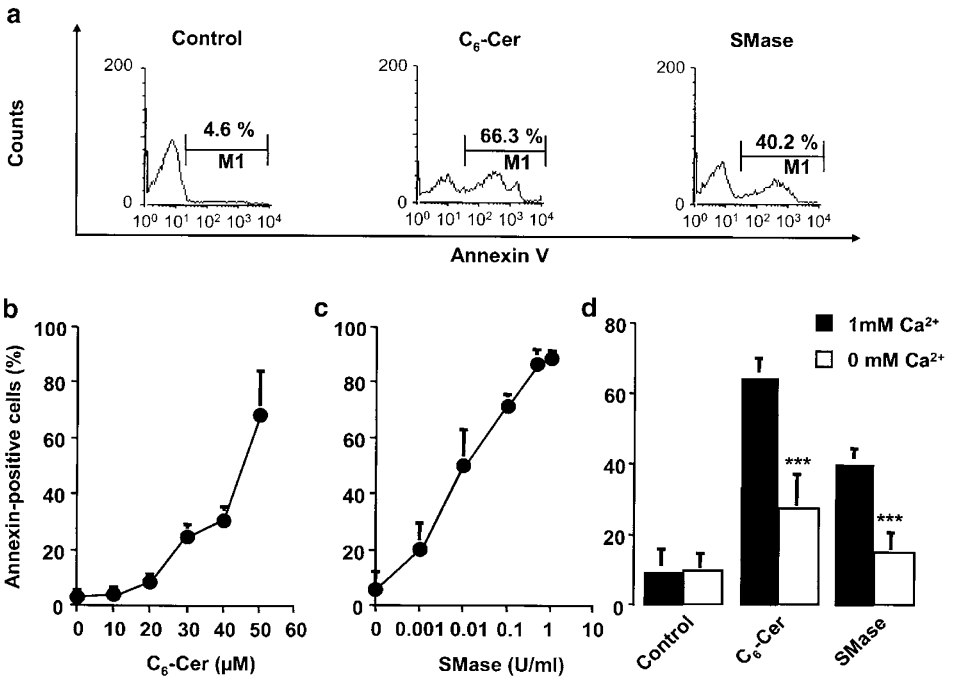


Figure 4 Ceramide and sphingomyelinase induce annexin binding of erythrocytes. (**a**) Histograms of representative experiments demonstrating annexin binding in control cells (left) or cells treated for 4 h with 50 μM C_6-ceramide (middle) or 0.01 U/ml SMase (right). (**b**) Annexin binding of erythrocytes after a 4-h treatment with different concentrations of ceramide. Arithmetic means $\pm$ S.E.M. (n=3) of apoptotic cells are given in % of the total population. (**c**) Annexin binding of erythrocytes after a 4-h treatment with different concentrations of SMase. Arithmetic means $\pm$ S.E.M. (n=3) of apoptotic cells are given in % of the total population. (**d**) Annexin binding of erythrocytes after a 4-h treatment with Ringer's solution (control), 50 μM C_6-ceramide or 0.01 U/ml SMase in the presence (black columns) or absence (open columns) of 1 mM Ca^{2+} in the extracellular fluid. Arithmetic means $\pm$ S.E.M. (n=3) of apoptotic cells are given in % of the total population. ***Significantly different from cells treated with C_6-ceramide or SMase in the presence of Ca^{2+} ($P<0.01$)

C_6-ceramide nor SMase enhanced intracellular Ca^{2+} activity in erythrocytes. $[Ca_T]_i$ values were 1.41 ± 0.13 μmol/10^{13} cells (n=4) for control cells, 1.20 ± 0.22 μmol/10^{13} cells (n=4) for cells treated with 50 μM C_6-ceramide and 1.30 ± 0.29 μmol/10^{13} cells (n=4) for cells treated with 0.01 U/ml SMase (Figure 7a). Even high concentrations of C_6-ceramide (100 μM) did not increase intracellular Ca^{2+} concentration (Figure 7b). In contrast, hyperosmotic shock by exposure of the cells to 950 mOsm significantly enhanced erythrocyte Ca^{2+} concentration (Figure 7a), a finding confirming earlier observations.[17] $[Ca_T]_i$ values were 1.26 ± 0.10 μmol/10^{13} cells (n=4) for control cells and 3.1 ± 0.14 μmol/10^{13} cells (n=4) for cells exposed to 950 mOsm. To further determine the role of cation channels, osmotic shock was applied in the presence of the cation channel blocker amiloride. The blocker partially blunted phosphatidylserine exposure after osmotic shock (Figure 7c). However, neither C_6-ceramide- nor SMase-induced annexin binding was significantly reduced by 1 mM amiloride (Figure 7c), indicating that cation channels do not play an essential role in ceramide-induced death signaling.

Osmotic shock stimulates the formation of ceramide

As ceramide stimulates annexin binding, it might participate in the breakdown of phosphatidylserine asymmetry following osmotic shock. We thus tested whether exposure to hypertonic extracellular fluid influences ceramide formation. As shown in Figure 8a, exposure of erythrocytes to osmotic shock (950 mOsm) increased binding of anti-ceramide antibody, which is reflected by a significant shift of the fluorescence of the cells. The fluorescence shift was already observed after 30 min and persisted for 15 h. For comparison and as a positive control, the effect of a 5-min exposure of erythrocytes to 1 U/ml SMase is shown (Figure 8a). The fluorescence shift after SMase treatment was in the same range as in the case of osmotic shock. Furthermore, nonspecific binding of antibodies to erythrocytes after osmotic shock was ruled out by the use of an isotype matched unspecific antibody that did not show enhanced binding to erythrocytes (Figure 8b). Detailed analysis revealed that the mean fluorescence of anti-ceramide FITC staining after treatment of erythrocytes with 950 mOsm was increased approximately four-fold as compared with control erythrocytes that were incubated in the presence of 300 mOsm (Figure 8c).

Osmotic shock induces sphingomyelin breakdown

Formation of ceramide by a sphingomyelinase should be paralleled by a decline of cellular sphingomyelin content, as *de novo* synthesis of ceramide in erythrocytes is quite unlikely.[22] To test for sphingomyelin breakdown, we labeled erythrocytes with radioactive choline and measured the incorporation of [*methyl*-^{3}H]choline into erythrocyte lipids. As shown in Figure 9a, significant label was detected in

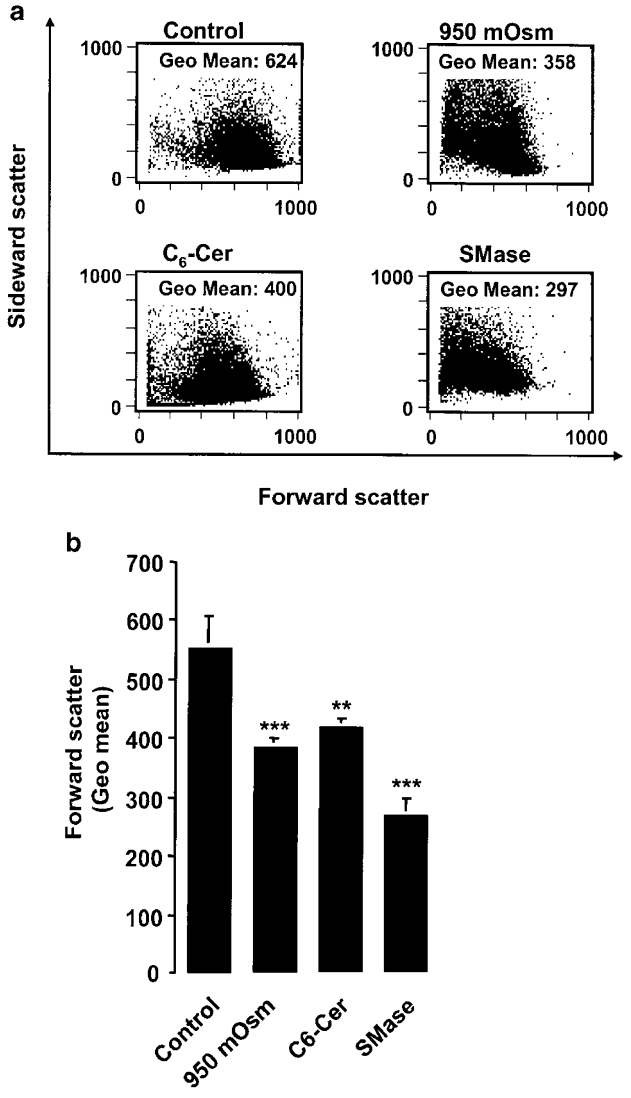


Figure 5 Osmotic shock, ceramide and sphingomyelinase induce erythrocyte shrinkage. (**a**) After 8 h of incubation, cells were measured in the FACS calibur for forward scatter and side scatter. Dot plots of representative experiments for control cells (upper left) or cells treated for 8 h with 950 mOsm (upper right), 50 μM C_6-ceramide (lower left) or 0.01 U/ml SMase (lower right) are shown. Geometric mean values are given for the single experiment. (**b**) Forward scatter analysis of erythrocytes after an 8-h treatment with 950 mOsm, 50 μM C_6-ceramide or 0.01 U/ml SMase. Control refers to erythrocytes exposed to an isotonic buffer solution (300 mOsm) for 8 h. Arithmetic means $\pm$ S.E.M. (n=3) of the geometric mean of the forward scatter are given. **Significantly different from control treated cells ($P<0.02$). ***Significantly different from control treated cells ($P<0.01$)

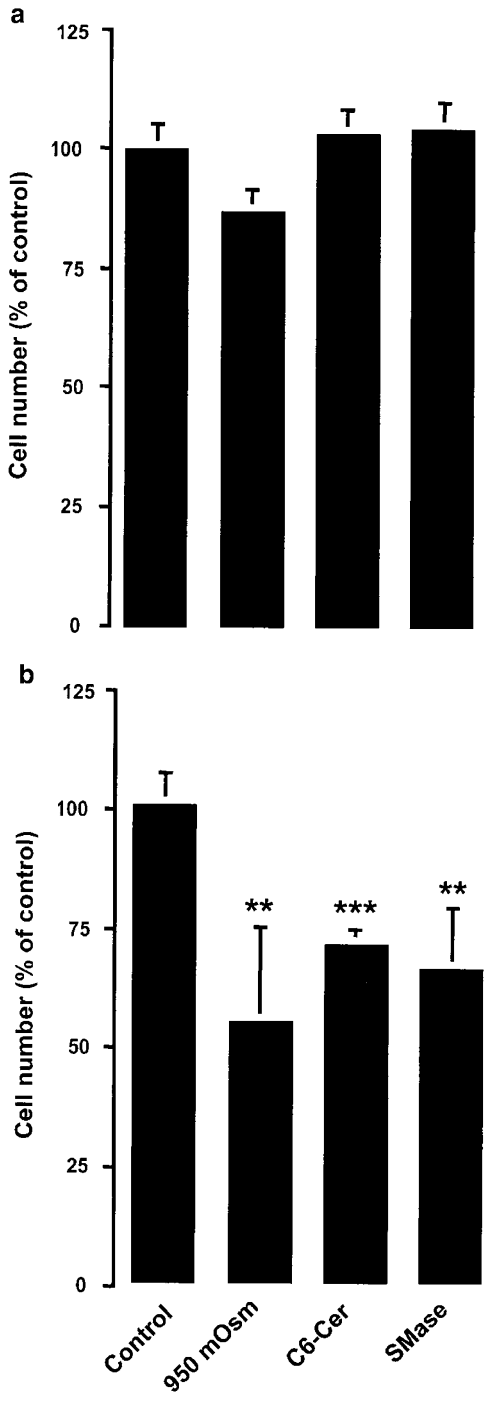
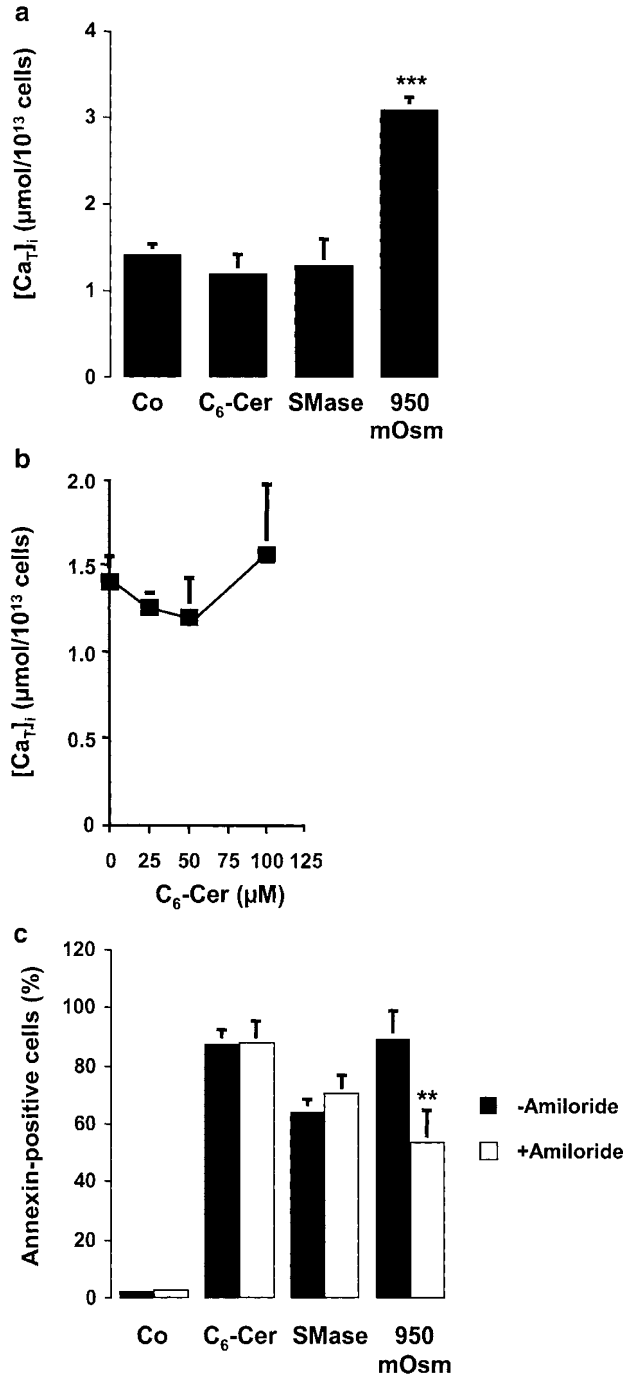


Figure 6 Influence of osmotic shock, ceramide and sphingomyelinase on erythrocyte cell number after 8 or 24 h of incubation. (**a**) After the 8-h treatment with 950 mOsm, 50 μM C_6-ceramide or 0.01 U/ml SMase, cells were counted in a hemocytometer. Control refers to erythrocytes exposed to an isotonic buffer solution (300 mOsm) for 8 h. (**b**) After 24-h treatment with 950 mOsm, 50 μM C_6-ceramide or 0.01 U/ml SMase, cells were counted in a hemocytometer. Control refers to erythrocytes exposed to an isotonic buffer solution (300 mOsm) for 24 h. Arithmetic means $\pm$ S.E.M. (n=3) of the cell numbers are given in % of control. Erythrocyte numbers under control conditions were $(1.3 \pm 0.1) \times 10^7$ cells/ml. **Significantly different from control treated cells ($P<0.02$). ***Significantly different from control treated cells ($P<0.01$)

erythrocyte lipids after 48 h of incubation, which reached 21800 ± 1200 d.p.m./10^9 cells after 72 h of incubation. More importantly, we could demonstrate that approximately 10% of the total lipid-bound radioactivity (1980 ± 100 d.p.m./10^9 cells) could be removed by the use of bacterial SMase (Figure 9a), thereby confirming that labeled choline was incorporated into SM of erythrocytes. Additionally, the efficiency of the bacterial SMase was checked. For this purpose, erythrocyte membranes were labeled with radioactive SM, and lipid-bound radioactivity was removed by incubation for 2 h with SMase.

As expected, 96% of the radioactivity of the total lipids was removed by SMase treatment (Figure 9b).

We now tested whether treatment of erythrocytes with 950 mOsm induced SM breakdown. As shown in Figure 9c, SM breakdown already occurred after 1 h of hypertonic shock when SM levels reached approximately 60% of control. SM levels increased again after 6 h but failed to reach the control level. This might be attributed to the low metabolic activity of erythrocytes.



3,4-Dichloroisocoumarin and knockout of aSMase but not fumonisin B1 blunts the annexin binding induced by osmotic shock

3,4-Dichloroisocoumarin has been shown to inhibit activation of SMase and apoptosis triggered by daunorubicin.[23] We thus used this inhibitor to further investigate the involvement of SMase and ceramide in osmotic shock-induced annexin binding of erythrocytes. As shown in Figure 10, annexin binding induced by osmotic shock was concentration-dependently blunted by 3,4-dichloroisocoumarin. At a concentration of 50 μM, annexin binding in the presence and absence of Ca^{2+} was reduced by 35 and 33%, respectively. In contrast, the annexin binding of erythrocytes following exposure to 1 μM ionomycin or 50 μM C_6-ceramide was not significantly modified in the presence of 50–200 μM 3,4-dichloroisocoumarin, thereby ruling out that the inhibitor interfered with the Ca^{2+} pathway or signaling downstream of ceramide formation.

In a second approach, we used erythrocytes from aSMase knockout mice. Osmotic shock-induced phosphatidylserine exposure was significantly reduced by 42% in erythrocytes from aSMase (−/−) mice as compared with the erythrocytes from wild-type littermates (Figure 11a). These data confirm the hypothesis that breakdown of sphingomyelin plays a role in osmotic shock-induced erythrocyte death.

Fumonisin B1 is an inhibitor of ceramide synthesis and has been shown to antagonize ceramide-mediated apoptosis at concentrations of 20–50 μM.[24] However, fumonisin B1 did not influence annexin binding of erythrocytes following treatment with 950 mOsm (Figure 11b), thereby ruling out that the elevated ceramide originates from the biosynthetic pathway.

Ceramide sensitizes the erythrocytes to osmotic shock

Since ceramide is released upon osmotic shock and triggers annexin binding, we investigated the combined effect of osmotic shock and ceramide on erythrocyte apoptosis. As illustrated in Figure 12a, the sublethal concentration of 20 μM C_6-ceramide clearly potentiated annexin binding induced by 950 mOsm after 4 h of combined treatment. Similarly,

Figure 7 Osmotic shock, but neither ceramide nor sphingomyelinase increase intracellular Ca^{2+} of erythrocytes. (**a**) $^{45}Ca^{2+}$ entry into erythrocytes was measured as described in Materials and Methods. Erythrocytes were bathed in uptake solution without (control) or with 50 μM C_6-ceramide (C_6-cer) or 0.01 U/ml sphingomyelinase (SMase). Additionally, erythrocytes were exposed to uptake solution adjusted to 950 mOsm. Arithmetic means ± S.E.M. (n=3) of intracellular calcium are given in μmol/10^{13} cells. ***Significantly different from control treated cells ($P<0.01$). (**b**) $^{45}Ca^{2+}$ entry into erythrocytes was measured in the presence of different concentrations of C_6-ceramide. Arithmetic means ± S.E.M. (n=3) of intracellular calcium are given in μmol/10^{13} cells. (**c**) Annexin binding of erythrocytes after 8 h of incubation with 50 μM C_6-ceramide (C_6-cer), 0.01 U/ml sphingomyelinase (SMase) or 950 mOsm in the absence (black bars) or presence of 1 mM amiloride (open bars). Control refers to erythrocytes exposed to an isotonic buffer solution (300 mOsm) for 8 h. Arithmetic means ± S.E.M. (n=3) of annexin-positive cells are given in % of the total population. **Significantly different from cells treated with 950 mOsm in the absence of amiloride ($P<0.02$)

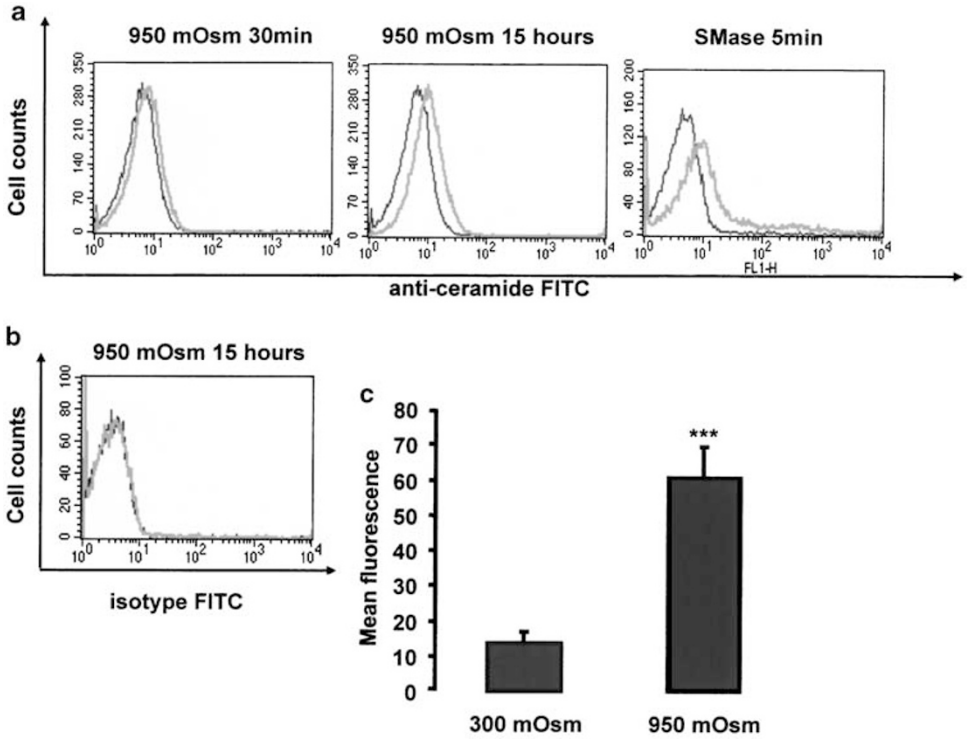


Figure 8 Stimulation of ceramide formation in erythrocytes by osmotic shock. (**a**) Ceramide was detected by anti-ceramide antibody and FACS analysis as described in Materials and Methods. Histograms of representative experiments of control cells treated with Ringer's solution (black line in all histograms) and cells exposed for 30 min (grey line, left histogram) or for 15 h (grey line, middle histogram) to osmotic shock of 950 mOsm are shown. As a positive control, erythrocytes were exposed for 5 min to 1 U/ml SMase (grey line, right histogram). (**b**) Histogram of a representative experiment demonstrating the binding of isotype matched anti-mouse antibody to control cells treated with Ringer's solution (black line) and cells exposed for 15 h to osmotic shock of 950 mOsm (grey line). (**c**) Arithmetic means $\pm$ S.E.M. (n=4) of the mean fluorescence of erythrocytes after treatment with Ringer's solution (300 mOsm) or after osmotic shock (950 mOsm). ***Significantly different from cells treated with 300 mOsm ($P<0.01$)

formation of intracellular ceramide by addition of 0.005 U/ml SMase to the culture solution sensitized erythrocytes to osmotic shock after 4 h of combined treatment (Figure 12b).

Discussion

The breakdown of phosphatidylserine asymmetry is a well-known signal for the elimination of erythrocytes by macrophages *in vivo*.[25,26] Phosphatidylserine exposure itself is stimulated by elevation of intracellular Ca^{2+}[14,15,17] and subsequent activation of a Ca^{2+}-dependent scramblase.[18,19,27] The present study confirms that osmotic shock triggers erythrocyte shrinkage and annexin binding. The osmolarity utilized is similar to the osmolarity prevailing in kidney medulla. On an average, each erythrocyte passes the hypertonic renal medulla 10 times a day. However, the dwelling time is normally too short to trigger significant breakdown of phosphatidylserine asymmetry. This may be different in acute renal failure where erythrocytes are indeed trapped in the kidney medulla.[28] Thus, at least in some conditions, hyperosmolarity may play a role in erythrocyte death. More importantly, though, similar mechanisms are triggered by other challenges of erythrocyte survival, such as oxidative stress and energy depletion.[17,29]

The effects of osmotic shock, oxidative stress and energy depletion have been shown to be partially mediated by unselective cation channels allowing the passage of Ca^{2+}.[30,31] However, we show here that osmotic shock-induced erythrocyte annexin binding is not completely blocked in the absence of extracellular Ca^{2+}. These data point to the existence of additional signaling mechanisms leading to annexin binding of erythrocytes. First of all, by using a pan-caspase inhibitor and by Western blot analysis of caspase-3 activation, we ruled out a functional role of caspases in erythrocyte death following osmotic shock, which is in accordance with the results of other groups showing that caspases in erythrocytes are not activated during cellular stress.[14,15] Since activation of SMase with subsequent formation of ceramide has been demonstrated as an important cellular response to different stress conditions,[20,32–34] we specifically investigated the effect of ceramide. Indeed, we could demonstrate that C_6-ceramide as well as treatment with bacterial SMase trigger annexin binding.

Hyperosmotic shock and ceramide induce erythrocyte shrinkage and phosphatidylserine exposure, both clear hallmarks of apoptosis in nucleated cells. The absence of hemoglobin release points to the integrity of the cell membrane, another crucial feature of apoptosis. In contrast, necrosis typically leads to cell swelling and eventual

disrupture of the cell membrane with subsequent release of intracellular proteins.[24] Thus, even though several features of apoptosis in nucleated cells, that is, caspase activation, mitochondrial depolarization and DNA fragmentation, are missing, the erythrocytes apparently undergo an apoptosis-like programmed cell death following exposure to osmotic shock. The ability of C_6-ceramide to induce this kind of erythrocyte death was somewhat surprising, as erythrocytes lack mitochondria, crucial elements in the ceramide-triggered signaling cascade in nucleated cells.[35–37] Thus, at least in erythrocytes, C_6-ceramide must trigger annexin binding through a pathway distinct from that described in nucleated cells. Furthermore, this pathway should be independent from oxidative phosphorylation since erythrocytes generate their energy by glycolysis. Interestingly, it has been shown that regulation of the glycolytic pathway may directly influence apoptotic signaling, especially after growth factor withdrawal.[38] Along those lines, the capacity of C_6-ceramide to trigger mitochondria-independent annexin binding in erythrocytes discloses the presence of an alternative pathway that may exist in nucleated cells as well.

Ceramides have been reported to form large channels in the outer mitochondrial membrane allowing the release of intermembrane space proteins with a molecular weight cutoff of about 60 000 Da.[39] However, it is shown here that C_6-ceramide and treatment with SMase do not enhance intracellular Ca^{2+} activity in erythrocytes. A simple channel-based mechanism of the ceramide effect is therefore rather unlikely. Moreover, the ceramide-induced erythrocyte annexin binding is blunted but not abolished in the nominal absence of Ca^{2+}. Thus, C_6-ceramide induced annexin binding is probably not secondary to increase of cytosolic Ca^{2+} activity. Instead, the effect of C_6-ceramide adds to or even potentiates the effects of Ca^{2+} entry. Accordingly, in the presence of C_6-ceramide the annexin binding following osmotic shock is accelerated.

With the exception of the description of a *P. falciparum* SMase during infection,[40] no data are available about the role of ceramide in erythrocyte (patho-) physiology. In the present study, five lines of evidence are provided that ceramide is involved in erythrocyte death signaling following osmotic shock: (i) accumulation of ceramide is observed after osmotic shock, (ii) osmotic shock induces breakdown of SM, (iii) ceramide induces annexin binding even in the absence of osmotic shock, (iv) osmotic shock-induced annexin binding is inhibited by 3,4-dichloroisocoumarin, which is known to efficiently prevent activation of SMase[23] and (v) knockout of aSMase impairs phosphatidylserine exposure after osmotic

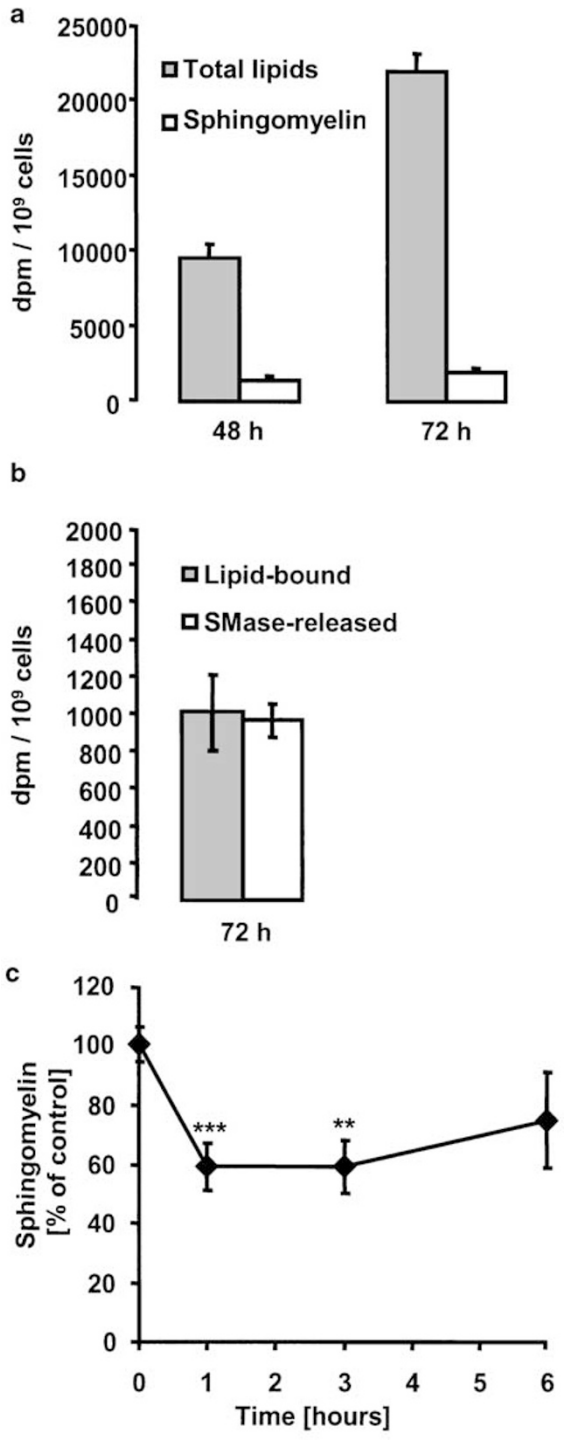


Figure 9 Osmotic shock induces sphingomyelin breakdown in erythrocytes. (**a**) Erythrocytes were labeled by addition of [*methyl*-^{3}H]choline chloride for 48 or 72 h. Then, the incorporation of radioactivity into cellular lipids or sphingomyelin was determined as described in Materials and Methods. Values are given in d.p.m./10^9 cells $\pm$ S.E.M. (n=3). (**b**) Erythrocytes were labeled by addition of [*methyl*-^{3}H]sphingomyelin for 72 h and the incorporation of radioactivity into cellular lipids was determined as described in Materials and Methods (lipid-bound). Then, sphingomyelin was digested using bacterial SMase and the release of water-soluble radioactivity was determined (SMase-released). Values are given in d.p.m./10^9 cells $\pm$ S.E.M. (n=3). Note that the lipid-bound radioactivity was completely released by the use of bacterial SMase. (**c**) Erythrocytes were labeled by addition of [*methyl*-^{3}H]choline chloride for 72 h. Then, erythrocytes were exposed to 950 mOsm for different time periods. Control erythrocytes were treated in parallel with Ringer's solution. After incubation, sphingomyelin was determined by the use of bacterial SMase as described in Materials and Methods. Sphingomyelin levels of osmotic shock-treated cells are given in % of control $\pm$ S.E.M. (n=6). **Significantly different from control treated cells ($P<0.02$). ***Significantly different from control treated cells ($P<0.01$)

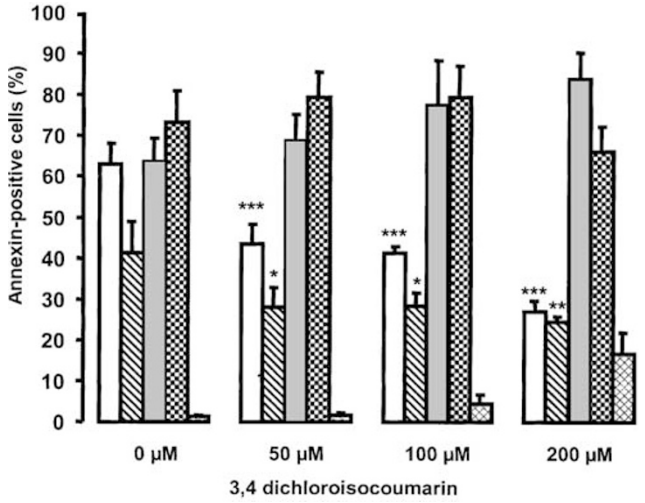


Figure 10 3,4-Dichloroisocoumarin blunts osmotic shock-induced annexin-binding, but not ionomycin- or C_6-ceramide-induced annexin-binding of erythrocytes. Erythrocytes were exposed to 950 mOsm in the presence (open columns) or absence (striped columns) of 1 mM Ca^{2+}, to 50 μM C_6-ceramide (filled columns), to 1 μM ionomycin (black and white columns) or treated with Ringer's solution (control, hatched columns) for 8 h. Annexin-binding was measured in the absence or presence of different concentrations of 3,4-dichloroisocoumarin. Arithmetic means $\pm$ S.E.M. (n=3) of annexin-positive cells are given in % of the total population. *Significantly different from cells treated with 950 mOsm (Ca^{2+}-free) in the absence of 3,4-dichloroisocoumarin ($P<0.05$). **Significantly different from cells treated with 950 mOsm (Ca^{2+}-free) in the absence of 3,4-dichloroisocoumarin ($P<0.02$). ***Significantly different from cells treated with 950 mOsm in the absence of 3,4-dichloroisocoumarin ($P<0.01$)

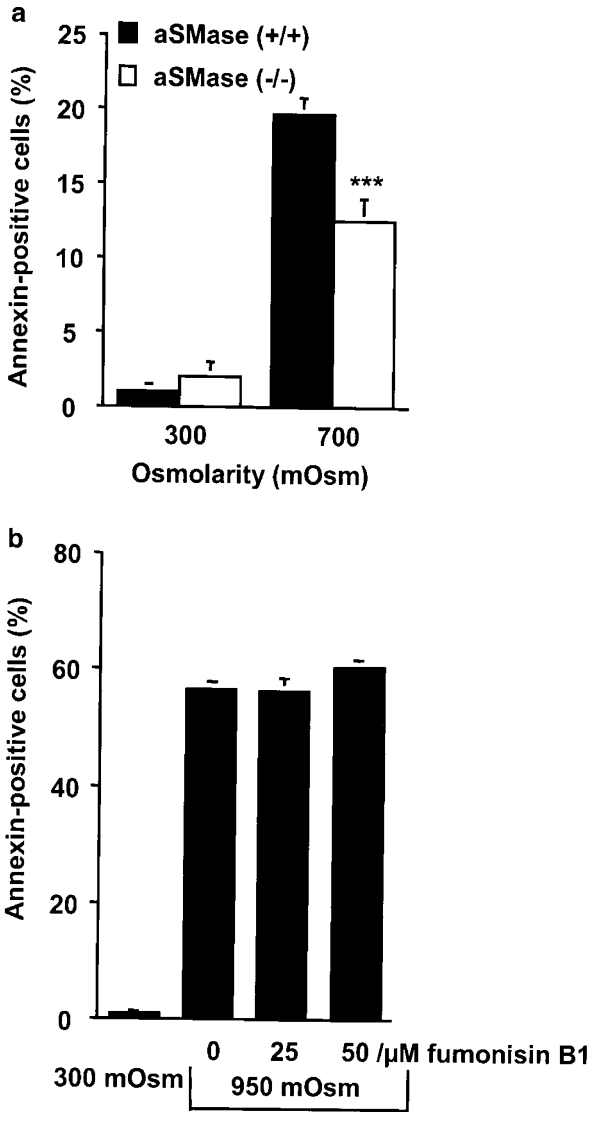


Figure 11 Knockout of aSMase but not treatment with fumonisin B1 blunts osmotic shock-induced annexin binding of erythrocytes. (**a**) Erythrocytes from aSMase knockout mice (aSMase (−/−), open bars) or corresponding littermates (aSMase (+/+), black bars) were exposed to 300 or 700 mOsm for 24 h. Arithmetic means $\pm$ S.E.M. (n=3) of annexin-positive cells are given in % of the total population. ***Significantly different from aSMase (+/+) cells treated with 700 mOsm ($P<0.01$). (**b**) Annexin binding of erythrocytes after 8 h of incubation with 950 mOsm in the absence or presence of 25 or 50 μM fumonisin B1. Control refers to erythrocytes exposed to an isotonic buffer solution (300 mOsm) for 8 h. Arithmetic means $\pm$ S.E.M. (n=3) of annexin-positive cells are given in % of the total population

shock. These data clearly demonstrate that erythrocytes are able to generate ceramide, presumably via breakdown of SM. Under normal conditions, erythrocytes lack considerable SMase activity, at least when enzyme activity is assayed in cell-free extracts.[41] Distinct stress conditions, however, such as increase of extracellular osmolarity, lead to activation of SMase and generation of ceramide.

The effect of osmotic shock is blunted by the sphingomyelinase inhibitor 3,4-dichloroisocoumarin suggesting that the effect of osmotic shock is sensitive to ceramide release. In contrast, the effects of neither C_6-ceramide nor ionomycin are inhibited by 3,4-dichloroisocoumarin, indicating that the drug exerts its inhibitory effect by interference with the signaling upstream of ceramide and cytosolic Ca^{2+}. Thus, ceramide formation could play a mediating or a permissive role in the triggering of annexin binding of erythrocytes. The experiments utilizing the ceramide antibody demonstrate that osmotic cell shrinkage stimulates the formation of ceramide. These observations strongly suggest the presence of a cell volume-sensitive SMase in erythrocytes that forms ceramide and thus sensitizes the erythrocytes for apoptosis.

Among the functions of ceramide is clustering of receptors. It has been shown that clustering of the CD95 receptor is mediated via ceramide-rich membrane rafts.[42] As a matter of fact, apoptosis of nucleated cells following osmotic shock[3–5] has been attributed to ligand-independent clustering of multiple growth factor and cytokine receptors, such as tumor necrosis factor receptor.[6] Thus, it seems reasonable that ceramide might play a similar role in erythrocytes, for example, in the clustering of channels or enzymes, such as scramblase.

In summary, similar to nucleated cells, erythrocytes undergo annexin binding following exposure to C_6-ceramide. Moreover, ceramide represents an important signaling molecule after hyperosmotic shock and sensitizes the erythrocytes for proapoptotic stimuli. The generation of ceramide may therefore participate in the regulation of erythrocyte survival.

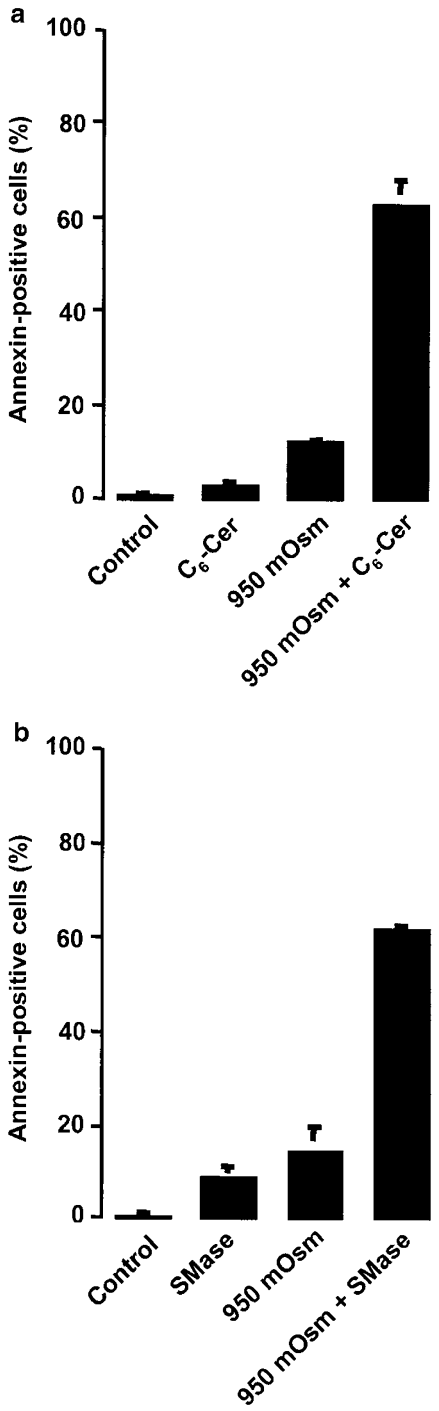


Figure 12 Ceramide and sphingomyelinase sensitize the erythrocytes to the apoptotic action of osmotic shock. (**a**) Annexin binding of erythrocytes after a 4-h treatment with Ringer's solution (control), 20 μM C_6-ceramide (C_6-Cer), exposure to hypertonic solution (950 mOsm) or combined treatment with 20 μM C_6-ceramide and hypertonic solution. Arithmetic means $\pm$ S.E.M. (n=3) of apoptotic cells are given in % of the total population. (**b**) Annexin binding of erythrocytes after a 4-h treatment with Ringer's solution (control), 0.005 U/ml SMase, exposure to hypertonic solution (950 mOsm) or combined treatment with 0.005 U/ml SMase and hypertonic solution. Arithmetic means $\pm$ S.E.M. (n=3) of apoptotic cells are given in % of the total population

Materials and methods

Cells

Erythrocytes were drawn from healthy volunteers or from aSMase knockout mice and the corresponding wild-type littermates. aSMase knockout mice[43,44] and littermates were a kind gift of Dr Verena Jendrossek (University of Tübingen, Germany) and were originally obtained from Dr R Kolesnick (Sloan Kettering Cancer Memorial Center, NY, USA). Erythrocytes were either used without purification or after separation by centrifugation for 25 min; 2000 *g* over Ficoll (Biochrom KG, Berlin, Germany). Experiments with nonpurified or experiments with Ficoll-separated erythrocytes yielded the same results (data not shown).

In some control experiments, erythrocytes were infected with *P. falciparum* as described previously.[45]

Jurkat T-cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 0.56 g/l L-glutamine, 100 000 U/l penicillin and 0.1 g/l streptomycin. Where indicated, osmolarity was increased to 700 or 950 mOsm by adding sucrose.

Solutions

Experiments were performed at 37°C in Ringer's solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 5 mM Glucose, 1 mM $CaCl_2$, pH=7.4. For the nominally calcium-free solution $CaCl_2$ was replaced by 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). Where indicated, osmolarity was increased to 950 mOsm by adding sucrose. Ionomycin (Sigma; Taufkirchen, Germany) was used at a concentration of 1 μM. D-erythro-*N*-hexanoylsphingosine (C_6-ceramide) was dissolved in dimethyl sulfoxide (DMSO) to give a 50 mM stock solution and further diluted in Ringer's solution containing 0.1% bovine serum albumin. The maximum concentration of DMSO was in all cases 0.1%, a concentration that did not induce annexin binding (data not shown). Fumonisin B1 was dissolved in methanol to give a 50 mM stock solution and further diluted in Ringer's solution containing 0.1% bovine serum albumin. D-erythro-*N*-palmitoylsphingosine (C_{16}-ceramide) was dissolved in ethanol/dodecane (98 : 2, v/v) at a concentration of 177 μM. The solution was then added to Ringer's solution and sonicated for 30 min before use. Vehicle was present at 0.3% and was added to controls. Sphingomyelinase from *Staphylococcus aureus*, rabbit polyclonal anti-caspase-3 antibody (raised against human recombinant caspase-3 and recognizing the proenzyme and the active 17 kDa subunit), C_6-ceramide and 3,4-dichloroisocoumarin were purchased from Biomol GmbH (Hamburg, Germany). C_{16}-ceramide, fumonisin B1, monoclonal antibody to ceramide (clone MID 15B4; isotype IgM) and isotype pure mouse IgM were from Alexis (Grünberg, Germany). The pan-caspase inhibitor zVAD-fmk (in which z stands for benzyloxycarbonyl and fmk for fluoromethyl ketone) was from Calbiochem (Bad Soden, Germany) and was dissolved in DMSO to give a 20 mM stock solution. This inhibitor was synthesized as a methyl ester to enhance cell permeability. $^{45}Ca^{2+}$ was from ICN Biomedicals GmbH (Eschwege, Germany) and delivered as $CaCl_2$ in aqueous solution (specific activity: 0.185–1.11 TBq/g Ca).

Determination of cell numbers and hemolysis

Erythrocytes were suspended at 0.15% hematocrit and incubated under different control and stress conditions (osmotic stress, C_6-ceramide and SMase treatment). After incubation, the cell number was determined using a hemocytometer as described previously.[46] Additionally, hemolysis was

determined by photometric measurement of hemoglobin release. The hemoglobin concentration in the supernatant was determined quantitatively by photometry (absorbance at 546 nm after oxidation to *cyanomet*-hemoglobin). The absorbance of the supernatant of completely lysed erythrocytes was set as 100% hemolysis.

FACS analysis

FACS analysis was performed essentially as described.[47] After incubation, cells were washed in Annexin-binding buffer containing 125 mM NaCl, 10 mM HEPES (pH=7.4), 5 mM $CaCl_2$. Erythrocytes were stained with Annexin-Fluos (Böhringer Mannheim, Germany) at a 1 : 100 dilution. After 15 min, samples were diluted 1 : 5 and measured by flow cytometric analysis on a FACS-Calibur (Becton Dickinson; Heidelberg, Germany). Cells were analyzed by forward and side scatter and annexin-fluorescence intensity was measured in FL-1. In the case of Jurkat cells and *P. falciparum*-infected erythrocytes, the cells were counterstained using propidium iodide (5 μg/ml). Propidium iodide-fluorescence was measured in FL-3.

For determination of ceramide, cells were stained for 1 h at 4°C with 1 μg/ml anti-ceramide antibody or 1 μg/ml isotype matched pure mouse antibody in phosphate-buffered saline (PBS) containing 1% FCS at a dilution of 1 : 5 as described recently.[42] After three washes with PBS/1% FCS, cells were stained with polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig specific antibody (Pharmingen, Hamburg, Germany) in PBS/1% FCS at a dilution of 1 : 50 for 30 min. Unbound secondary antibody was removed by washing the cells two times with PBS/1% FCS and samples were analyzed by flow cytometric analysis on a FACS-Calibur. FITC-fluorescence intensity was measured in FL-1.

Western blot analysis

Western blot analysis was performed as described.[48] After incubation, cells were washed twice with PBS and lysed in buffer containing 20 mM Tris/HCl, pH 7.4, 300 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% Na dodecylsulfate (SDS), 2.5 mM EDTA, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined using the Bio-Rad protein assay from Biorad (München, Germany) and equal amounts of protein were separated by SDS-PAGE.[49] Then, blotting of proteins onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) was performed exactly as described.[48] After blotting, the membrane was blocked for 1 h in PBST (PBS, 0.05% Tween-20) containing 3% nonfat dry milk and incubated with primary anti-caspase-3 antibody at a dilution of 1 : 1000 for 1 h. After the membrane had been washed three times in PBST, secondary donkey anti-rabbit horseradish peroxidase-linked whole antibody (Amersham Biosciences; Freiburg, Germany) at a dilution of 1 : 1000 in PBST was applied for 1 h. Finally, the membrane was washed in PBST again and the ECL enhanced chemiluminescence system from Amersham Biosciences was used to visualize the protein bands in question.

Microscopy

Fluorescence microscopy was performed essentially as described.[24] After incubation, erythrocytes were stained with Annexin-Fluos as described above. After washing the cells with annexin buffer, 10 μl of the suspension were applied to a slide and covered with a cover glass. Note that the staining and washing solutions after hyperosmotic shock were also adjusted to 950 mOsm by addition of sucrose. Finally, the cells were analyzed under a fluorescence microscope (Nikon; Düsseldorf, Germany) and digital pictures were taken using a digital imaging system (Visitron Systems; Puchheim, Germany) equipped with the Metaview software.

Calcium measurements

Intracellular calcium was measured as described in detail elsewhere.[50,51] Erythrocytes were washed four times by centrifugation (2000 *g* for 5 min) and resuspension in five volumes of solution A containing in mM: 80 KCl, 70 NaCl, 10 HEPES, 0.2 $MgCl_2$, 0.1 EGTA; pH 7.5 to remove extracellular Ca^{2+}. The cell pellet was then washed twice in solution B to remove EGTA from the medium. Solution B had the same composition as solution A but without EGTA. The cells were suspended at 10% hematocrit and preincubated for 20 min at 37°C in the final incubation solution B supplemented with 10 mM inosine and 1 mM sodium orthovanadate. Then $^{45}Ca^{2+}$ was added from a 100 mM $CaCl_2$ stock solution with a specific activity of about 10^7 c.p.m./μmol to reach a final concentration of 150 μM. After 10 min, 100 μl aliquots were delivered into 1.2 ml of ice-cold solution B with 0.2 mM $CoCl_2$ and 1 mM amiloride. The cells were collected by centrifugation in an Eppendorf centrifuge (14 000 r.p.m. for 0.5 min, 4°C) and the cell pellet was washed twice using 1 ml of the same medium. The supernatant was discarded and the cells were lysed and the proteins precipitated by addition of 0.6 ml 6% trichloroacetic acid. After a further spin (14 000 r.p.m. for 2 min, 4°C), 0.5 ml of clear supernatant was used for measuring $^{45}Ca^{2+}$ radioactivity by scintillation counting. $^{45}Ca^{2+}$ specific activity was determined by addition of 0.6 ml 6% trichloroacetic acid to 100 μl suspension samples and centrifugation as described above. Then, 100 μl of clear supernatant were taken for scintillation counting. The total calcium content of the cells $[Ca_T]_i$ was calculated by dividing the activity of the samples by the specific activity of $^{45}Ca^{2+}$ and by the number of cells. Different concentrations of C_6-ceramide or sphingomyelinase were added to the cell suspensions together with $^{45}Ca^{2+}$. Exposure of erythrocytes to 950 mOsm was achieved by the addition of sucrose to solution B during 20 min of preincubation and 10 min of $^{45}Ca^{2+}$ uptake. Note that the delivery medium for washing the cells after radioactive labeling was also adjusted to 950 mOsm by the addition of sucrose.

Choline labeling of erythrocytes

Erythrocytes were washed two times with Ringer's solution and then grown at 10% hematocrit in RPMI 1640 (Life Technologies; Karlsruhe, Germany) containing 50 mg/l gentamycin (Life Technologies), 2 mM L-glutamine, 20 mM HEPES, 20 mg/l hypoxanthine (Sigma; Taufkirchen, Germany), 1 mM glucose monohydrate, 5 g/l Albumax II (Life Technologies) and 10% heat-inactivated human serum AB^+. The cells were incubated for 72 h in the presence of 7.4×10^4 Bq/ml [*methyl*-^{3}H]choline chloride (Amersham Pharmacia Biotech; Braunschweig, Germany). Postlabeling, cells were washed twice with Ringer's solution, reseeded at 2×10^8 cells/ml in control Ringer's solution or in Ringer's solution adjusted to 950 mOsm by adding sucrose for 1, 3 and 6 h. After incubation, cells were harvested by centrifugation in the Eppendorf centrifuge (14 000 r.p.m. for 0.5 min, 4°C) and the cell pellet was washed twice using 1 ml of the same medium. Then, lipids were extracted by a modified method of Bligh and Dyer as described earlier.[52] Briefly, cell pellets were resuspended in 50 μl methanol, 25 μl chloroform and 20 μl water. Samples were stirred for 10 min on a vortex mixer and centrifuged at 13 000 $\times$ *g* for 2 min. Phase separation was accomplished by the addition of 25 μl chloroform and 25 μl water. The suspension and centrifugation steps were repeated. Volumes of 20 μl of the chloroform phases were

taken for scintillation counting, and 50 μl of the chloroform phases were dried under nitrogen and used for SM measurement as described below.

Bacterial SMase

SM was quantified using bacterial sphingomyelinase to release [^{3}H]phosphocholine as described.[53] Briefly, cellular lipid was resuspended in 100 μl of assay buffer (100 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 0.1% Triton X-100). Samples were sonicated for 5 min and 1 U/ml *Streptomyces* sp. SMase (Sigma; Taufkirchen, Germany) was added. Reaction mixtures were incubated for 2 h at 37°C. Reactions were stopped by addition of 1.0 ml of chloroform/methanol (2 : 1, v/v). Phase separation was completed by addition of 100 μl of water. SM was quantitated by counting the upper, aqueous phase, containing the liberated [^{3}H]phosphocholine and phosphatidylcholine was quantitated by drying and counting the lower, organic phase. Where appropriate, SM was normalized using phosphatidylcholine measurements. Blank reactions contained no SMase. The radioactivity of control samples normally reached 1500 d.p.m./10^9 cells and was set as 100%. Subsequently, SM in the samples of hyperosmotic shock-treated cells was calculated as % of control.

Optimization studies illustrated that the above conditions yielded maximal SM hydrolysis (100%) (Figure 9b).

Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was made by paired or unpaired *t*-test, where appropriate.

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