Effect of Anandamide on Erythrocyte Survival

Peter J. Bentzen and Florian Lang

Department of Physiology, University of Tübingen

Key Words

Arachidonylethanolamide • Apoptosis • Calcium • Oxidative stress • Cyclooxygenase

Abstract

The endocannabinoid anandamide (Arachidonylethanolamide, AEA) is known to induce apoptosis in a wide variety of nucleated cells. The present study explored whether anandamide induces suicidal death of erythrocytes or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Eryptotic cells are phagocytosed and thus cleared from circulating blood. Triggers of eryptosis include increase of cytosolic Ca^{2+} activity, formation of PGE_2 , oxidative stress and excessive cell shrinkage. Erythrocyte Ca^{2+} activity was estimated from Fluo3 fluorescence, phosphatidylserine exposure from annexin V binding, and erythrocyte volume from forward scatter in FACS analysis. Exposure of erythrocytes to anandamide ($= 2.5 \mu\text{M}$) increased cytosolic Ca^{2+} activity, enhanced the percentage of annexin V binding erythrocytes and decreased erythrocyte forward scatter, effects significantly blunted in the presence of cyclooxygenase inhibitors acetylsalicylic acid ($50 \mu\text{M}$) or ibuprofen

($100 \mu\text{M}$) and in the nominal absence of extracellular Ca^{2+} . Anandamide further enhanced the stimulating effects of hypertonic (addition of 550 mM sucrose) or isotonic (isosmotic replacement of Cl^- with gluconate) cell shrinkage on annexin V binding. The present observations demonstrate that anandamide increases cytosolic Ca^{2+} activity, thus leading to cell shrinkage and cell membrane scrambling of mature erythrocytes.

Copyright © 2007 S. Karger AG, Basel

Introduction

Anandamide, the most important endogenous cannabinoid, has previously been shown to induce apoptosis in a wide variety of cells [1] including liver cells [2-4] colorectal cancer cells [5], transformed glioma cells [6, 7], neurons [8], neuroblastoma [9, 10], neuroglioma [11], PC12 cells [12, 13], blastocysts [14], uterine cervix cancer cells [15], Chinese Hamster Ovary cells [16], prostatic cancer cells [17], endothelial cells [18], dendritic cells [19], lymphocytes [20] and lymphoma cells [10, 21, 22]. On the other hand, cannabinoids have been shown to protect primary neurons, astrocytes and oligodendrocytes from apoptosis [7, 23], to stimulate cell proliferation of cancer cells [24] and to enhance the pro-

liferative effect of erythropoietin [25].

Mechanisms involved in anandamide induced apoptosis of nucleated cells include Ca^{2+} and calpain [8, 10], ceramide [3, 17], lipid rafts [9], G-proteins [26], ROS production [4, 12, 16, 27], p38 kinase pathway [2, 13], Nuclear Factor kappaB [19] and cyclooxygenase 2 [5, 11]. Even though anandamide was able to activate caspases [12, 28], the apoptotic effect of anandamide was not significantly blunted by caspase inhibition [8].

Erythrocytes are similarly able to undergo suicidal cell death or eryptosis [29]. Eryptosis is triggered by activation of Ca^{2+} -permeable erythrocyte cation channels [30–32] and Ca^{2+} entry with subsequent activation of Ca^{2+} -sensitive K^+ channels, KCl exit, osmotic water loss and cell shrinkage [33, 34]. Ca^{2+} further triggers Ca^{2+} -sensitive scrambling of the cell membrane [35] with phosphatidylserine exposure at the cell surface [30]. Eryptosis is further elicited by ceramide (acylsphingosine) [36].

Phosphatidylserine exposure favors engulfment by macrophages equipped with phosphatidylserine receptors [37] and subsequent elimination from circulating blood [38]. Accordingly, similar to erythrocyte senescence [39–41] and neocytolysis [42], eryptosis leads to disposal of affected erythrocytes [29]. Enhanced eryptosis has been observed following treatment of erythrocytes with PGE_2 [43], platelet activating factor [44], paclitaxel [45], methylglyoxal [46], chlorpromazine [47], cyclosporine [48], amyloid peptides [49], mercury [50], lead [51] and hemolysin [43]. Moreover, eryptosis is favoured by phosphate depletion [52], iron deficiency [38], Hemolytic Uremic Syndrome [53], sepsis [54], malaria [55], Wilson disease [56], as well as genetic erythrocyte disorders, such as sickle cell anemia, thalassemia and glucose 6 phosphate dehydrogenase deficiency [57]. Eryptosis is inhibited by erythropoietin [58], urea [59], catecholamines [32] and several channel blockers [31, 34].

Hitherto nothing is known about effects of anandamide on erythrocyte survival. Thus, the present experiments explored whether anandamide induces suicidal death of erythrocytes and aimed to elucidate underlying mechanisms.

Materials and Methods

Erythrocytes, solutions and chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers. The volunteers provided informed consent. The study has been approved by the ethics committee of the University of Tübingen (184/2003V).

Ringer solution contained (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. Where indicated, chloride was replaced by gluconate or osmolarity increased by addition of 550 mM sucrose.

Anandamide was used at concentrations ranging from 2.5 to 10 μM , ionomycin at a concentration of 1 μM , N-acetyl-cysteine at 0.1 mM to 1.5 mM, acetylsalicylic acid at 50 μM and ibuprofen at 100 μM . Where applicable, the final concentration of the solvent dimethyl sulfoxide (DMSO) was 0.1%. Ionomycin, N-acetyl-cysteine, acetylsalicylic acid and ibuprofen were purchased from Sigma (Taufkirchen, Germany), anandamide and the Ca^{2+} dye Fluo-3/AM from Calbiochem (Bad Soden, Germany).

FACS analysis of annexin V binding and forward scatter

FACS analysis was performed as described [60]. After incubation in the presence or absence of anandamide, cells were washed in annexin V binding buffer containing (in mM): 125 NaCl, 10 HEPES/NaOH (pH 7.4), and 5 CaCl_2 . Erythrocytes were suspended in a solution composed of annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin V buffer (dilution of 1:50). After 10 min. of incubation, samples were finally diluted 1:5 in annexin V binding buffer and measured by flow cytometric analysis on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Cells were analysed by forward scatter and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+} activity

Intracellular Ca^{2+} measurements were performed as described previously [43]. Erythrocytes were loaded with Fluo-3/AM (Calbiochem) by addition of 2 μl of a Fluo-3/AM stock solution (2.0 mM in DMSO) to 1 ml erythrocyte suspension (0.16% hematocrit in Ringer). Cells were incubated at 37°C for 15 min under protection from light. Subsequently, an additional 2 μl aliquot of Fluo-3/AM stock solution was added, and the cells were incubated for 25 min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1000 g for 5 min at 22°C and washed two times with Ringer solution containing 0.5% bovine serum albumin (Sigma) and one time with Ringer. Fluo-3/AM-loaded erythrocytes were re-suspended in 0.5 ml Ringer solution (0.16% hematocrit) containing different concentrations of anandamide, and incubated for different time periods at 37°C. Then, Ca^{2+} -dependent fluorescence intensity was measured by FACS analysis in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a positive control for enhanced Ca^{2+} activity, Fluo-3-labelled erythrocytes were incubated in Ringer containing 1 μM of ionomycin.

Determination of hemolysis

In order to control for hemolysis, cells were suspended (0.3%) and incubated for 24h in Ringer's solution containing different concentrations of anandamide. After incubation, hemoglobin concentration in the supernatant was determined

Fig. 1. Stimulation of phosphatidylserine exposure by anandamide. A. Histograms of annexin V binding in a representative experiment of erythrocytes from healthy volunteers exposed for 24 hours to Ringer solution without (left panel) or with (right panel) 10 μ M anandamide. B. Dose dependence of the anandamide effect on phosphatidylserine exposure and hemolysis. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage annexin V binding erythrocytes (black bars) exposed for 24 hours to Ringer solution without (Ringer) or with anandamide at concentrations ranging from 2.5 to 10 μ M. For comparison, the percentage of hemolysis (white bars) is shown. * indicates significant difference from control values (ANOVA; $P < 0.05$).

by photometric measurement (Tecan Elisa Reader, at 405 nm). The respective values are expressed in percentage of haemoglobin concentration following complete lysis of the cells by exposure to pure water (set as 100% hemolysis). The absorption of anandamide has been determined concurrently and was subtracted from the absorption of the supernatant.

Statistics

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

Fig. 2. Effects of anandamide on erythrocyte forward scatter. A. Histograms of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 24 hours to Ringer solution without (left panel) or with (right panel) 10 μ M anandamide. B. Effect of anandamide on forward scatter. Arithmetic means $\pm$ SEM ($n = 4$) of the forward scatter of erythrocytes exposed for 24 hours to Ringer solution without (Ringer, open bar) or with anandamide (AEA, closed bars) at concentrations ranging from 2.5 to 10 μ M. * indicates significant difference from Ringer treated controls (ANOVA; $P < 0.05$).

Results

Phosphatidylserine exposure was determined from binding of FITC-labelled annexin V. Annexin binding in the absence of anandamide was low. Incubation of erythrocytes in Ringer solution for 24 h resulted in an average percentage of annexin V exposing erythrocytes of $1.6 \pm 0.1\%$ ($n = 4$). The addition of anandamide ($= 5 \mu$ M) increased within 24 h significantly the percentage of annexin V binding cells (Fig. 1A,B).

Fig. 3. Effects of anandamide on cytosolic Ca^{2+} activity and effects of Ca^{2+} removal from the medium on anandamide induced phosphatidylserine exposure and reduction of forward scatter. A. Arithmetic means $\pm$ SEM ($n = 4$) of Fluo3-dependent fluorescence in erythrocytes incubated for 60 min (white bars), 180 min (grey bars) or 360 min (black bars) in Ringer solution without or with anandamide at concentrations ranging from 2.5 to 10 μM , or with 1 μM ionomycin (Iono) as a positive control. * indicates significant difference from Ringer treated controls (ANOVA; $P < 0.05$). B. Effect of anandamide on annexin V binding in the presence and absence of Ca^{2+} in the incubation medium. Arithmetic means $\pm$ SEM ($n = 8$) of erythrocyte annexin V binding after incubation in Ringer solution with (Ringer) or without (0 Ca^{2+}) Ca^{2+} for 24 hours in the absence (open bars) or presence (closed bars) of anandamide at a concentration of 10 μM . * indicates significant difference from Ringer-treated controls (ANOVA; $P < 0.05$). # indicates significant difference from the presence of Ca^{2+} in the medium (ANOVA; $P < 0.05$). C. Effect of anandamide on erythrocyte forward scatter in the presence and absence of Ca^{2+} in the incubation medium. Arithmetic means $\pm$ SEM ($n = 8$) of erythrocyte forward scatter after incubation in Ringer solution with (Ringer) or without (0 Ca^{2+}) Ca^{2+} for 24 hours in the absence (open bars) or presence (closed bars) of anandamide at a concentration of 10 μM . * indicates significant difference from Ringer-treated controls (ANOVA; $P < 0.05$). # indicates significant difference from the presence of Ca^{2+} in the medium (ANOVA; $P < 0.05$).

Cell volume was estimated from forward scatter in FACS analysis after an incubation time of 24 h. The exposure to anandamide ($= 2.5 \mu\text{M}$) was followed by a dose dependent significant decrease of the forward scatter pointing to erythrocyte shrinkage (Fig. 2A,B).

Fluo3-dependent fluorescence was employed to determine cytosolic Ca^{2+} activity. As shown in Fig. 3A, anandamide ($= 2.5 \mu\text{M}$) increased the cytosolic free Ca^{2+} concentration after an incubation time of 6 hours. For comparison, the effect of the Ca^{2+} ionophore ionomycin (1 μM) on the Fluo3-dependent fluorescence is shown. The increase of the cytosolic Ca^{2+} concentration could at least partially account for the stimulation of phosphatidylserine exposure and cell shrinkage following anandamide treatment. In order to further test for a role of Ca^{2+} in the stimulation of phospholipid scrambling, additional experiments were performed comparing the effects of 10 μM anandamide on annexin V binding and forward scatter in the presence and absence of Ca^{2+} in the incubation medium. As shown in Fig 3B, the removal of Ca^{2+} significantly blunted the percentage of annexin V binding erythrocytes after an incubation time of 24 h. Anandamide induced cell shrinkage was likewise significantly reduced by the removal of Ca^{2+} from the medium (Fig 3C).

To examine whether the stimulation of annexin V binding by anandamide was dependent on formation of reactive oxygen species, erythrocytes were preincubated for 60 minutes with several concentrations of the antioxidant N-acetyl cysteine followed by the addition of 10 μM of anandamide with further incubation for 24 hours. The preincubation with N-acetyl cysteine ($>0.1 \text{ mM}$) significantly blunted annexin V binding (Fig. 4), but did not significantly blunt the decrease of forward scatter following exposure to anandamide (data not shown).

Fig. 4. Effects of anandamide on erythrocyte phosphatidylserine exposure following preincubation with N-acetylcysteine (NAC). Arithmetic means $\pm$ SEM ($n = 6$) of erythrocyte annexin V binding after preincubation in Ringer solution without (open and closed bar) or with N-acetyl cysteine (grey bars) for 60 minutes at concentrations ranging from 0.1 mM to 1.5 mM followed by the addition of 10 μ M of anandamide and continued incubation for 24 hours. * indicates significant difference from Ringer-treated controls (ANOVA; $P < 0.05$). # indicates significant difference from the absence of preincubation with N-acetyl cysteine (ANOVA; $P < 0.05$).

Eryptosis may be triggered by stimulation of PGE_2 formation [43]. Therefore, additional experiments were performed in the presence and absence of the cyclooxygenase inhibitors acetylsalicylic acid (50 μ M) and ibuprofen (100 μ M). As shown in Fig. 5A/B, both, acetylsalicylic acid (50 μ M) and ibuprofen (100 μ M) significantly blunted the effect of anandamide on erythrocyte annexin V binding and erythrocyte forward scatter.

Further experiments were performed to elucidate, whether anandamide modified the effect of known triggers of eryptosis, such as hyperosmotic or isotonic cell shrinkage [29]. As shown in Fig. 6, hyperosmotic shock significantly increased the percentage of annexin V binding erythrocytes after an incubation time of 6 h, an effect significantly augmented in the presence of anandamide ($= 2.5 \mu\text{M}$). The exposure of erythrocytes to hyperosmotic solutions further decreased the forward scatter, an effect reflecting cell shrinkage (Fig. 7). In hypertonic extracellular fluid the forward scatter was similar in the presence and absence of anandamide. Thus, in hypertonic medium anandamide did not further shrink erythrocytes.

Fig. 5. Effects of anandamide on erythrocyte phosphatidylserine exposure and forward scatter in the presence and absence of cyclooxygenase-inhibitors. A. Arithmetic means $\pm$ SEM ($n = 6$) of the annexin V binding of erythrocytes exposed for 24 hours to Ringer solution without (open bars) or with anandamide (closed bars) at a concentration of 10 μ M in the absence (Ringer) or presence of cyclooxygenase (COX) antagonists acetylsalicylic acid (ASA, 50 μ M) and ibuprofen (Ibu, 100 μ M). * indicates significant difference from Ringer-treated controls (ANOVA; $P < 0.05$). # indicates significant difference from the absence of COX-inhibitors (ANOVA; $P < 0.05$). B. Arithmetic means $\pm$ SEM ($n = 6$) of the forward scatter of erythrocytes exposed for 24 hours to Ringer solution without (open bars) or with anandamide (closed bars) at a concentration of 10 μ M in the absence (Ringer) or presence of COX-antagonists acetylsalicylic acid (50 μ M) and ibuprofen (100 μ M). * indicates significant difference from Ringer treated controls (ANOVA; $P < 0.05$). # indicates significant difference from the absence of COX-inhibitors (ANOVA; $P < 0.05$).

Isotonic cell shrinkage could be accomplished by Cl^- removal. Thus, additional experiments have been performed on the effects of anandamide following Cl^- removal. As illustrated in Fig. 8, Cl^- removal significantly

Fig. 6. Effects on annexin V binding of hyperosmotic shock in the presence or absence of anandamide. A. Histograms of annexin V binding in a representative experiment of erythrocytes exposed for 6 hours to either isotonic (290 mOsm) Ringer solution (upper panels) or hypertonic (850 mOsm) Ringer solution (lower panels) in the absence (left panels) or in the presence (right panels) of 5 μ M anandamide. B. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage annexin V binding erythrocytes following incubation for 6 hours in isotonic (290 mOsm; white bars) or hypertonic (850 mOsm; black bars) Ringer solution either in the absence (0 μ M) or presence of anandamide at concentrations from 2.5 to 10 μ M. * indicates significant difference from isotonic Ringer solution (ANOVA; $P < 0.05$), # indicates significant difference from the respective values in the absence of anandamide (ANOVA; $P < 0.05$).

increased the percentage of annexin V binding erythrocytes after an incubation time of 24h, an effect significantly augmented in the presence of anandamide ($= 2.5 \mu$ M). Cl^- removal further decreased the forward scatter, again pointing to erythrocyte shrinkage (Fig. 9).

Fig. 7. Effects on forward scatter of hyperosmotic shock in the presence or absence of anandamide. A. Histograms of forward scatter in a representative experiment of erythrocytes exposed for 6 hours to either isotonic (290 mOsm) Ringer solution (upper panels) or hypertonic (850 mOsm) Ringer solution (lower panels) in the absence (left panels) or in the presence (right panels) of 5 μ M anandamide. B. Arithmetic means $\pm$ SEM ($n = 4$) of forward scatter of erythrocytes incubated for 6 hours in isotonic (290 mOsm; white bars) or hypertonic (850 mOsm; black bars) Ringer solution either in the absence (0 μ M) or presence of anandamide at concentrations from 2.5 to 10 μ M. * indicates significant difference from isotonic Ringer solution (ANOVA; $P < 0.05$), # indicates significant difference from the respective values in the absence of anandamide (ANOVA; $P < 0.05$).

Similar to the observations during hyperosmotic shock, the decrease of forward scatter following Cl^- removal was not augmented in the presence of anandamide. Accordingly, anandamide did not further shrink erythrocytes in the absence of Cl^- .

Fig. 8. Effects on annexin V binding of Cl⁻ removal in the presence or absence of anandamide. A. Histograms of annexin V binding in a representative experiment of erythrocytes exposed 24 hours to either Ringer solution (upper panels) or to Cl⁻ free solution (lower panels) either in the absence (left panels) or presence (right panels) of 5 μ M anandamide. B. Arithmetic means $\pm$ SEM ($n = 4$) of annexin V binding in % of the total population of erythrocytes incubated for 24 hours in chloride containing (white bars) or chloride free (black bars) solution either in the absence or presence of anandamide at concentrations from 2.5 to 10 μ M. * indicates significant difference from Cl⁻ containing Ringer solution (ANOVA; $P < 0.05$), # indicates significant difference from the respective control values in the absence of anandamide (ANOVA; $P < 0.05$).

Discussion

The present study discloses a novel effect of the endocannabinoid anandamide, i.e. the triggering of eryptosis. The concentrations required for this effect are almost three orders of magnitude higher than those in circulating plasma of healthy individuals but are well in the range of local concentrations. The plasma anandamide

Fig. 9. Effects on forward scatter of chloride removal in the presence or absence of anandamide. A. Histograms of forward scatter in a representative experiment of erythrocytes exposed 24 hours to either Ringer solution (upper panels) or chloride-free solution (lower panels) either in the absence (left panels) or presence (right panels) of 5 μ M anandamide. B. Arithmetic means $\pm$ SEM ($n = 4$) of erythrocyte forward scatter following incubation for 24 hours in chloride containing (white bars) or chloride free (black bars) solution either in the absence or presence of anandamide at concentrations from 2.5 to 10 μ M. * indicates significant difference from Cl⁻ containing Ringer solution (ANOVA; $P < 0.05$), # indicates significant difference from the respective control values in the absence of anandamide (ANOVA; $P < 0.05$).

concentrations approach in healthy individuals 4 nM but may be substantially higher in disease states such as acute hepatitis [2]. It has been estimated that local concentrations may approach values as high as 60 μ M in disease [2].

The present experiments further allow some insight into the mechanisms mediating the anandamide effect. As evidenced from Fluo3 fluorescence, anandamide

stimulates Ca^{2+} entry after 6 h of incubation. The increase of cytosolic Ca^{2+} is expected to activate Ca^{2+} -sensitive K^+ channels with subsequent K^+ exit, hyperpolarization, and exit of Cl^- and osmotically obliged water, eventually leading to cell shrinkage [33, 34]. Moreover, Ca^{2+} may modify the cytoskeleton [61, 62] and activate a wide variety of Ca^{2+} sensitive enzymes such as transglutaminase [63], phospholipases [64], calpain [63], protein kinases as well as phosphatases [65, 66]. The degradation of membrane proteins by calpain leads to cell membrane blebbing, another hallmark of eryptosis [67, 68]. Anandamide induced apoptosis of nucleated cells similarly involves Ca^{2+} and calpain [8, 10].

The effect of anandamide is blunted in the nominal absence of extracellular Ca^{2+} , a finding further highlighting the role of Ca^{2+} in anandamide induced phospholipid scrambling of erythrocyte cell membranes.

The Ca^{2+} permeable erythrocyte cation channels are activated by the cyclooxygenase product PGE_2 [43]. Accordingly, the formation of PGE_2 largely accounts for the stimulation of the cation channels and subsequent eryptosis following osmotic shock [43]. Thus, we have tested for effects of anandamide in the presence of cyclooxygenase inhibitors acetylsalicylic acid and ibuprofen. As a matter of fact, both drugs significantly blunted the effect of anandamide on phospholipid scrambling and forward scatter. The anandamide induced apoptosis of nucleated cells has previously been shown to depend on cyclooxygenase 2 [5, 11]. Moreover, in nucleated cells anandamide treatment has been shown to trigger the release the PGE_2 precursor arachidonic acid [69].

Triggers of eryptosis further include oxidative stress [57]. Preincubation of erythrocytes with the anti-oxidant N-acetyl cysteine indeed significantly blunted the anandamide induced phosphatidylserine exposure, suggesting a role of reactive oxygen species in the

anandamide induced phosphatidylserine exposure of erythrocytes. Previous studies [4, 12, 16, 27] suggested that the pro-apoptotic effect of anandamide in nucleated cells is in part due to the generation of reactive oxygen species.

Taken together, the present observation suggest that anandamide stimulates the formation of PGE_2 , which in turn activates the Ca^{2+} permeable cation channels leading to entry of Ca^{2+} with subsequent phospholipid scrambling and activation of Ca^{2+} -sensitive K^+ channels, KCl exit, water loss and cell shrinkage [33, 34].

The phosphatidylserine exposure at the cell surface favours phagocytotic removal of apoptotic or eryptotic cells by phagocytes equipped with phosphatidylserine receptors [70], which allow engulfment of defective cells [37]. Thus, phosphatidylserine exposing erythrocytes are rapidly sequestered from circulating blood [38].

In conclusion, anandamide does not only trigger apoptosis of nucleated cells but similarly induces eryptosis, the suicidal death of erythrocytes. Anandamide is effective at least in part through cyclooxygenase dependent stimulation of Ca^{2+} entry. The effects observed in erythrocytes may be similarly operative in nucleated cells.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Lejla Subasic and Jasmin Bühringer. This study was supported by the Deutsche Forschungsgemeinschaft, Nr. La 315/4-3 and La 315/13-1, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research), the Else-Übelmesser-Stiftung and the Biomed program of the EU (BMH4-CT96-0602).

References

- 1 Maccarrone M, Finazzi-Agro A: The endocannabinoid system, anandamide and the regulation of mammalian cell apoptosis. *Cell Death Differ* 2003;10:946-955.
- 2 Biswas KK, Sarker KP, Abeyama K, Kawahara K, Iino S, Otsubo Y, Saigo K, Izumi H, Hashiguchi T, Yamakuchi M, Yamaji K, Endo R, Suzuki K, Imaizumi H, Maruyama I: Membrane cholesterol but not putative receptors mediates anandamide-induced hepatocyte apoptosis. *Hepatology* 2003;38:1167-1177.
- 3 Giuliano M, Calvaruso G, Pellerito O, Portanova P, Carlisi D, Vento R, Tesoriere G: Anandamide-induced apoptosis in Chang liver cells involves ceramide and JNK/AP-1 pathway. *Int J Mol Med* 2006;17:811-819.

- 4 Siegmund SV, Seki E, Osawa Y, Uchinami H, Cravatt BF, Schwabe RF: Fatty acid amide hydrolase determines anandamide-induced cell death in the liver. *J Biol Chem* 2006;281:10431-10438.
- 5 Patsos HA, Hicks DJ, Dobson RR, Greenhough A, Woodman N, Lane JD, Williams AC, Paraskeva C: The endogenous cannabinoid, anandamide, induces cell death in colorectal carcinoma cells: a possible role for cyclooxygenase 2. *Gut* 2005;54:1741-1750.
- 6 Contassot E, Wilmotte R, Tenan M, Belkouch MC, Schnuriger V, de Tribolet N, Burkhardt K, Dietrich PY: Arachidonyl ethanolamide induces apoptosis of human glioma cells through vanilloid receptor-1. *J Neuropathol Exp Neurol* 2004;63:956-963.
- 7 Galve-Roperh I, Aguado T, Rueda D, Velasco G, Guzman M: Endocannabinoids: a new family of lipid mediators involved in the regulation of neural cell development. *Curr Pharm Des* 2006;12:2319-2325.
- 8 Movsesyan VA, Stoica BA, Yakovlev AG, Knoblach SM, Lea PM, Cernak I, Vink R, Faden AI: Anandamide-induced cell death in primary neuronal cultures: role of calpain and caspase pathways. *Cell Death Differ* 2004;11:1121-1132.
- 9 Bari M, Battista N, Fezza F, Finazzi-Agro A, Maccarrone M: Lipid rafts control signaling of type-1 cannabinoid receptors in neuronal cells. Implications for anandamide-induced apoptosis. *J Biol Chem* 2005;280:12212-12220.
- 10 Maccarrone M, Lorenzon T, Bari M, Melino G, Finazzi-Agro A: Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. *J Biol Chem* 2000;275:31938-31945.
- 11 Hinz B, Ramer R, Eichele K, Weinzierl U, Brune K: Up-regulation of cyclooxygenase-2 expression is involved in R(+)-methanandamide-induced apoptotic death of human neuroglioma cells. *Mol Pharmacol* 2004;66:1643-1651.
- 12 Sarker KP, Obara S, Nakata M, Kitajima I, Maruyama I: Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3. *FEBS Lett* 2000;472:39-44.
- 13 Sarker KP, Biswas KK, Yamakuchi M, Lee KY, Hahiguchi T, Kracht M, Kitajima I, Maruyama I: ASK1-p38 MAPK/JNK signaling cascade mediates anandamide-induced PC12 cell death. *J Neurochem* 2003;85:50-61.
- 14 Maccarrone M, Falciglia K, Di Rienzo M, Finazzi-Agro A: Endocannabinoids, hormone-cytokine networks and human fertility. *Prostaglandins Leukot Essent Fatty Acids* 2002;66:309-317.
- 15 Contassot E, Tenan M, Schnuriger V, Pelte MF, Dietrich PY: Arachidonyl ethanolamide induces apoptosis of uterine cervix cancer cells via aberrantly expressed vanilloid receptor-1. *Gynecol Oncol* 2004;93:182-188.
- 16 Sarker KP, Maruyama I: Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts. *Cell Mol Life Sci* 2003;60:1200-1208.
- 17 Mimeault M, Pommery N, Watzet N, Bailly C, Henichart JP: Anti-proliferative and apoptotic effects of anandamide in human prostatic cancer cell lines: implication of epidermal growth factor receptor down-regulation and ceramide production. *Prostate* 2003;56:1-12.
- 18 Yamaji K, Sarker KP, Kawahara K, Iino S, Yamakuchi M, Abeyama K, Hashiguchi T, Maruyama I: Anandamide induces apoptosis in human endothelial cells: its regulation system and clinical implications. *Thromb Haemost* 2003;89:875-884.
- 19 Do Y, McKallip RJ, Nagarkatti M, Nagarkatti PS: Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. *J Immunol* 2004;173:2373-2382.
- 20 Schwarz H, Blanco FJ, Lotz M: Anandamide, an endogenous cannabinoid receptor agonist inhibits lymphocyte proliferation and induces apoptosis. *J Neuroimmunol* 1994;55:107-115.
- 21 Flygare J, Gustafsson K, Kimby E, Christensson B, Sander B: Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma. *FEBS Lett* 2005;579:6885-6889.
- 22 Gasperi V, Fezza F, Spagnuolo P, Pasquariello N, Maccarrone M: Further insights into the regulation of human FAAH by progesterone and leptin implications for endogenous levels of anandamide and apoptosis of immune and neuronal cells. *Neurotoxicology* 2005;26:811-817.
- 23 Maccarrone M, Piccirilli S, Battista N, Del Duca C, Nappi G, Corasaniti MT, Finazzi-Agro A, Bagetta G: Enhanced anandamide degradation is associated with neuronal apoptosis induced by the HIV-1 coat glycoprotein gp120 in the rat neocortex. *J Neurochem* 2004;89:1293-1300.
- 24 Hart S, Fischer OM, Ullrich A: Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. *Cancer Res* 2004;64:1943-1950.
- 25 Valk P, Verbakel S, von Lindern M, Lowenberg B, Delwel R: Enhancement of erythropoietin-stimulated cell proliferation by Anandamide correlates with increased activation of the mitogen-activated protein kinases ERK1 and ERK2. *Hematol J* 2000;1:254-263.
- 26 Bari M, Paradisi A, Pasquariello N, Maccarrone M: Cholesterol-dependent modulation of type 1 cannabinoid receptors in nerve cells. *J Neurosci Res* 2005;81:275-283.
- 27 Siegmund SV, Uchinami H, Osawa Y, Brenner DA, Schwabe RF: Anandamide induces necrosis in primary hepatic stellate cells. *Hepatology* 2005;41:1085-1095.
- 28 Sancho R, de I, V, Appendino G, Di M, V, Macho A, Munoz E: The CB1/VR1 agonist arvanil induces apoptosis through an FADD/caspase-8-dependent pathway. *Br J Pharmacol* 2003;140:1035-1044.
- 29 Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, Lang F: Mechanisms of Suicidal Erythrocyte Death. *Cell Physiol Biochem* 2005;15:195-202.
- 30 Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, Wieder T, Huber SM: Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ* 2003;10:249-256.
- 31 Lang KS, Myssina S, Tanneur V, Wieder T, Huber SM, Lang F, Duranton C: Inhibition of erythrocyte cation channels and apoptosis by ethylisopropylamiloride. *Naunyn Schmiedebergs Arch Pharmacol* 2003;367:391-396.
- 32 Lang PA, Kempe DS, Akel A, Klarl BA, Eisele K, Podolski M, Hermle T, Niemoeller OM, Attanasio P, Huber SM, Wieder T, Lang F, Duranton C: Inhibition of erythrocyte "apoptosis" by catecholamines. *Naunyn Schmiedebergs Arch Pharmacol* 2005;372:228-235.
- 33 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca²⁺-activated K⁺ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003;285:C1553-C1560.
- 34 Myssina S, Lang PA, Kempe DS, Kaiser S, Huber SM, Wieder T, Lang F: Cl⁻ channel blockers NPPB and niflumic acid blunt Ca(2+)-induced erythrocyte 'apoptosis'. *Cell Physiol Biochem* 2004;14:241-248.
- 35 Woon LA, Holland JW, Kable EP, Roufogalis BD: Ca²⁺ sensitivity of phospholipid scrambling in human red cell ghosts. *Cell Calcium* 1999;25:313-320.
- 36 Lang KS, Myssina S, Brand V, Sandu C, Lang PA, Berchtold S, Huber SM, Lang F, Wieder T: Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. *Cell Death Differ* 2004;11:231-243.
- 37 Boas FE, Forman L, Beutler E: Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc Natl Acad Sci U S A* 1998;95:3077-3081.

- 38 Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM, Wieder T, Lang F: Enhanced programmed cell death of iron-deficient erythrocytes. *FASEB J* 2006;20:368-370.
- 39 Arese P, Turrini F, Schwarzer E: Band 3/ complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. *Cell Physiol Biochem* 2005;16:133-146.
- 40 Barvitenko NN, Adragna NC, Weber RE: Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell Physiol Biochem* 2005;15:1-18.
- 41 Bosman GJ, Willekens FL, Werre JM: Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem* 2005;16:1-8.
- 42 Rice L, Alfrey CP: The negative regulation of red cell mass by neocytolysis: physiologic and pathophysiologic manifestations. *Cell Physiol Biochem* 2005;15:245-250.
- 43 Lang PA, Kempe DS, Myssina S, Tanneur V, Birka C, Laufer S, Lang F, Wieder T, Huber SM: PGE(2) in the regulation of programmed erythrocyte death. *Cell Death Differ* 2005;12:415-428.
- 44 Lang PA, Kempe DS, Tanneur V, Eisele K, Klarl BA, Myssina S, Jendrosseck V, Ishii S, Shimizu T, Waidmann M, Hessler G, Huber SM, Lang F, Wieder T: Stimulation of erythrocyte ceramide formation by platelet-activating factor. *J Cell Sci* 2005;118:1233-1243.
- 45 Lang PA, Huober J, Bachmann C, Kempe DS, Sobiesiak M, Akel A, Niemoeller OM, Dreischer P, Eisele K, Klarl BA, Gulbins E, Lang F, Wieder T: Stimulation of erythrocyte phosphatidylserine exposure by paclitaxel. *Cell Physiol Biochem* 2006;18:151-164.
- 46 Nicolay JP, Schneider J, Niemoeller OM, Artunc F, Portero-Otin M, Haik G, Jr., Thornalley PJ, Schleicher E, Wieder T, Lang F: Stimulation of suicidal erythrocyte death by methylglyoxal. *Cell Physiol Biochem* 2006;18:223-232.
- 47 Akel A, Hermle T, Niemoeller OM, Kempe DS, Lang PA, Attanasio P, Podolski M, Wieder T, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by chlorpromazine. *Eur J Pharmacol* 2006;532:11-17.
- 48 Niemoeller OM, Akel A, Lang PA, Attanasio P, Kempe DS, Hermle T, Sobiesiak M, Wieder T, Lang F: Induction of eryptosis by cyclosporine. *Naunyn Schmiedeberg Arch Pharmacol* 2006;374:41-49.
- 49 Nicolay JP, Gatz S, Liebig G, Gulbins E, Lang F: Amyloid induced suicidal erythrocyte death. *Cell Physiol Biochem* 2007;19:175-184.
- 50 Eisele K, Lang PA, Kempe DS, Klarl BA, Niemoeller O, Wieder T, Huber SM, Duranton C, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by mercury ions. *Toxicol Appl Pharmacol* 2006;210:116-122.
- 51 Kempe DS, Lang PA, Eisele K, Klarl BA, Wieder T, Huber SM, Duranton C, Lang F: Stimulation of erythrocyte phosphatidylserine exposure by lead ions. *Am J Physiol Cell Physiol* 2005;288:C396-C402.
- 52 Birka C, Lang PA, Kempe DS, Hoefling L, Tanneur V, Duranton C, Nammi S, Henke G, Myssina S, Krikov M, Huber SM, Wieder T, Lang F: Enhanced susceptibility to erythrocyte "apoptosis" following phosphate depletion. *Pflügers Arch* 2004;448:471-477.
- 53 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med* 2006;84:378-388.
- 54 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. *J Mol Med* 2007;85:269-277.
- 55 Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, Lang F: Dependence of Plasmodium falciparum in vitro growth on the cation permeability of the human host erythrocyte. *Cell Physiol Biochem* 2003;13:347-356.
- 56 Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, Koka S, Eisele K, Klarl BA, Rubben H, Schmid KW, Mann K, Hildenbrand S, Heftner H, Huber SM, Wieder T, Erhardt A, Haussinger D, Gulbins E, Lang F: Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. *Nat Med* 2007;13:164-170.
- 57 Lang KS, Roll B, Myssina S, Schittenhelm M, Scheel-Walter HG, Kanz L, Fritz J, Lang F, Huber SM, Wieder T: Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cell Physiol Biochem* 2002;12:365-372.
- 58 Myssina S, Huber SM, Birka C, Lang PA, Lang KS, Friedrich B, Risler T, Wieder T, Lang F: Inhibition of erythrocyte cation channels by erythropoietin. *J Am Soc Nephrol* 2003;14:2750-2757.
- 59 Lang KS, Myssina S, Lang PA, Tanneur V, Kempe DS, Mack AF, Huber SM, Wieder T, Lang F, Duranton C: Inhibition of erythrocyte phosphatidylserine exposure by urea and Cl⁻. *Am J Physiol Renal Physiol* 2004;286:F1046-F1053.
- 60 Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM: Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J Biol Chem* 1990;265:4923-4928.
- 61 Nunomura W, Takakuwa Y, Tokimitsu R, Krauss SW, Kawashima M, Mohandas N: Regulation of CD44-protein 4.1 interaction by Ca²⁺ and calmodulin. Implications for modulation of CD44-ankyrin interaction. *J Biol Chem* 1997;272:30322-30328.
- 62 Takakuwa Y, Mohandas N: Modulation of erythrocyte membrane material properties by Ca²⁺ and calmodulin. Implications for their role in regulation of skeletal protein interactions. *J Clin Invest* 1988;82:394-400.
- 63 Anderson DR, Davis JL, Carraway KL: Calcium-promoted changes of the human erythrocyte membrane. Involvement of spectrin, transglutaminase, and a membrane-bound protease. *J Biol Chem* 1977;252:6617-6623.
- 64 Allan D, Billah MM, Finean JB, Michell RH: Release of diacylglycerol-enriched vesicles from erythrocytes with increased intracellular (Ca²⁺). *Nature* 1976;261:58-60.
- 65 Cohen CM, Gascard P: Regulation and post-translational modification of erythrocyte membrane and membrane-skeletal proteins. *Semin Hematol* 1992;29:244-292.
- 66 Minetti G, Piccinini G, Balduini C, Seppi C, Brovelli A: Tyrosine phosphorylation of band 3 protein in Ca²⁺/A23187-treated human erythrocytes. *Biochem J* 1996;320 (Pt 2):445-450.
- 67 Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF, Schulze-Osthoff K, Wesselborg S: Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ* 2001;8:1197-1206.
- 68 Bratosin D, Estaquier J, Petit F, Arnoult D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC: Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell Death Differ* 2001;8:1143-1156.
- 69 Someya A, Horie S, Murayama T: Arachidonic acid release and prostaglandin F(2alpha) formation induced by anandamide and capsaicin in PC12 cells. *Eur J Pharmacol* 2002;450:131-139.
- 70 Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM: A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 2000;405:85-90.