Aus dem Institut für Physiologie der Universität Tübingen
Abteilung Physiologie I
Direktor: Professor Dr. F. Lang

The Regulatory Role of Temperature and Endothelin-B
Receptor in Erythrocyte Programmed Cell Death

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Syed Minnatullah Qadri

aus

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Dekan: Professor Dr. I. B. Autenrieth

1. Berichterstatter: Professor Dr. Florian Lang

2. Berichterstatter: Professor Dr. K. Schulze-Osthoff

I dedicate this thesis to my beloved unlce Prof. Yahya and his wonderful family, my very loving father and mother, and my sweet sisters for their love and support throughout my life.

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1. Abbreviations

AIDS	Acquired immunodeficiency syndrome
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CFSE	Carboxyfluorescein-diacetate-succinimidyl-ester
cGK1	cGMP-dependent protein kinase type 1
CNQX	6-cyano-7-nitroquinoxaline-2,3-Dione
COX	Cyclooxygenase
CysLT	Cysteinyl-leukotriene receptor
DISC	Deat-inducing signaling complex
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EIPA	Ethylisopropylamiloride
ELISA	Enzyme linked immunosorbent assay
EP	E-prostanoid
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ETA	Endothelin A receptors
ETB	Endothelin B receptors
EPO	Erythropoietin
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FL-1	Fluorescence channel 1
FSC	Forward scatter
FITC	Fluorescein-isothiocyanate
GSH	Glutathione
G protein	Guanine-nucleotide-binding proteins
Hb	Hemoglobin

HCT	Hematocrit
HEPES	32-N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HGB	Hemoglobin concentration
HSP	Heat shock proteins
IL-1ß	Interleukin-1ß
IL-6	Interleukin-6
IL-10	Interleukin-10
LPS	Lipopolysaccharide
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
NBQX	1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(f)quinoxaline-7-sulfonamide
NHE	Na^+/H^+ exchanger
NO	Nitric oxide
NPP	Novel permeability pathways
PAF	Platelet activating factor
PBS	Phosphate-buffered saline
PKC	Protein kinase c
PGE_2	Prostaglandin E_2
POAH	Preoptic nuclei of the anterior hypothalamus
PS	Phosphatidylserine
RBC	Red blood cells, erythrocyte count
RNA	Ribonucleic acid
SEM	Standard error of mean
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Side scatter
TRAIL	TNF-related apoptosis –inducing ligand
TNF	Tumor necrosis factor
VSMC	Vascular smooth muscle cells

2. Introduction

Erythrocytes make up the most predominant and abundant cells in humans. Erythrocytes account for a quarter of the total cell number in adult humans and their number is estimated to be around 30 trillion. Their gross volume exceeds 2 litres, which is about 10% of the total cell volume. As a clinical reference, the erythrocyte or red blood cell count is projected to be 5 million cells per cubic millimetre of blood. They were first microscopically visualised and described by Anton van Leeuwenhoek in 1658. Erythrocytes have a life span of 100-120 days in the circulation, after which they undergo senescence only to be recognised by macrophages and undergo phagocytosis. Erythrocytes are designated to the paramount function of oxygen transportation from the lungs to tissues and carbon dioxide back. To a lesser extent they also transport hydrogen ions. Considering, that erythrocytes are unable to divide to replenish their loss, they are produced from the red bone marrow by a process called erythropoiesis. The hormone erythropoietin stimulates erythrocyte formation from undifferentiated pluripotent stem cells which continuously divide and give rise to various blood cells (Bessis et al., 1981).

2.1. Morphology and ionic transport in erythrocytes

Erythrocytes have a unique flat and biconcave disc shape with a diameter of 8 μm, 1 μm thick in the middle and 2 μm thick at the outside edges. This unique structure provides the red blood cells with a larger surface area for oxygen diffusion and its thinness ensures the rapid movement of oxygen from the exterior to the innermost regions. Unlike other cells, erythrocytes are devoid of important cell organelles like the nucleus and mitochondria. This not only provides more room for hemoglobin molecules in the cytosol of erythrocytes but also contributes to the uniqueness in its biological function. Without DNA, RNA and ribosomes, erythrocytes cannot synthesize proteins for cell repair, growth, division and renewing enzyme supplies. The hemoglobin molecules consist of two portions. The globin portion is a protein made of four highly folded polypeptide chains. The iron containing heme group is bound to the polypeptide chains. Hemoglobin plays a key role in oxygen and carbon dioxide transport and contributes to the pH buffering capacity in the blood. It also helps in the vasodilation of arterioles by binding with nitric oxide (NO) (Bessis and Delpech, 1981; Mohandas et al., 2008).

The erythrocyte membrane structure facilitates the maintenance of structural integrity while it undergoes various deformative changes during its life time. A complex network of skeletal proteins and a composite lipid bilayer give it stability and flexibility. The lipid bilayer consists of cholesterol and phospholipids. Unlike the membrane phospholipids, cholesterol is evenly distributed in the bilayer. The outer monolayer is composed mainly of phophatidylcholine and sphingomyelin. The inner bilayer consists of phosphatidylethanolamine, phosphatidylserine (PS) and to a lesser extent, phosphoinositide constituents. Different phospholipids transport proteins such as scramblases, flippases and floppases have been implicated in the movement of phospholipids dependent or independent of energy (Zwaal et al., 1997).

Over fifty erythrocyte membrane proteins have been characterised and documented so far in detail. A large number of these proteins form various blood group antigens. Diversified functions of membrane proteins include: transport, adhesion and signaling receptors. Many functions of membrane proteins still remain elusive. Some of the important membrane proteins are: band 3, Glut 1, Kidd antigen protein, aquaporin 1, Na^+ -K^+ -ATPase, Ca^{2+} ATPase, Na^+ -K^+ -$2Cl^-$ cotransporter, Na^+ -Cl^- cotransporter, Na^+ -K^+ cotransporter, K^+ -Cl^- cotransporter, and Gardos channel. ICAM-4 and laminin binding protein function as adhesion proteins. The membrane proteins that contribute to the structural integrity of the erythrocyte membrane consist of 2 macro-molecular complexes. The first complex is ankyrin based while the other is protein 4.1R based (Figure. 1). Membrane proteins form linkages with skeletal proteins and prevent from membrane vesiculation. The skeletal proteins comprise of a 2-dimensional spectrin-based membrane network containing alpha and beta spectrin, actin, protein 4.1R, adducin, dermatin, tropomyosin and tropomodulin. The spectrin dimer-dimer interaction and the spectrin actin - protein 4.1R junctional complex are key regulators of membrane mechanical stability and play a critical role in withstanding shear stresses in the circulation (Bennett, 1983; Nicolas et al., 2003; Reid et al., 1990).

The ionic balance in the erythrocyte is regulated by various transporters (Figure. 2). Of particular importance, in the present study is the Gardos channel. The Gardos channel, or the Ca^{2+} -activated K^+ -channel, is a major route for cellular loss of potassium from erythrocytes. Dehydration in erythrocytes follows the loss of K^+, Cl^- and water from the cell (Maher et al., 2003). In sickle cell disease, two transport systems have been described for erythrocyte dehydration, the Gardos channel and

the K^+ / Cl^- cotransporter. The Gardos channel can be inhibited by venom-derived charbybdotoxin and Ca^{2+} channel blocker nitrendipine (Brugnara, 1995; Ellory et al., 1992). The most widely used agent, however, is the anti-fungal agent, clotrimazole (Alvarez et al., 1992). A new agent Tram 34 is also known to inhibit the Gardos channel (Maher and Kuchel, 2003).
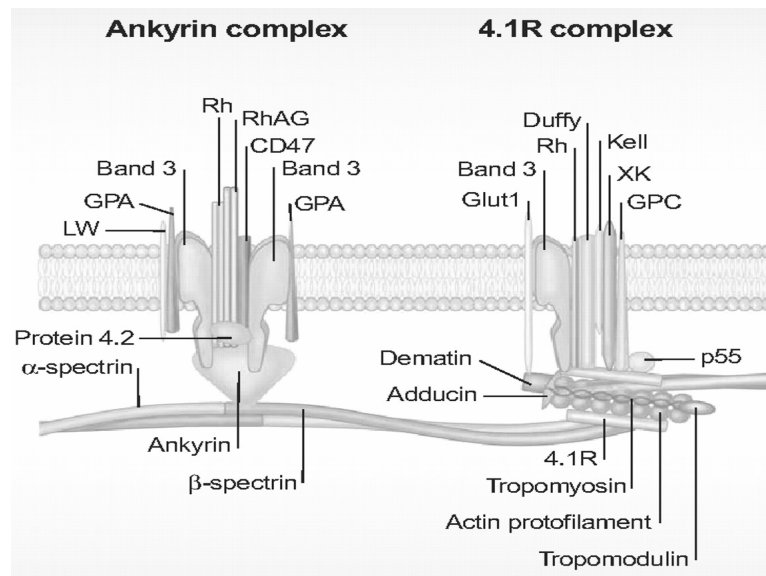

Figure 1. Structural proteins in the erythrocyte membrane and their linkages to skeletal proteins. (Mohandas and Gallagher 2008)
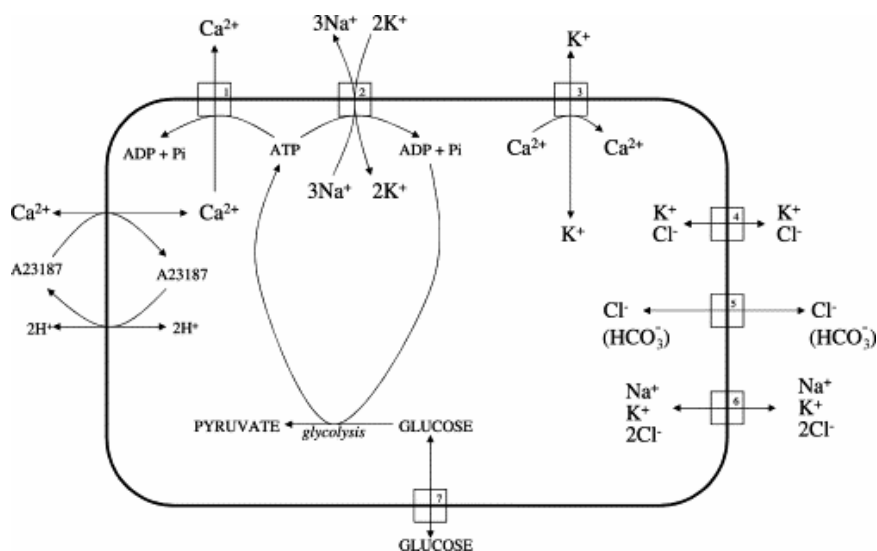

Figure 2. A schema of important ionic transport pathways in erythrocytes. (Maher and Kuchel 2003)

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2.2. Apoptosis

Apoptosis or programmed cell death is a highly regulated and organised process for the maintenance of tissue homeostasis in the organism. It is an essential physiological regulatory mechanism for the control of cell number in development and throughout the organism's life. On the other side of the paradigm, apoptosis may be involved in various pathological processes like neurodegeneration, cardiovascular diseases, immunological diseases, acquired immunodeficiency syndrome (AIDS) and cancer (Fadeel et al., 2005). Apoptosis plays an integral part in many biological events which ensures cell turnover, and the removal of harmful cells. Several human diseases may be attributed directly or indirectly to a derangement of apoptosis, resulting in either cell accumulation, in which cell eradication or cell turnover is impaired; or cell loss, in which the apoptotic programme is inadvertently triggered. Apoptosis prevents the deleterious effects of necrosis which is characterized by cell rupture and release of noxious cellular contents and regional inflammation. Other forms of cell death that have been described in the literature include autophagy, paraptosis, necroptosis and oncosis. Apoptosis does not induce inflammation or tissue scarring and facilitates normal cell turnover during embryogenesis and in adult tissues (Leist et al., 2001;Okada et al., 2004;Shintani et al., 2004;Shintani and Klionsky, 2004;Degterev et al., 2005)

2.3. Apoptosis in nucleated cells

The suicidal death machinery of nucleated cells has been well documented and characterized. A cascade of events that lead to apoptosis in nucleated cells has been studied with a similar mechanism in most cells. The morphological events that characterize apoptosis include nuclear and cytoplasmic condensation, blebbing of the plasma membrane, DNA fragmentation, loss of adhesion, rounding (in adherent cells) and cell shrinkage. Important features of apoptosis are the activation of aspartate-specific proteases, caspases, and the externalization of phosphatidylserine (PS) on the cell surface. The apoptotic bodies are rapidly recognized, ingested and degraded by phagocytes. Apoptosis can be induced by different ways such as ultraviolet irradiation, X-rays, growth factor withdrawal and chemotherapeutic drugs (Wyllie et al., 1980; Rich et al., 1999).

Two major pathways have been described in apoptosis, the extrinsic (death receptor-mediated) signaling and the intrinsic (mitochondria-dependent) pathways. Ligation of

death receptors on the cell such as FAS is followed by the formation of death-inducing signaling complex (DISC). The TNF-related apoptosis –inducing ligand (TRAIL) works in a similar way as the FAS pathway. Caspase 8 activation, in turn, triggers the activation of downstream caspases such as caspase 3. This further induces the degradation of cell's chromosomal DNA and leads to fragmentation and cell death (Zimmermann et al., 2001; Luo et al., 1998; Li et al., 1998;Scaffidi et al., 1998;LeBlanc et al., 2003).

The intrinsic pathway is mitochondria-dependent as the mitochondria play a central role in some apoptotic pathways. The mitochondrial pathway involving BCL-2 family members, mitochondria, cytochrome c, APAF-1 and caspase 9 is distinct from that of the extrinsic pathway (Schultz et al., 2003). Ceramide is another factor that induces cytochrome C release from mitochondria followed by the activation of sphingomyelinase (Ghafourifar et al., 1999). Cells lacking caspase 8 do not respond to death ligands but may undergo apoptosis by other agents like stress. Similarly, cells lacking caspase 9 are incapable of undergoing apoptosis by such stressors, but readily die in response to death ligands (Fadeel and Orrenius, 2005). Besides, the extrinsic and intrinsic pathways there may be other apoptosis signaling pathways like those triggered by dependence receptors. A common example is the hedgehog signaling pathway (Yang et al., 2010).

2.4. Apoptosis of erythrocytes "Eryptosis"

Until recently erythrocytes were considered to be conspicuous units with the unique function of transporting oxygen. With the lack of nucleus, mitochondria and cellular organelles, erythrocytes were considered to lack the essential machinery of nucleated cells. When erythrocytes reached the end of their ageing cycle they were believed to undergo processes other than apoptosis. As in apoptosis of nucleated cells, erythrocytes show certain hallmarks that are characteristics of apoptosis. Like nucleated cells they show cell shrinkage with loss of intracellular potassium through the activation of the Gardos channel. Cell membrane blebbing is another characteristic which is caused by the Ca^{2+}-sensitive scramblase. When erythrocytes are exposed to a calcium ionophore (ionomycin) the influx of calcium into the erythrocyte triggers the exposure of phosphatidylserine on the outer leaflet. This theoretically may also result from Ca^{2+} sensitive and ATP-dependent aminophospholipid translocase. Unlike apoptosis of nucleated cells, the metamorphosis of erythrocytes on ionomycin treatment does not require the action of

caspases. The term "Eryptosis" has been used to describe and distinguish suicidal death of erythrocytes from conventional suicidal death of nucleated cells (apoptosis). The uniqueness of the erythrocyte suicidal death machinery justifies us to use the term eryptosis. The exposure of PS on the outer leaflet enables the recognition of apoptotic erythrocytes and they are eliminated from circulation after being engulfed by macrophages (Foller et al., 2008c; Foller et al., 2009b; Lang et al., 2008; Lang et al., 2005b).

2.5. Signaling pathways in eryptosis

Erythrocyte membranes may exhibit minimal channel activity due to their inert composition. However, non-selective cation channels may be activated by osmotic cell shrinkage as erythrocytes are permeable to Cl^- ions. These non-selective cation channels could also be activated by oxidative stress (Huber et al., 2001; Duranton et al., 2002b). In conditions of energy deprivation to the cell, there may be a deficient replenishment of GSH and the weakening of antioxidative defence. These conditions could also potentiate the activity of non-selective cation channels (Bilmen et al., 2001; Mavelli et al., 1984). These non-selective channels are also permeable to divalent cations like Ca^{2+} (Kaestner et al., 2000; Duranton et al., 2002a). On the exposure of erythrocytes to osmotic shock or oxidative stress the uptake of Ca^{2+} is triggered. This increase in intracellular Ca^{2+} activity, in turn, starts a new cascade of events. Ca^{2+} activates scramblase leading to the breakdown of phosphatidylserine asymmetry.

The non-selective cation channels are inhibited by intracellular and extracellular Cl^-. Cl^- removal may result in cell shrinkage which is followed by the exit of K^+ and Cl^- ions. This shrinkage may lead to the opening of non-selective cation channels with the influx of calcium. In patch clamp experiments, in order to study non selective channel activity, Cl^- ions are removed from the medium (Huber, Gamper, and Lang, 2001). The Ca^{2+} channel TRPC6 may contribute to the Ca^{2+} entry (Foller et al., 2008d). The stimulation of the cation channel may be secondary to PGE_2 formation and can be pharmacologically inhibited by phopholipase-A2 inhibitors: quinacrine and palmitoyl-trifluoro-methyl-ketone and cyclooxygenase inhibitors: acetylsalicylic acid and diclophenac (Lang, Gulbins, Lerche, Huber, Kempe, and Foller, 2008).

Increased cytosolic Ca^{2+} stimulates the Ca^{2+} -sensitive K^+ channels (Gardos channels). The efflux of K^+ ions follows the exit of Cl^- and osmotically obliged water

loss from the cell leads to cell shrinkage. This further potentiates cell membrane scrambling. Inhibitors of the Gardos channel like charbybdotoxin and clotrimazole not only blunt cell shrinkage but would also inhibit PS exposure on the outer leaflet, thus attenuating eryptosis. Furthermore, PGE_2 could also activate the Gardos channel in addition to facilitating the influx of Ca^{2+} in the cell (Bookchin et al., 1987; Brugnara et al., 1993; Lang et al., 2003).

It has previously been shown that prostaglandins are one of the key players in eryptosis. Hyperosmotic shock and Cl^- -removal trigger the release of PGE_2. PGE_2 further activates the Ca^{2+} dependent cystein endopeptidase calpain. This effect, however, is not required for PS exposure. Platelet activating factor (PAF) which is involved in thrombosis, and cardiovascular function may cause cell shrinkage in erythrocytes. PAF also contributes to the disintegration of sphingomyelin and release of ceramide from erythrocytes. PAF further triggers the PS exposure of erythrocytes. Eryptosis could be inhibited by antagonizing PAF signaling by ABT491. PAF, like PGE_2, is also known to activate the Gardos channels. (Lang et al., 2005d; Lang et al., 2005e)

Ceramide formation serves as an alternative pathway for eryptosis or it may participate in conjunction to the non-selective cation channels and the Gardos channels. Sphingomyelinase can be stimulated by platelet activating factor. Hyperosmotic shock induces eryptosis with the involvement of Ca^{2+} ions. However, induction of eryptosis is not entirely blunted in the absence of calcium from the hypertonic medium. This signifies the role of ceramide in eryptosis. It has been shown that C6-ceramide and bacterial sphingomyelinase may induce eryptosis. Eryptosis in hyperosmotic conditions may be blunted by antagonizing sphingomyelinase using 3, 4-dichloroisocoumarin.

Eryptosis induced by C6-ceramide is also shown to potentiate the effects of Ca^{2+} entry on phosphatidylserine exposure (Lang, Lang, Bauer, Duranton, Wieder, Huber, and Lang, 2005b)

Deprivation of energy to erythrocytes involves the activation of PKC and PKC-dependent phosphorylation of membrane proteins with subsequent phosphatidylserine exposure and cell shrinkage. Eryptosis by energy depletion can be simulated by stimulation of PKC with phobolesters or inhibition of protein phosphatases like okadaic acid. Erythrocytes express various PKCs which phosphorylate the cytoskeletal proteins and the Na^+/H^+ antiporter NHE1 (Klarl et al.,

2006; Andrews et al., 2002; Bourikas et al., 2003). Oxidative stress acts partially by activating the Ca^{2+} permeable cation channels and further by Cl^- channels which cause shrinkage of erythrocytes (Huber et al., 2002; Tanneur et al., 2006).

Eryptosis has been shown to be inhibited by nitric oxide. The mechanism may be dependent on the activation of cGMP-dependent protein kinases. Mice deficient of the cGMP-dependent protein kinase type 1 (cGK1) have an enhanced eryptosis phenotype which points out to the essential role of nitric oxide in preventing suicidal erythrocyte death (Foller et al., 2008a). Erythrocytes participate in the regulation of NO formation. NO release from deoxygenated erythrocytes contributes to vasodilation and counteracts suicidal erythrocyte death in hypoxic tissue (Nicolay et al., 2007b).

Leukotrienes have been shown to stimulate suicidal erythrocyte death. Antagonism of the cysteinyl-leukotriene receptor CysLT1 by cinalukast or inhibition of 5-lipoxygenase by BW B70C inhibits eryptosis induced by energy depletion (Foller et al., 2009d). AMPA receptor antagonists NBQX and CNQX have been shown to blunt eryptosis pointing out the role of glutamate receptors in suicidal erythrocyte death (Foller et al., 2009c). AMP-activated protein kinase, the energy sensing enzyme, has also shown been shown to regulate suicidal erythrocyte death. Erythrocytes drwan from AMP-activated protein kinase deficient mice show enhanced susceptibility to eryptosis in conditions of energy depeltion (Foller et al., 2009e). Figure 3, illustrates a a synopsis of the major pathways involved in eryptosis signaling.
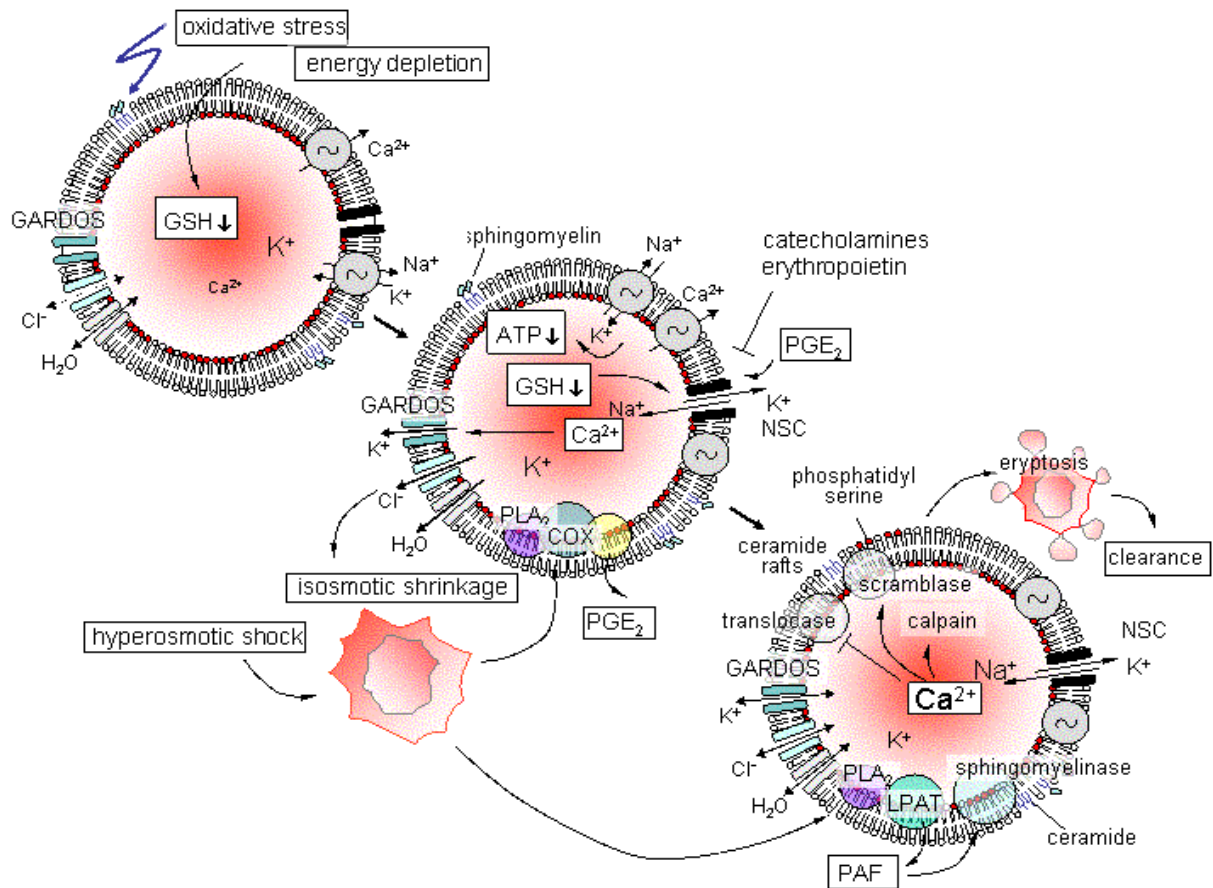
Figure 3. Summary of eryptosis signaling pathways.

2.6. **Stimulators and inhibitors of eryptosis**

A wide variety of endogenous mediators, xenobiotics and clinical conditions have been shown to influence suicidal erythrocyte death. Several drugs and chemicals have been studied to show their influence on eryptosis where a majority of them may induce suicidal erythrocyte death by a diverse array of mechanisms. Some known xenobiotic stimulators of eryptosis are as follows: aluminium (Niemoeller et al., 2006b), amantadine (Foller et al., 2008b) , amiodarone (Nicolay et al., 2007a), amphotericin b (Mahmud et al., 2009c), arsenic (Mahmud et al., 2009a), cadmium (Sopjani et al., 2008b), CD95/FAS ligand (Mandal et al., 2005), bismuth (Braun et al., 2009), chlorpromazine (Akel et al., 2006), ciglitazone (Niemoeller et al., 2008b), curcumin (Bentzen et al., 2007) , cyclosporine (Niemoeller et al., 2006a), gadolinium (Foller et al., 2009f), gold (Sopjani et al., 2008a), hemin (Gatidis et al., 2009), hemolysin (from Vibrio parahaemolyticus) (Lang et al., 2004b), lead (Kempe et al., 2005), listeriolysin (Foller et al., 2007b), lithium (Nicolay et al., 2009), methyldopa (Mahmud et al., 2008), menadione (vitamin K3) (Qadri et al., 2009a), methylglyoxal

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(Nicolay et al., 2006), paclitaxel (Lang et al., 2006b), phytic acid (Eberhard et al., 2010), retinoic acid (Niemoeller et al., 2008a), selenium (Sopjani et al., 2008c), silver (Sopjani et al., 2009), thymoquinone (Qadri et al., 2009d), tin (Nguyen et al., 2009), vanadate (Foller et al., 2008e), and zinc (Kiedaisch et al., 2008). Majority of the xenobiotics induce eryptosis by augmenting the intracellular calcium activity. However, certain drugs like methyldopa and menadione may contribute to eryptosis by stimulating ceramide formation (Mahmud, Foller, and Lang, 2008; Qadri, Eberhard, Mahmud, Foller, and Lang, 2009a). Phytic acid may cause suicidal erythrocyte death by decreasing the cellular ATP content alone (Eberhard, Foller, and Lang, 2010). Thymoquinone induced suicidal erythrocyte death by the stimulation of protein kinase C but with no appreciable stimulation of ceramide or intracellular calcium activity (Qadri, Mahmud, Foller, and Lang, 2009d).

Certain endogenous mediators and xenobiotics have also been studied to inhibit suicidal erythrocyte death. Mechanisms implicated for the inhibition of Ca^{2+} permeable cation channels include attenuation of PGE_2 formation and antioxidant activity. Eryptosis is inhibited by erythropoietin, which enhances the life span of circulating erythrocytes not only by inhibiting the apoptosis of erythrocyte progenitor cells but similarly slowing the clearance of mature erythrocytes (Myssina et al., 2003). Paradoxically, erythrocytes from erythropoietin- over expressing mice have been observed to undergo apoptosis faster ex vivo (Foller et al., 2007a). Catecholamines epinephrine, dopamine and isoproterenol have also been shown to inhibit suicidal erythrocyte death (Lang et al., 2005c). Eryptosis is also inhibited by flufenamic acid (Kasinathan et al., 2007), caffeine (Floride et al., 2008), zidovudine (Kucherenko et al., 2008), vitamin C (Mahmud et al., 2010), resveratrol (Qadri et al., 2009b), thymol (Mahmud et al., 2009b) and xanthohumol (Qadri et al., 2009c). Adenosine is also an inhibitor of eryptosis (Niemoeller et al., 2007). Pharmacological inhibition of eryptosis can be achieved by inhibiting protein kinase C by staurosporine (Klarl, Lang, Kempe, Niemoeller, Akel, Sobiesiak, Eisele, Podolski, Huber, Wieder, and Lang, 2006). Urea can blunt suicidal erythrocyte death by abrogating ceramide formation (Lang et al., 2004a).

2.7. Clinical implications and physiological benefits of eryptosis

Eryptosis has been to shown to participate in the limitation of erythrocyte survival in various clinical dysfunctions and normal physiological processes. In ageing of erythrocytes, the cytosolic calcium activity is increased, which is a hall mark of

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suicidal death (Kiefer et al., 2000; Romero et al., 1999). Erythrocytes in sickle cell disease and glucose-6-phosphate dehydrogenase deficiency are more susceptible to the effects of osmotic shock, energy depletion and oxidative stress and may undergo eryptosis faster than healthier erythrocytes (Lang et al., 2002). Such haematological disorders could significantly reduce the life span of erythrocytes in circulation. There is enough evidence pointing to the benificial role of eryptosis in limiting the intraerythrocyte survival of the malaria pathogen, Plasmodium falciparum. The parasite induces novel permeability pathways (NPP) in the intact cell membrane allowing the exchange of nutrients and disposal of waste products (Kirk, 2001). The parasite activates host cell channels by oxidation of the cell membrane and these channels constitute the NPP. Cation channels, which increase cytosolic Ca^{2+} and Na^+, are required by the parasite for survival and consequently the same cation channel activation leads to eryptosis. However, at present it is not substantiated whether phospatidylserine exposing erythrocytes are are beneficial for the host or the pathogen. Eryptosis favours the recognition of apoptotic erythrocytes and facilitates their clearance by macrophages, thereby limiting the life span of the infected cells. The erythrocyte infected with plasmodium is prone to prevent suicidal erythrocyte by sequestration of Ca^{2+} and lowering the activity of the Ca^{2+} pump. Moreover, the premature hemolysis of the infected erythrocyte is also prevented and by a decrease in the colloid osmotic pressure of the erythrocyte cytosol. This is done by excess hemoglobin digestion and export of the hemoglobin derived acids through the NPP. As a consequence, in the host-pathogen interaction machinery, plasmodial infection leads to a breakdown in the phospholipid asymmetry and exposure of phosphatidylserine (Foller et al., 2009a).

Eryptosis is an important mechanism which prevents hemolysis of erythrocytes. Depletion of energy, defective Na^+/K^+ ATPase and enhanced leakiness of the cell membrane leads to a gain of Na^+ and Cl^- and osmotically obliged water which cause cell swelling (Lang et al., 1998). Increasing the cell volume with the compensation of K^+ loss and Na^+ influx favours the entry of Cl^- leading to an increased cell volume and thereafter the cell ruptures with cellular release of hemoglobin in the circulation. Increased intracellular Ca^{2+} activity indicates the inability of the cell to maintain its electrolyte gradient. Increased Ca^{2+} enhances scramblase activity leading to phosphatidylserine exposure at the cell surface and activation of the Gardos channel which delays cell swelling and disruption of erythrocyte membranes. Eryptosis may

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thus play an important role in prevention of the detrimental consequences of hemolysis in the circulation (Lang et al., 2005a).

Eryptosis participates in the pathophysiology of several clinical conditions which include sepsis (Kempe et al., 2007), hemolytic uremic syndrome (Lang et al., 2006a), renal insufficiency (Myssina, Huber, Birka, Lang, Lang, Friedrich, Risler, Wieder, and Lang, 2003), malaria (Foller, Bobbala, Koka, Huber, Gulbins, and Lang, 2009a), sickle cell anemia (Wood et al., 1996), beta thalessemia, glucose-6-phosphate dehydrogenase deficiency (Lang, Roll, Myssina, Schittenhelm, Scheel-Walter, Kanz, Fritz, Lang, Huber, and Wieder, 2002), phosphate depletion (Birka et al., 2004), and Wilson's disease (Lang et al., 2007).

2.8. Hyperthermia

2.8.1. Thermoregulation in humans

The normal body temperature exhibits a circadian rhythm with a variation from an approximately low of 36.4°C in the morning to a hi gh of 36.9°C in the late afternoon. Temperature regulatory mechanisms include a complex network of neural connections which include the hypothalamus, limbic sytem, lower brainstem, the reticular formation, spinal cord, and the sympathetic ganglia. The "preoptic area" which includes the preoptic nuclei of the anterior hypothalamus (POAH) and the septum is vital in thermoregulation. Temperature is modulated by balanced activities of temperature-sensitive neurons which integrate afferent messages regarding core body and peripheral or skin temperatures and thus evoke behavioral and physiologic responses and controlling heat production and dissipation (Aronoff et al., 2001).

2.8.2. Pathophysiology of fever

A regulated rise in body temperature after an increase in the hypothalamic set point describes fever. Many mediators underlying pyrexia have been described. Éndogenous pyrogens which include polypeptide cytokines are involved in the regulation of inflammatory response to tissue injury and infection. Interleukin-1ß (IL-1ß), tumor necrosis factor (TNF) and interleukin-6 (IL-6) act directly on the hypothalamus, thus, eliciting fever. Certain exogenous pyrogens like microbial surface components evoke pyrexia by stimulating pyrogenic cytokines. Endotoxin or the lipopolysaccharide (LPS) of the outer bacterial membrane however, can induce

pyrexia at the hypothalamic level mimicking the IL-1ß (Dinarello et al., 1999). Other mediators are triggered by these signals which include prostaglandin E_2 (PGE_2). PGE_2 is believed to be the proximal mediator of pyrexia. Preoptic neurons bearing E-prostanoid (EP) receptors alter their intrinsic firing rates in response to PGE_2 and evoke an elevation in thermoregulatory set point. Four cellular receptors (EP1, EP2, EP3 and EP4) are known for PGE_2, although the exact subtype eliciting pyrexia is unknown. EP3 deficient mice have been shown to have an impaired pyrexic response to endogenous pyrogens and endotoxins (Ushikubi et al., 1998). Fever is strongly regulated by the immune response. Inflammatory stimuli trigger propyretic messages and consquently a release of endogenous antipyretic substances such as arginine vasopressin and glucocorticoids (Kluger et al., 1998). Interleukin-10 (IL-10) and epoxyeiconasoids generated by cytochrome P-450 enymes play a role in limiting fever and inflammation (Kozak et al., 2000). PGE_2 is synthesized from arachidonic acid which is released from cell membrane lipid by phospholipase. Arachidonic acid is metabolized by the two isoform of the COX enzyme, COX-1 and COX-2. COX-2 is the key provider of PGE_2 during pyrexia and thus a target for COX antagonism therapy (Schwartz et al., 1999). Microbes, after invading tissues trigger the inflammatory response and activate local vascular endothelial cells and leukocytes. Activated leukocytes release the pyrogenic cytokines. These evoke PGE_2 formation in the central nervous system and the signals activate the POAH neurons orchestrating the febrile response (Aronoff and Neilson, 2001).

2.8.3. Hyperthermia and apoptosis

Hyperthermia has been shown to induce both apoptosis and necrosis in vitro in a temperature-dependent manner. Many different types of cells die by apoptosis in response to hyperthermia is now a well established fact (O'Neill et al., 1998b). However, the mechanisms that lead to programmed cell death following hyperthermia are poorly understood. In general, cancer cells exposed to temperatures >42°C will undergo cell death but the percentage of cells undergoing apoptosis dramatically decreases and the percentage undergoing necrosis increases (Milleron et al., 2007). Apoptosis induced by hyperthermia in nucleated cells generally may have a variation depending on factors such as prior exposure to heat, type of tumor, cell line, and the stage of cell cycle (Samali et al., 1999). Previous studies have shown that both the intrinsic and extrinsic pathways play a role in heat-shock induced apoptosis. There is also evidence of synergism between death receptors, pathways and apoptosis. For

instance, heat shock sensitizes the cell to FasL by downregulating FLIP, a dominant negative inhibitor of caspase-8 activation within the DISC (Tran et al., 2003). Figure 5 shows a schematic representation of hyperthermia induced apoptosis signaling.

Hyperthermia or the stress of heat shock involves the rapid synthesis of an evolutionarily conserved family of proteins called "heat shock proteins" (HSP). The HSPs enable the cell to antagonize the stress element of heat until it is removed and thereby enhance cell survival. Upon stressor removal, the level of intacellular HSPs return to normal. However, if the source of stress remains over a prolonged period of time or if the intensity of heat is augmented the presence of HSPs may be insufficient to protect the cells further. Consequently, the synthesis of HSPs declines and enhancement of apoptotic processes occur (O'Neill et al.,1998a). HSP27 and HSP70 are by far the most important HSPs in hyperthermia inuced apoptosis pathways. Elevated expression and chaperoning function of HSPs are not restricted to elevated temperatures alone. They can also be observed in various stress conditions, and some HSPs carry out similar functions during regular protein synthesis as long as some amino acids have not developed complex structures. Taken together, there exists to be a strong correlation between HSP expression and inhibition of hyperthermic cell death.

Hyperthermia affects fluidity and stability of cellular membranes and impedes the function of transmembranal transport proteins and cell surface receptors in vitro. Membrane alterations are an important target in hyperthermic cell death (Coss et al., 1996). Earlier studies suggest the changes in membrane potential, intacellular pH, elevated intracellular sodium and calcium content, as well as an elevation of potassium-efflux under hyperthermia (Hildebrandt et al., 2002). Furthermore, hyperthermia has been demonstrated to induce a variety of changes in the cytoskeletal organization (cell shape, mitotic apparatus, intracytoplasmatic membranes such as endoplasmatic reticulum and lysosome) (Jung, 1986). Hyperthermia induces cell blebbing in different cell lines, a characteristic of programmed cell death. Hyperthermia has also been shown to induce apoptotic DNA fragmentation in different cell lines (Fairbairn et al., 1995). In HepG2 cells, hyperthermia has been shown to operate independently of p53 pathway but occurs by alternative signaling like the Notch pathway. In thermosensitive cells, the induction of apoptosis and the involvement of apoptosis family genes Bax and Bcl-2 occur independently of p53. In thermoresistant cells the level of apoptosis is low and the

main consequence of hyperthermia is the reduction of cell proliferation associated with a fast and durable expression of p53 (Basile et al., 2008). Figure 4, provides an overview of the interrelationship between hyperthermia and apoptosis and their signaling pathways.
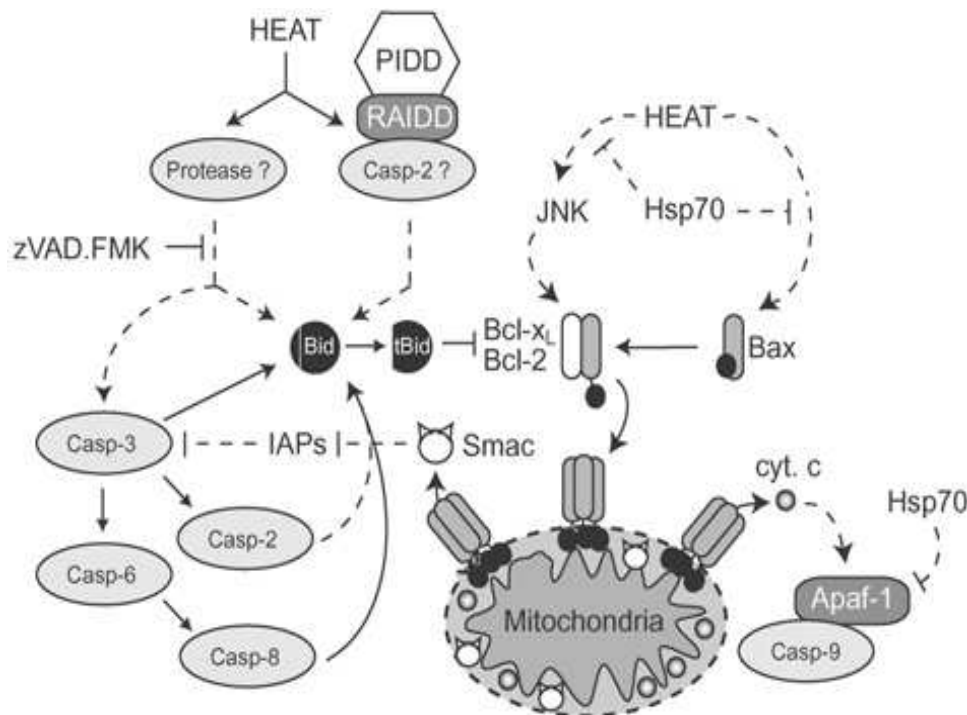

Figure 4. Schema of hyperthermia-induced apoptosis in nucleated cells (Milleron and Bratton, 2007)

2.8.4. Fever and anemia

Anemia has a multifactorial etiology. Some important factors contributing to anemia include parasitic infections, HIV infection, chronic inflammatory disorders, micronutrient deficiencies, and genetic disorders. Under a wide variety of clinical conditions, hyperthermia or fever is paralleled by anemia (Lee, 1983). The coincidence of hyperthermia and anemia is considered to result largely from a common cause, such as release of inflammatory mediators, which modify the setpoint of temperature regulation and by the same token compromise erythrocyte formation or survival (Cronstein, 2007). Many cytokines that are involved in chronic acute phase response have an inhibitory activity on erythroid precursors colony formation *in vitro*. These factors are concomitant with low erythropoetin levels

pointing to anemia. Fever and anemia are obviously connected in malaria, where the destruction of erythrocytes coincides with the inflammatory response against the parasite (Grobusch et al., 2005). Fever and anemia are both closely related to malarial outbreaks according to cross-sectional studies (Takem et al., 2010).

2.9. Endothelins

2.9.1. Endothelin structure

A family of peptides was isolated and identified in 1985 when peptidergic activity was produced in endothelial cells that caused coronary vasoconstriction. The three members of the family are endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). They are produced in a variety of tissues where they act as modulators of vasomotor tone, cell proliferation, and hormone production. They are essential in vascular physiology and disease. Endothelin-1 is present in many mammalian species, including humans (Levin, 1995). Endothelin-2 and endothelin-3 are encoded by separate genes and are isoforms of endothelin-1 which show a high degree of primary amino acid sequence identity. They are all 21-amino acid polypeptides that contain two intramolecular disulfide bonds. The three endothelins have a structural and functional resemblance to sarafotoxins, a family of isopeptides isolated from the venom of the snake *Atractaspis engaddensis*, and it suggests a common evolutionary origin (Miyauchi et al., 1999). The primary sequences of ET-1, ET-2, ET-3 and sarafotoxin are illustrated in figure 5.

Vascular endothelial cells are a major source of ET-1, the genes that encode the three endothelin isopeptides are expressed in a wide variety of cell types, including cardiomyocytes, vascular smooth cells, renal tubular epithelium, glomerular mesangium, glia, the pituitary, macrophages, mast cells, etc. suggesting their particpation in complex regulatory mechansisms in various organs. Moreover, endothelins have a variety of pharmacological actions both in the cardiovascular system and in other tissues (Simonson, 1993; Rubanyi et al., 1994).
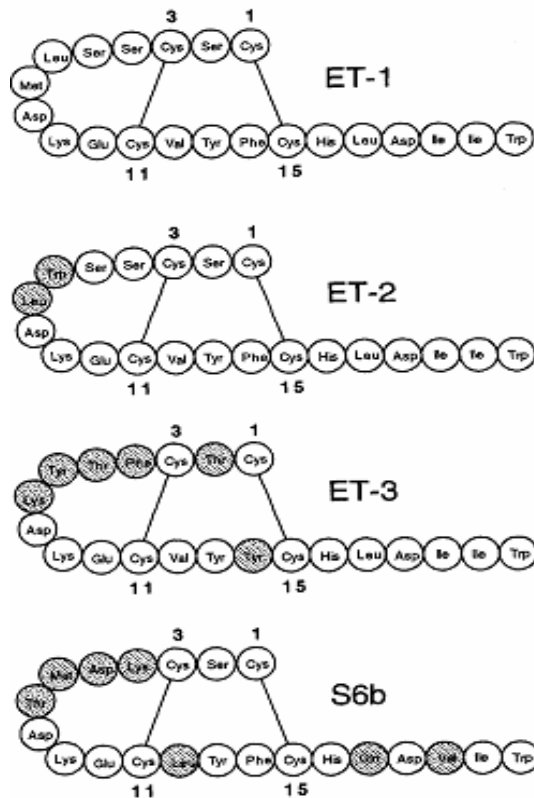
Figure 5. Primary sequences of ET-1, ET-2, ET-3 and sarafotoxin. (Takuwa et al., 1989)

2.9.2. Endothelin receptors

Endothelin receptors are members of the superfamily of receptors linked with guanine-nucleotide-binding (G) proteins and range from 45,000 to 50,000 Daltons in size in various tissues. Two types of (A and B) endothelin receptors are bound by all the three types of endothelins. The two receptors have a 50% similarity in their amino acid structure and each type is highly conserved across mammalian species (Levin, 1995).

Endothelin A (ETA) receptors have ten times more affinity to bind to ET-1 than ET-3 and are expressed abundantly on vascular smooth muscle cells and cardiomyocytes. These receptors mediate vasoconstriction by the action of ET-1, but it is known that in some vascular structures endothelin B (ETB) receptors may contribute to this action. Endothelin activated receptors stimulate phospholipase C and trigger the formation of inositol 1,4,5-triphosphate and diacylglycerol which enhances the intracellular calcium concentration and therby causes vasoconstriction. The elevated intracellular calcium causes the persistence of vasoconstriction when ET-1 is removed. Nitric oxide, on the other hand, shortens the duration of vasoconstriction by facilitating intracellular calcium to return to the basal level. Diacylglycerol and calcium

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stimulate protein kinase C which mediates the mitogenic action of ET-1. ETA receptors, by the action of ET-1, inhibit activation of chloride channels and activate some potassium channels attenuating the proarrhythmic effetcs of catecholamines in the myoocardium (Simonson et al., 1993; Ono et al., 1994).

Figure 6, depicts the mechanisms of signaling induced by ET1 through the ETA recpetor. ETB receptors are predominantly expressed on endothelial cells and to a lesser extent on vascular smooth cells. ETB receptors bind to both ET-1 and ET-3 receptors with a similar affinity. The effects of the activation of ETB receptors are similar to the activation of ETA receptors in stimulating the activation of phospholipase C, and further triggering the same pathway of inositol 1,4,5-triphosphate and diacylglycerol and thus calcium. ETB receptor activation by ET-3 is implicated in the normal development of cells derived from neural-crest precursors (Aramori et al., 1992). Epidermal melanocytes and colon ganglionic neurons do not develop with a functional deficiency of ETB receptors. Endothelin receptor production is in concordance with endothelins. Epidermal growth factor, basic fibroblast growth factor, cyclic AMP and estrogen upregulate ETA receptors in some tissues. C-type natriuretic hormone, angiotensin II and basic fibroblast growth factor upregulate ETB receptors. On the other hand, endothelins, angiotensin II, and platelet-derived growth factor, and transforming growth factor ß downregulate ETA receptors while cyclic AMP and catecholamines downregulate ETB receptors (Levin, 1995).
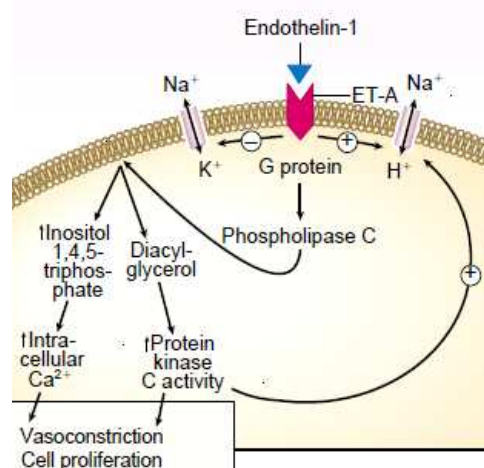

Figure 6. Mechanisms of ETA receptor stimulation by ET-1 (Levin, 1995)

Tissue specific differences in the expressions of ETA and ETB receptors mediate different actions by the three endothelins. ETA receptors in the kidney are expressed

mostly in the vasa recta and arcurate arteries while ETB receptors are found in collecting ducts which implies to their different roles in modulation of salt and water reabsorption. However, both receptors are found in the glomeruli. Mice deficient of ETB receptors phenotypically show aganglionic megacolon and spotting which is suggestive of Hirschsrung´s disease in humans. Knock out of ET-3 also expressed a similar phenotype which underlines the role of close interaction between ET-3 and ETB receptors in the development of colon ganglionic neurons (Baynash et al., 1994; Hosoda et al., 1994).

2.9.3. Physiology and pathophysiology of endothelins

The plasma concentration of endothelins is very low and it may be defined as a paracrine/autocrine mediator. Endothelin-1 production is simulated by a wide range of factors which includes angiotensin II, arginine vasopressin, thrombin, high-density and low-density lipoproteins, insulin, transforming growth factor ß, insulin-like growth factor I, epidermal growth factor, and basic fibroblast growth factor. Nitric oxide and prostacyclin inhibit the production of ET-1 through the generation of cyclic guanosine monophosphate. ET-3 probably acts through ETB receptors stimulating NO production by endothelial cells. ET-1 and ET-3 stimulate the production of several prostaglandins (Levin, 1995).

In cardiovascular pathology, ET-1 plasma levels are increased following myocardial infarction and congestive heart failure (Battistini et al., 1993). This may induce bronchospasms as ETA receptors are expressed on the bronchial smooth muscle cells. Retention of salt and water is also contributed by its effects on adrenal aldosterone production. Plasma ET-1 may be normal in hypertension but is increased in preeclampsia. In nephrological pathophysiology, endothelin may play a role due to its vascular related functions (Branch et al., 1991). In cyclosporine nephrotoxicity, ET-1 production is increased (Bunchman et al., 1991). High levels of ET-1 were detected in patients with pulmonary hypertension (Macquin-Mavier et al., 1989). Endothelin has also been implicated in developmental disorders such as cardiovascular malformations and ET-1 knockout mice die prematurely due to severe craniofacial maldevelopment pointing to the essential role of ET-1 in differentiation and development of tissues derived from the neural crest. Plasma ET-1 concentrations are also markedly increased after cerebrovascular ischemia and cerebral infarction (Kurihara et al., 1994).

2.9.4. Endothelin and apoptosis

There is increasing evidence that endothelin may contribute to tumor growth by protecting cells from apoptosis. ET-1 has been shown to protect rat fibroblasts and human endothelial cells from serum deprivation-induced apoptosis in vitro (Wu-Wong et al., 1997). It is also documented that ET-1 is a survival factor for rat colon carcinoma cells against FasL-mediated apoptosis (Eberl et al., 2000). It is therefore, conclusive that ET-1 may influence tumor growth by influencing both cell proliferation and cell death (Shichiri et al., 1997). Furthermore, ET-1 may influence malignant tissue growth by favoring angiogenesis and ET-1 has also shown to be a mitogen, and favour metastases (Grant et al., 2003).

2.9.5. Endothelin and erythrocytes

Both, human and murine erythrocytes express the ETB receptor (Rivera et al., 1999; Rivera et al., 2002; Rivera, 2007). Acute exposure to endothelin activates Ca^{2+}-sensitive K^+ channels receptor, an effect expected to shrink erythrocytes thus favouring cell membrane scrambling (Schneider et al., 2007). Modulation of erythrocyte Gardos channel activity by ET-1 might play an important role in the dehydration of sickle cell erythrocytes. ET-1 receptor activation causes intracellular Ca^{2+} activity increase and activation of PKC, resulting in K^+ and water loss and formation of denser erythrocyte (Rivera, Rotter, and Brugnara, 1999).

3. Aim of the study

The objectives of the present study are to investigate the role and mechanisms involved in the regulation of erythrocyte survival and suicidal erythrocyte death by temperature changes and endothelin-B receptor stimulation.

In the first section of the study, experiments were performed to elucidate the role of temperature changes on erythrocyte survival and apoptosis. The following were measured: intracellular calcium activity, phosphatidylserine exposure, cell volume changes, ceramide formation, cytosolic ATP concentration changes, presence of hemolysis concomitantly with enhanced eryptosis, and the inhibition of hyperthermia-induced cell death by CysLT1 receptor antagonism. Using FACS analysis, photometric measurement, and luminometry, it was indeed shown that enhanced eryptosis parallels graded increase in temperature.

In the second section of the study, *in vitro* and *in vivo* experiments were performed to understand the role of endothelin-B receptor stimulation by endothelin-1 in erythrocyte survival. To this end, endothelin and its agonist sarafotoxin were used to show their blunting effects on erythrocyte suicidal death. Intracellular Ca^{2+} activity and phosphatidylserine cell surface exposure were analysed using FACS and *in vivo* studies on ETB knockout (etb$^{-/-}$) and wild type mice (etb$^{+/+}$) were performed to show the significance of ETB receptor in inhibiting suicidal erythrocyte death. With the application of FACS analysis, western blotting, confocal microscopy, and various *in vivo* experiments it is shown that etb$^{-/-}$-mice are comparatively more susceptible to eryptosis than wild type mice.

4. Materials and methods

4.1. Chemicals, solutions and reagents

The experiments were performed with the aid of standard laboratory equipment, reagents and conditions at the Physiology Institute, University of Tübingen.

Incubation of erythrocytes was done in an isotonic medium. The Ringer solution was prepared in the laboratory and consisted of:

- NaCl   125 mM
- KCl   5 mM
- $MgSO_4$ 1.2 mM
- HEPES   32.2 mM
- Glucose   5mM
- $CaCl_2$ 1 mM

The pH was regulated by NaOH to 7.4 and the osmolarity was measured using VAPRO 5520 vapor pressure osmometer (Wescor, Utah, USA). The Ringer solution was then filtered by passing through a sterile filter (Millipore, Cork, Ireland).

To experimentally induce eryptosis, glucose-depleted Ringer was used where glucose was removed from the isotonic ringer solution and this consisted of the following:

- NaCl   125 mM
- KCl   5 mM
- $MgSO_4$ 1.2 mM
- HEPES   32.2 mM

Similar to the Ringer solution, the glucose-depleted Ringer solution was also adjusted to pH 7.4, osmolarity adjusted and filtered.

Phosphate buffered saline (pH 7.4) was used in FACS analysis of ceramide abundance and as an unstained control for reticuolocyte determination. The constituition of PBS is as follows:

- NaCl   137 mM

- ◆ KCl   2.7 mM
- ◆ Na_2HPO_4 10 mM
- ◆ KH_2PO_4 1.76 mM,

For FACS analysis, a special buffer was used to wash and measure the samples "annexin binding buffer" similar to the Ringer solution but with additional calcium chloride. The constitution of annexin binding buffer is as follows:

- ◆ NaCl   125 mM
- ◆ HEPES   10 mM
- ◆ $CaCl_2$ 5 mM

For the isolation of erythrocytes, CDP buffer and SAG-M solution were used which included the following constituents:

CDP buffer

◆ Citric acid	3.27 mg/ml
◆ Sodium citrate	26.3 mg/ml
◆ Sodium hydrogenphosphate dihydrate	2.5 mg/ml
◆ Dextrose monohydrate	25.5 mg/ml

SAG-M solution

◆ Sodium chloride	8.77 mg/ml
◆ Dextrose monohydrate	9 mg/ml
◆ Adenine	0.17 mg/ml
◆ Mannitol	5.25 mg/ml

For the present experiments, the following pharmacological agents were used:
1) Cinalukast 1µM (Sigma, Schnelldorf, Germany)
2) Endothelin 1 10-3000 nM (Sigma, Schnelldorf, Germany)
3) Endothelin 2 500 nM (Sigma, Schnelldorf, Germany)
4) Endothelin 3 500 nM (Sigma, Schnelldorf, Germany)
5) Sarafotoxin 6c 0.3-100 nM (Sigma, Schnelldorf, Germany)
6) Ionomycin 1 µM (Sigma, Schnelldorf, Germany)
7) Tert-butylhydroperoxide 0.1 mM (Sigma, Schnelldorf, Germany)

For FACS analysis and immunofluorescence confocal microscopy the following fluorescent dyes and antibodies were used:

1) Annexin V-Fluos (Roche, Mannheim, Germany)

2) Retic-Count reagent (Thiazole orange) (BD, San Jose, CA, USA)

3) Fluo-3/AM (Calbiochem, Bad Soden, Germany)

4) Anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany)

5) Polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany)

6) Carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) (Molecular Probes, Leiden, Netherlands)

7) Annexin V-APC (BD, Heidelberg, Germany)

4.2. Erythrocytes, sample preparation and incubation

The leukocyte-depleted banked erythrocytes were provided by the blood bank of the University of Tübingen. The volunteers providing erythrocytes gave informed consent. The study was approved by the Ethical commission of the University of Tübingen.

Isolation of human erythrocytes was done according to the following protocol:

1) To prepare a 500 ml erythrocyte concentrate an OptiPure RC quadruple blood pack set with a leukocyte depletion filter was used.

2) 500 ml blood was mixed with 70 ml of CDP-buffer.

3) It was centrifuged for 10 minutes at room temperature at 4795g.

4) The blood components were separated and filled in special blood packages.

5) The packaging procedure included addition of SAG-M stabilizing solution to the erythrocytes.

6) It was filtered through an integrated leukocyte depletion filter at room temperature.

7) The purified erythrocyte concentrates (30-90%) were stored at 4 ℃ until experiments were performed.

Temperature sensitivity of suicidal erythrocyte death

The present experiments involved human erythrocytes, the samples were made using 4 µl of erythrocytes in 1000 µl Ringer solution In experiments involving sustained temperatures of 37℃, erythrocytes were incubated in an incubator

(Heraeus, Germany). Where indicated, for temperatures between 38 and 41℃, the samples were incubated in a cryostat (Thermo, Waltham, US). CysLT1 leukotriene receptor antagonist cinalukast (1 µM) was added to the samples incubated at temperatures 37, 40 and 41℃ to check for amelioration in suicidal erythrocyte death. The time of incubation in all temperature-sensitive experiments was standardized to 24 hours.

Endothelin B receptor stimulation inhibits suicidal erythrocyte death

In in vitro experiments performed on human erythrocytes, 4 µl of erythrocytes were incubated in 1000 µl Ringer solution or glucose-depleted Ringer solution.

Where indicated, endothelin 1 (10-3000nM), endothelin 2 (500 nM), endothelin 3 (500 nM) and sarafotoxin 6C (0.3-100 nM) were incubated

Incubation times were between 48-72 hours for experiments involving human erythrocytes.

Ionomycin (1µM, 30 minutes incubation) was used along with endothelin-1 and sarafotoxin 6C to treat erythrocytes to determine whether endothelin influences the membrane scrambling activity of Ca^{2+}.

Experiments on murine erythrocytes were performed to test the effect of oxidation induced eryptosis. 2 µl or erythrocytes were incubated in 500 µl Ringer solution with or without 0.1 mM tert-butylhydroperoxide for 30 minutes.

To test for phosphatidylserine exposure immediately after blood retrieval, erythrocytes were not exposed to any incubation. They were directly stained and FACS analysis was performed.

4.3. Murine experiments

To study the role of endothelin-B receptor stimulation in suicidal death *in vivo*, experiments were performed in 9-16 week old male and female rescued ETB knockout mice (etb^{-/-}) and corresponding wild type mice (etb^{+/+}). The etb^{-/-}-mice have been described previously (Quaschning et al., 2005).

The animal experiments were performed according to the guidelines of the American Physiological Society and the German law for the welfare of animals and it was approved by the local authorities.

Blood collection was performed in heparin-containing tubes except for the blood counts.

To examine various blood parameters the following were analyzed:

1) Blood was collected in EDTA-containing tubes and erythrocyte counts, haemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration and mean corpuscular haemoglobin were determined using an electronic hematology particle counter (scil Vet abc, Weinheim, Germany).

2) Vitamin B_{12} and folic acid were determined in the blood by competitive immunoassays according to clinical standards in the laboratory of the University Hospital Tübingen.

3) Erythropoietin concentrations in the plasma were determined using an immunoassay kit according the the instructions of the manufacturer (R&D systems, Wiesbaden, Germany). For determination of erythropoietin, 50 µl of plasma was used. Plasma was collected by centrifugation of blood for 20 minutes, 2000 g, within 30 minutes of collection in heparinised tubes. Plasma was stored at -20℃. The absorbance was measured at 450 nm using a microplate reader (Tecan Sunrise, Crailsheim, Germany).

Clearance of fluorescence labelled-erythrocytes from the circulation was performed to check the significance of endothelin receptor in erythrocyte survival *in vivo*. The experiment was performed as follows:

1) Erythrocytes were obtained from 200 µl of blood. The erythrocytes were washed once in PBS and twice in Ringer solution.

2) The labelling solution was prepared by addition of adequate amounts of CFSE stock solution (10 mM in dimethyl sulfoxide) to PBS to yield a final concentration of 5 µM.

3) The cells were incubated with the labelling solution for 30 minutes at 37℃ protected from light.

4) Cells were pelleted by centrifugation at 400 g for 5 minutes and washed twice in PBS containing 1% FCS and pelleted at 400 g for 5 minutes.

5) The fluorescence labelled-erythrocytes pellet was resuspended in equal volume of pre-warmed Ringer solution.

6) 100 µl of fluorescence labelled-erythrocytes diluted in Ringer solution were injected intravenously into the same mice.

7) 2 µl of blood was retrieved at an interval of every 24 hours and the percentage of CFSE-labelled erythrocytes was measured as described in the section "FACS analysis"

8) The percentage of CFSE-positive erythrocytes was calculated in % of the total labelled fraction determined 5 min after injection.

To examine ratio between spleen and body weights, the weight of the mice was determined using a balance (Sartorius, Göttingen, Germany). The mice were then sacrificed and spleens were removed and weighed. A macroscopic photograph of the spleens from etb$^{+/+}$ and etb$^{-/-}$-mice was taken for comparison.

4.4. FACS analysis

Flow cytometry or FACS analysis is standard technique used for the qualitative and quantitative analysis of microscopic units like cells and chromosomes. In the present study, flow cytometry has been elaborately used to determine various parameters of suicidal erythrocyte death. In principle, cells-containing fluid is targeted by laser which then encrypts fluorescence intensity and scatter on an electronic detection apparatus. Four different types of fluorescence channels have been classified based on their emission wavelengths:

1) The FL1 channel with green fluorescence (515-545 nm)
2) The FL2 channel with orange fluorescence (564-606 nm)
3) The FL3 channel with red fluorescence (>670 nm)
4) The FL4 channel with red fluorescence (653-669 nm)

In the present study FACS analysis has been utilized to determine the following parameters:

1) Cell volume by forward scatter
2) Phosphatidylserine exposure by annexin V-binding
3) Reticulocyte counts by thiazole orange (Retic-Count) staining
4) Intracellular Ca^{2+} activity by Fluo-3 fluorescence
5) Ceramide measurement
6) Measurement of fluorescent labelled erythrocytes circulating in vivo

FACS analysis experiments were performed using the FACS-Calibur (BD; Heidelberg, Germany) (Figure, 7). The FACS analysis of human and murine erythrocytes can be measured with similar settings.

Figure 7. FACS-Calibur (BD; Heidelberg, Germany)

Changes in cell volume can be determined by analyzing changes in forward scatter. Measurement of forward scatter does not require staining and can be measured alone or concomitantly with other kinds of staining. In the present study, FSC was measured simultaneously with annexin V-binding. Figure 8 denotes a typical dot-plot image of FSC and SSC in human erythrocytes. Geometric mean of the FSC was measured for each sample. For measurement of FSC in erythrocytes the linear scale was used.
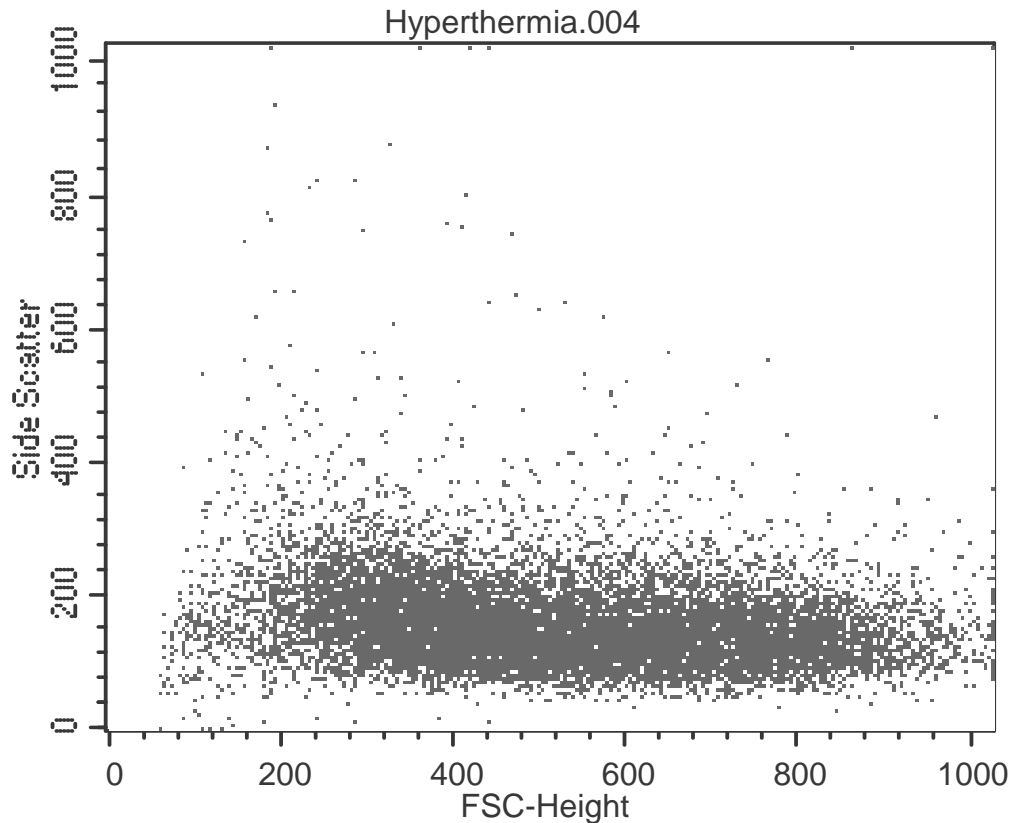
Figure 8. Forward scatter versus side scatter dot-plot image of erythrocytes on a linear scale

Annexin V-binding to measure phosphatidylserine exposure was performed as follows:

1) 50 µl of the sample was utilized to stain with Annexin V-Fluos (Roche, Mannheim, Germany)

2) The sample was washed once with annexin binding buffer and 150 µl dilution of 1:500 of Annexin V-Fluos in annexin binding buffer was resuspended.

3) After 15 minutes of incubation samples were measured on the FACS calibur.

4) Cells were analysed by forward scatter (automatically determined by Cell Quest software (BD), and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Reticulocyte counts in murine blood were measured using Retic-Count reagent (Thiazole orange) (BD, San Jose, CA, USA). Briefly, 2 µl of erythrocytes were incubated at 37℃ for 30 minutes (protected from li ght) in 500 µl of Retic-Count and simultaneously an unstained control was used with 2 µl blood and 500 µl PBS to

detect auto fluorescence. The forward scatter dot plot was changed to logarithmic scale (Figure 9) and erythrocytes were gated. The percentage of reticulocytes in whole blood was determined using a marker in the FL1 channel and the percentage generated by auto-fluorescence in unstained samples was subtracted from the corresponding stained samples.
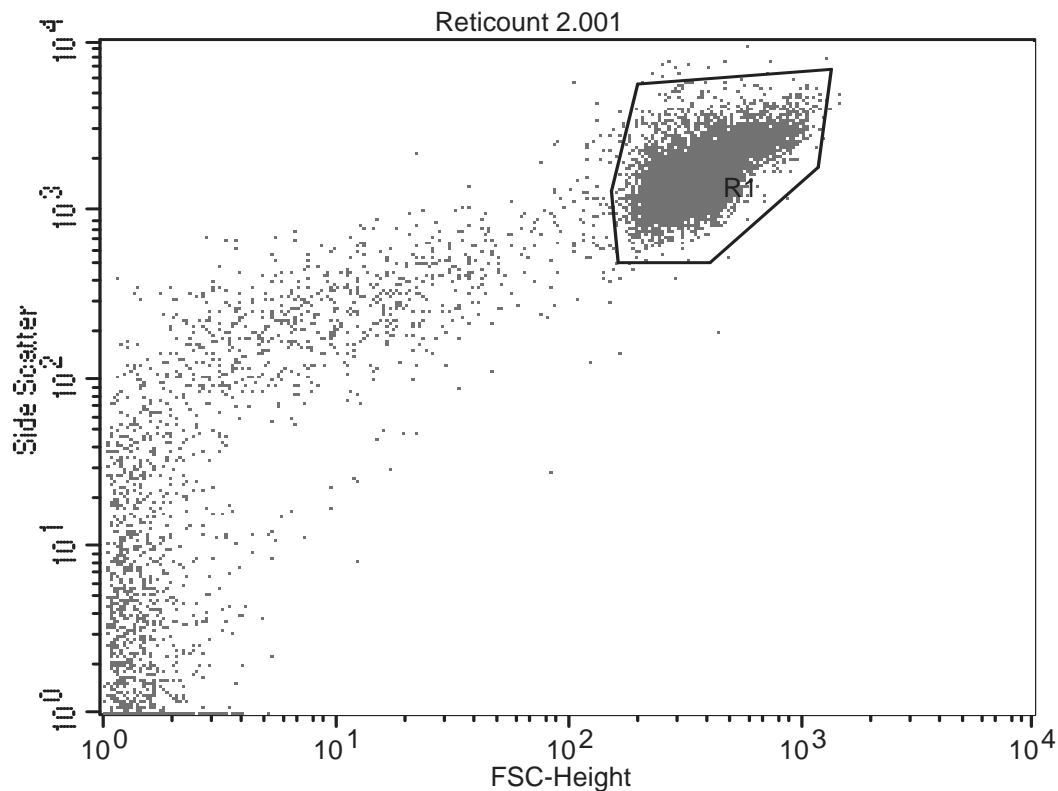

Figure 9 Forward scatter versus side scatter dot plot image of erythrocytes (within the gate R1) on logarithmic scale.

Intracellular Ca^{2+} activity was measured using cell membrane permeable Fluo-3/AM. Unlike all other indicators of Ca2+, Fluo-3/Am is non-fluorescent until its hydrolysis in the cell by cellular esterases. Fluo-3/AM (Calbiochem, Bad Soden, Germany) was used to measure intracellular calcium activity by FACS analysis as follows:

1) After incubation 50 µl erythrocyte suspension were washed in Ringer solution
2) They were loaded with Fluo-3/AM in annexin binding buffer (as for PS exposure analysis) and 2 µM Fluo-3/AM.
3) The cells were incubated at 37℃ for 20 min and washed twice in annexin binding buffer.

4) The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer.

5) Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

To analyse ceramide formation by FACS analysis, a monoclonal antibody-based assay was used.

1) After the desired time of incubation, erythrocytes were stained for 1 hour at 37℃ with 1 µg/ml of anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) (Sigma, Schnelldorf, Germany) at a dilution of 1:5.

2) The samples were washed twice with PBS containing 0.1% BSA and cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA.

3) Unbound secondary antibody was removed by repeated washing with PBS-BSA.

4) Finally, 200 µl of PBS-BSA was resuspended and the samples were then analysed by flow cytometric analysis in the channel FL-1.

FACS analysis was also used to measure fluorescence labelled erythrocytes. Carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) (Molecular Probes, Leiden, Netherlands) was used for labelling erythrocytes.
 2 µl of blood was collected in PBS + 0.5 mM EDTA and the fluorescence intensity was measured on FL-1. CFSE positive erythrocytes were distinguished on the FL1 histogram using a marker and the percentage of CFSE positive erythrocyte population was determined for each mouse.

4.5. Photometric determination of hemolysis and osmotic resistance

In hyperthermia-induced eryptosis samples were screened for the presence of hemolysis. To determine the presence of hemolysis in the samples, the samples were centrifuged for 3 minutes at 400 g, at room temperature. The supernatants were collected and or the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant

was determined photometrically at 405 nm on a microplate reader (Tecan sunrise, Crailsheim, Germany). The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis. Accordingly a standard curve (0%, 2.5%, 5%, 7.5%, 10%, 20% and 100%) was made to determine the percentage of hemolysis in the samples.

In the study involving the role of endothelin-1 in eryptosis, a difference in the osmotic resistances of erythrocytes from wild types and knock out mice was examined. For the determination of the resistance of erythrocyte to hemolysis under conditions of decreasing osmolarity, 2 µl of blood from $etb^{-/-}$-mice and $etb^{+/+}$-mice was added to 200 µl of PBS solutions of decreasing osmolarity. In order to create different osmotic conditions, PBS was diluted with distilled water in different proportions. The samples were centrifuged in a 96 well-plate for 5 minutes, 500g at room temperature. 50 µl of the supernatant was carefully transferred into another 96-well plate and the plate was read photometrically at 405 nm on a microplate reader (Tecan sunrise, Crailsheim, Germany). As a reference, erythrocytes that were lysed in purified distilled water were considered to be 100% hemolysed.

4.6. Estimation of intracellular ATP content

ATP depletion in the erythrocyte is an important factor in eryptosis signaling. To determine whether erythrocytes treated in sustained hyperthermic conditions underwent intracellular ATP depletion the following protocol was applied:

1) 90 µl of erythrocytes were incubated for 24 hours in the respective temperature conditions in Ringer solution with a final hematocrit of 5%.

2) As a positive control, glucose-depleted Ringer solution was used instead of Ringer solution for the incubation of erythrocytes.

3) After incubating of samples for 24 hours, all experiments were performed at 4℃ to prevent the degradation of ATP.

4) Erythrocytes were washed twice in PBS and after removal of the supernatant the pellets were lysed in distilled water.

5) Proteins were precipitated by the addition of 5% $HCLO_4$.

6) The samples were centrifuged, and an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by the addition of saturated $KHCO_3$ solution.

7) ATP concentrations of the aliquots were determined utilizing the luciferin-luciferase assay kit (Roche diagnostics) in accordance to the manufacturer's protocol.

8) The samples were measured on a luminometer (TD-20/20 Turner Designs, Sunnyvalde, CA, USA) (Figure 10) with a delay time of 60 seconds, and an integration time of 10 seconds after the addition of luciferase.

9) A standard curve was prepared to determine the correlation between light intensity and ATP concentration for the following concentrations of ATP: 0 nM (Blank), 125 nM, 250 nM, 500 nM and 1µM.

Figure 10. Luminometer for the intracellular ATP measurement by luciferin-luciferase assay (TD-20/20 Turner Designs, Sunnyvalde, CA, USA)

4.7. **Western blot analysis**

Western blotting was performed to determine the expression of ETA and ETB receptor protein in murine erythrocytes. The following experimental procedure was performed:

1) 150 µl of blood pellet was lysed in 50 ml of 20 mM of HEPES/NaOH with a pH of 7.4

2) Centrifugation (15,000 g for 20 minutes at 4℃) was done to pellet the ghost membranes.

3) The pellet was lysed in 100 µl of lysis buffer which contained:

  ♦ Tris-HCl   50 mM

  ♦ NaCl   150 mM

  ♦ Triton X-100   1%

- ◆ SDS   0.5%
- ◆ NaF   1mM
- ◆ Na_3VO_4 1mM
- ◆ ß-mercaptoethanol   0.4%

Protease inhibitor cocktail (Roche, Manheim, Germany) was added to the lysis buffer.

4) In Laemmli sample buffer, 80 µg of protein was dissolved at 95ºC for 5 minutes and it was resolved using 10% SDS-PAGE.

5) Immunoblotting of proteins was performed by the electro-transfer onto a PVDF membrane and it was blocked by 10% non fat milk in TBS-0.1% Tween 20 solution at room temperature for 1 hour.

6) The membrane was incubated at 4℃ overnight with ETA and ETB receptor antibody (Santa Cruz, CA, USA) in the dilutions of 1:2000 and 1:4000 respectively.

7) After washing with TBST, subsequent blocking was carried out.

8) The blots were incubated with secondary anti-rabbit antibody (Cell signaling) for 1 hour at room temperature. A dilution of 1:2000 was used for the secondary antibody.

9) Detection of antibody binding was carried out by the ECL detection reagent (Amersham, Freiburg, Germany) after washing.

4.8. Immunofluorescence and confocal microscopy

Eryptotic CFSE-labelled erythrocytes in the circulation of etb$^{-/-}$ and etb$^{+/+}$-mice were cleared and their presence in the spleen was detected using immunofluorescence.

The murine spleens were removed and mechanically homogenized in 1 ml of cold PBS. The suspension was centrifuged at 4ºC for 10 minutes, at 500 g.

To detect apoptotic erythrocytes, Annexin V-APC (BD, Heidelberg, Germany) was used. 5 µl of Annexin V-APC was added and the samples were incubated for 20 minutes, 37ºC, protected from light.

The suspension was transferred onto a glass slide and mounted with Prolong® Gold antifade reagent (Invitrogen). Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) was used to capture images. Water immersion Plan-Neofluar 63/1.3 NA DIC was used.

4.9. Statistical analysis

Data are expressed as arithmetic means ± SEM, and statistical analysis was made by paired or unpaired t-test, or by ANOVA, as appropriate, $p < 0.05$ was considered as statistically significant.

5. Results

5.1. Temperature sensitivity of suicidal erythrocyte death

One of the hallmarks of eryptosis is cell shrinkage. To determine, whether hyperthermia could trigger eryptosis in erythrocytes from healthy individuals, alterations of cell volume were depicted by measurement of forward scatter. As illustrated in figure 11, graded increases in the temperature from 37°C to 41°C resulted in a gradual decrease of the forward scatter.
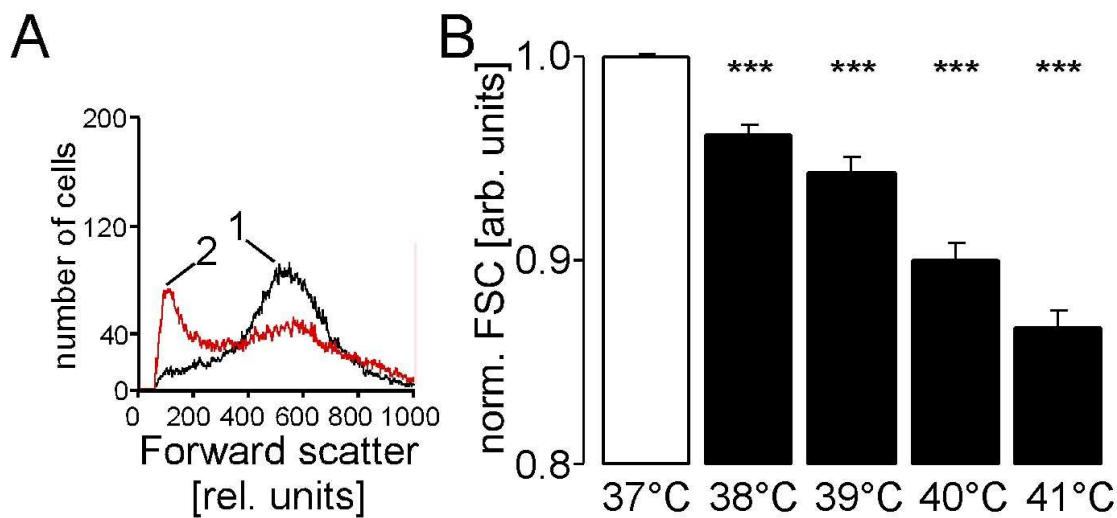
Figure 11. Effects of hyperthermia on erythrocyte forward scatter.

A. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 24 h to either 37°C (1, black line) or to 40°C (2, red line).

B. Forward scatter as a function of temperature. Arithmetic means ± SEM (n = 7-11 different erythrocyte specimens analysed in quadruplicates) of the normalized forward scatter of erythrocytes exposed for 24 h to 37 – 41°C. *** ($P < 0.001$) indicates significant difference from values at 37°C (ANOVA).

A further hallmark of eryptosis is cell membrane scrambling, leading to PS exposure at the cell surface. Annexin V-binding was employed to identify PS-exposing erythrocytes. As shown in figure 12, graded increases in the temperature from 37°C to 41°C increased the percentage of annexin V-binding erythrocytes. Thus, hyperthermia leads to cell membrane scrambling.
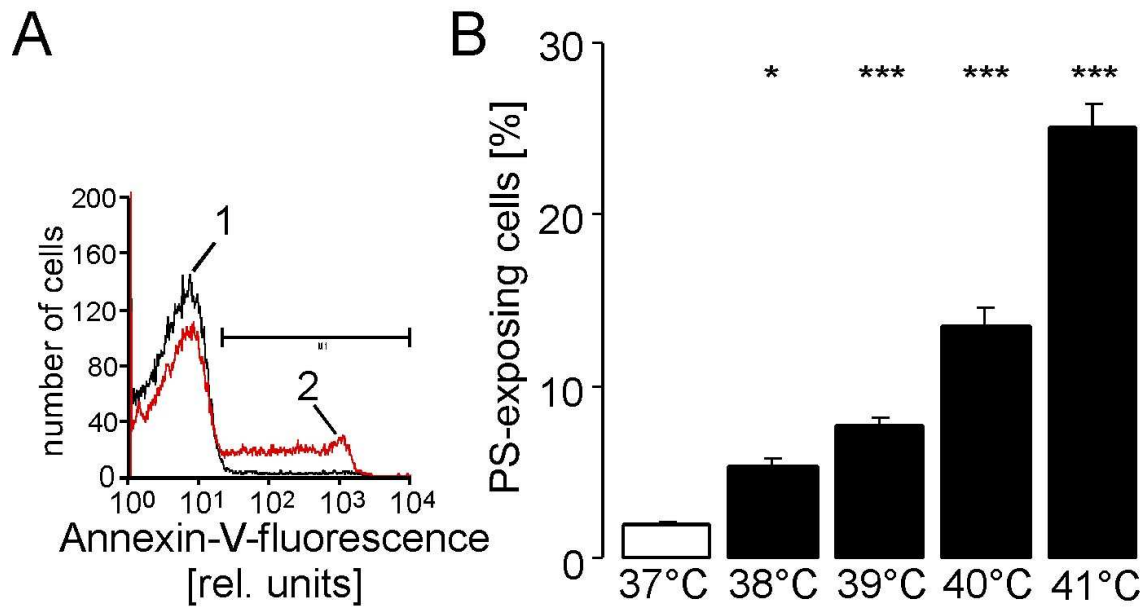
Figure 12. Effects of hyperthermia on erythrocyte PS exposure.

A. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 24 h to either 37℃ (1, blac k line) or 40℃ (2, red line).

B. Annexin V-binding as a function of temperature. Arithmetic means ± SEM (n = 7-14 different erythrocyte specimens analysed in quadruplicates) of the percentage of annexin V-binding erythrocytes following exposure for 24 h to 37℃ - 41℃. *, *** ($P < 0.05$, $P < 0.001$) indicate signifi cant difference from values at 37℃ (ANOVA).

The time dependence of the effect of hyperthermia on PS exposure and cell volume is shown in figure 13.

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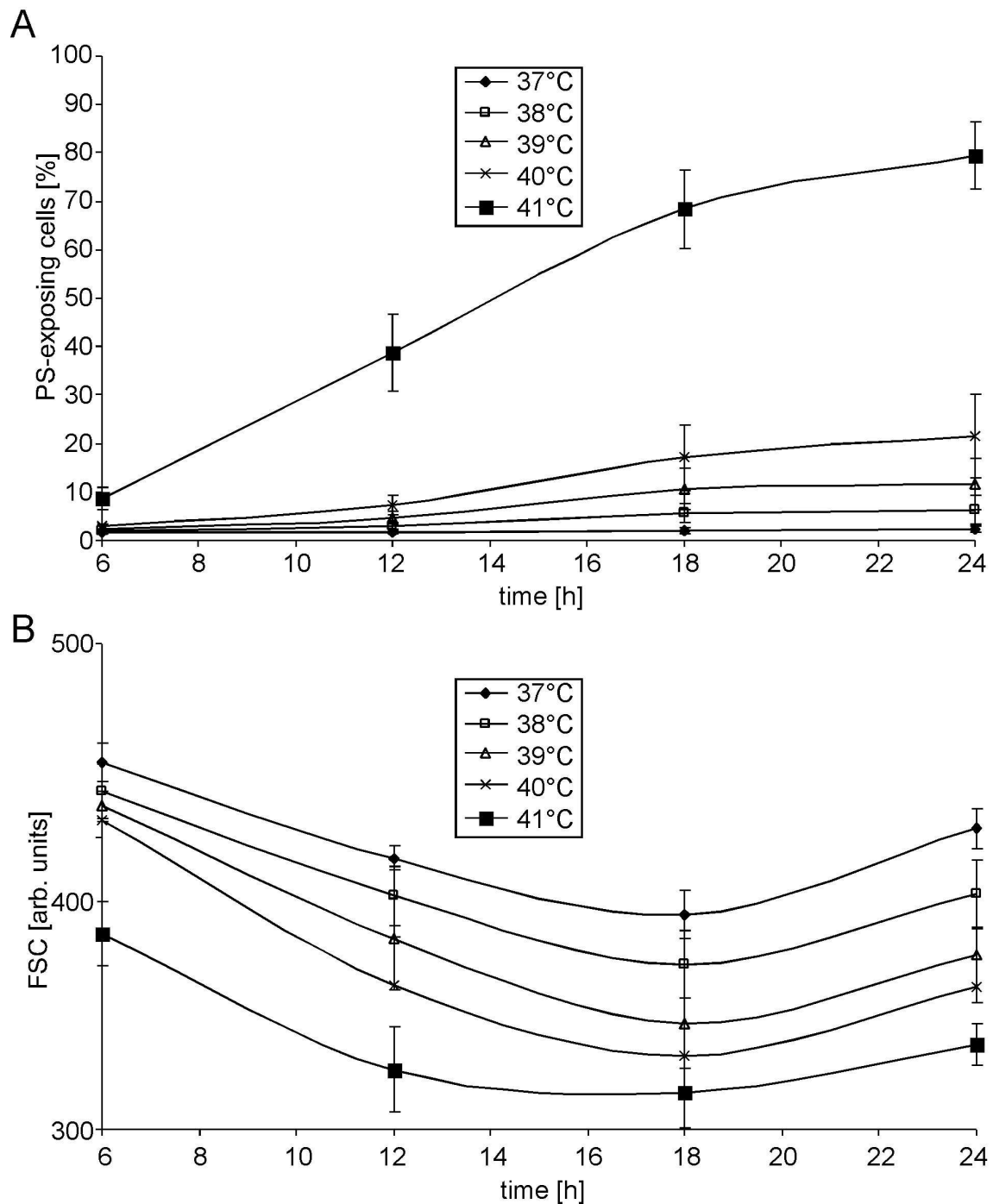
Figure 13. Time dependence of the effects of hyperthermia on erythrocyte PS exposure and forward scatter.

A. Annexin V-binding as a function of time. Arithmetic means ± SEM (n = 3-4 different erythrocyte specimens) of the percentage of annexin V-binding erythrocytes following exposure for 6 - 18 h to 37°C - 41°C.

B. Forward scatter as a function of time. Arithmetic means ± SEM (n = 3-4 different erythrocyte specimens) of the forward scatter of erythrocytes following exposure for 6 - 18 h to 37°C - 41°C.

Hyperthermia could cause direct lysis of erythrocytes. To test for this possibility, the percentage of lysed erythrocytes was determined following exposure to temperatures from 37℃ to 41℃ for 24 h. As shown in figure 14, hemolysis remained low up to 40℃ but increased sharply at 41℃.
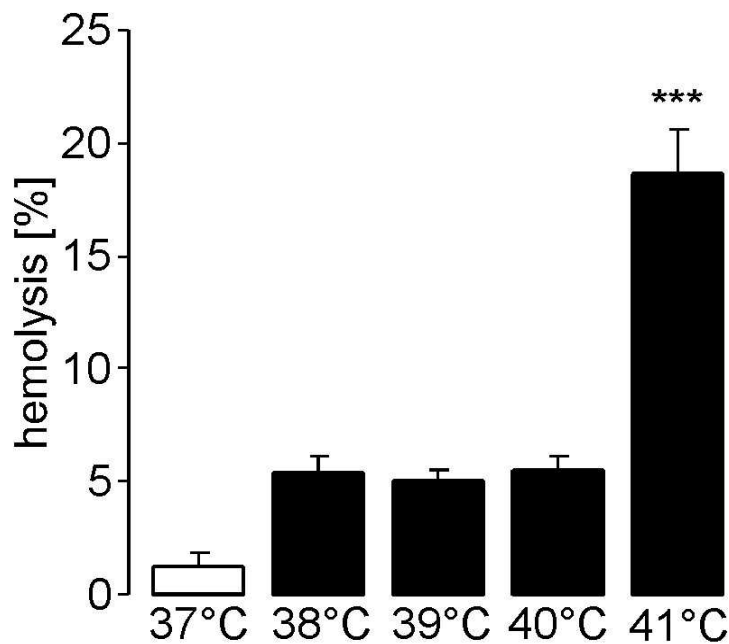

Figure 14. Effects of hyperthermia on hemolysis.

Hemolysis as a function of temperature. Arithmetic means ± SEM (n = 7-14) of the percentage of haemolysed erythrocytes following exposure for 24 h to 37℃ - 41℃. *** ($P < 0.001$) indicates significant difference from values at 37℃ (ANOVA)

Both, cell shrinkage and cell membrane scrambling could be triggered by increase in the cytosolic Ca^{2+} activity (Bratosin et al., 2001). Accordingly, Fluo3 fluorescence was exploited to estimate the cytosolic Ca^{2+} activity. As shown in figure 15 A,B, graded increases in the temperature from 37℃ to 41℃ incr eased the Fluo3 fluorescence indicating that hyperthermia increases the cytosolic Ca^{2+} activity.

Cell shrinkage and cell membrane scrambling could further be triggered by ceramide (Lang et al., 2004). Anti-ceramide antibodies were thus utilized to determine, whether hyperthermia was followed by ceramide formation. As shown in figure 15C, hyperthermia of 40℃ and 41℃ slightly but signific antly increased the ceramide abundance of human erythrocytes.
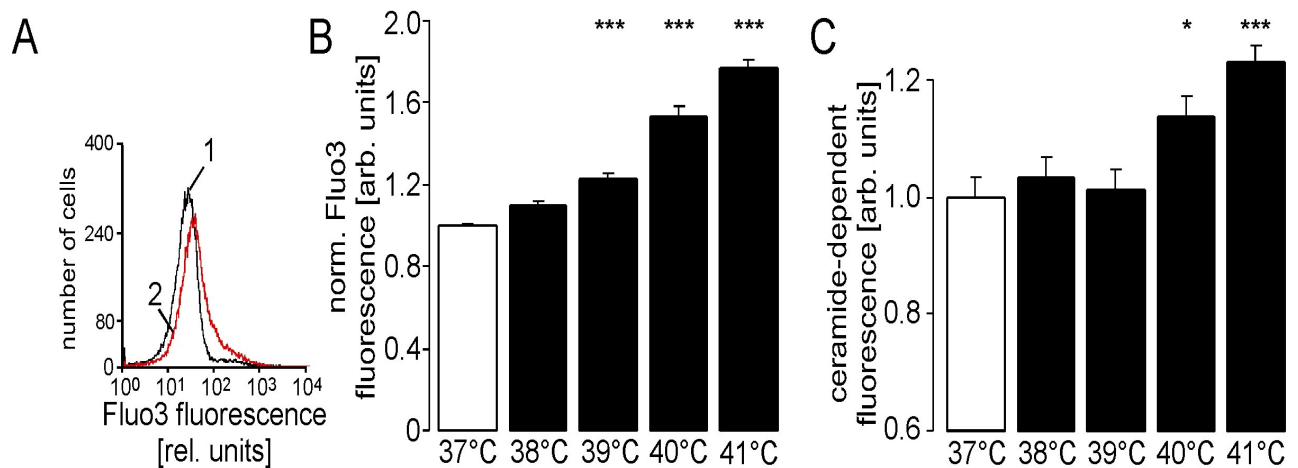
Figure 15. Effects of hyperthermia on cytosolic Ca^{2+} activity and ceramide abundance.

A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 24 h to either 37°C (1, blac k line) or 40°C (2, red line).

B. Fluo3 fluorescence as a function of temperature. Arithmetic means ± SEM (n = 7-14 different erythrocyte specimens analysed in quadruplicates) of the normalized Fluo3 fluorescence of erythrocytes following exposure for 24 h to 37°C - 41°C. *** (P < 0.001) indicates significant differe nce from values at 37°C (ANOVA).

C. Ceramide abundance as a function of temperature. Arithmetic means ± SEM (n = 3 different erythrocyte specimens analysed in quadruplicates) of the normalized ceramide-dependent fluorescence of erythrocytes following exposure for 24 h to 37°C - 41°C. *, *** (P < 0.05, P < 0.001) indicate significant difference from values at 37°C (ANOVA).

Eryptosis is further known to be triggered by energy depletion. Therefore, the cytosolic ATP content of erythrocytes was determined. As shown in figure 16A, graded increases in the temperature from 37°C to 41 °C significantly decreased the cytosolic ATP concentration.

Since energy depletion-induced eryptosis is inhibited by antagonizing the CysLT1 leukotriene receptor, the effect of the CysLT1 antagonist cinalukast (1 µM) on hyperthermia-induced eryptosis was tested. As shown in Figure 16B, cinalukast indeed significantly blunted hyperthermia-stimulated PS exposure.
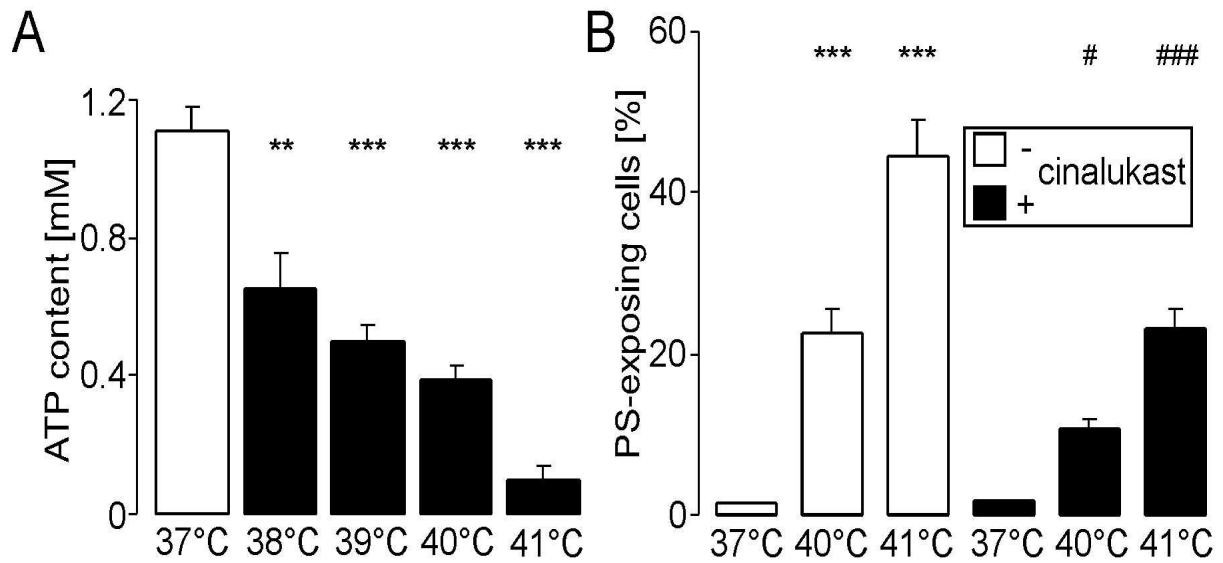
Figure 16. Effects of hyperthermia on ATP concent. Inhibitory effect of CysLT1 antagonism on hyperthermia-induced eryptosis.

A. Cytosolic ATP concentration as a function of temperature. Arithmetic means ± SEM (n = 4 different erythrocyte specimens analysed in quadruplicates) of the cytosolic ATP concentration of erythrocytes following exposure for 24 h to 37°C - 41°C . **, ** * (P < 0.01, P < 0.001) indicate significant difference from values at 37°C (ANOVA).

B. Arithmetic means ± SEM (n = 4 different erythrocyte specimens analysed in quadruplicates) of the percentage of annexin V-binding erythrocytes following exposure for 24 h to 37°C - 41°C in the absence (open bars) or presence of 1 µM CysLT1 antagonist cinalukast . *** (P < 0.001) indicates significant difference from values at 37°C (ANOVA). #, ### (P < 0.05, P < 0.001) indicate significant difference from absence of cinalukast (ANOVA).

5.2. Endothelin B receptor stimulation inhibits suicidal erythrocyte death

Fluo3 fluorescence was employed to determine whether endothelin 1 alters erythrocyte Ca^{2+} concentration following incubation in glucose-containing and glucose-free medium. As illustrated in figure 17, removal of glucose was followed by the expected significant increase in Fluo3 fluorescence. More importantly, both endothelin 1 (500 nM; figure 17 A,B) and the ETB agonist sarafotoxin 6c (10 nM; figure 17 C,D) significantly blunted the increase in the cytosolic Ca^{2+} activity following a 48 hours glucose depletion, whereas they had no influence on Fluo3 fluorescence in energy-repleted erythrocytes.
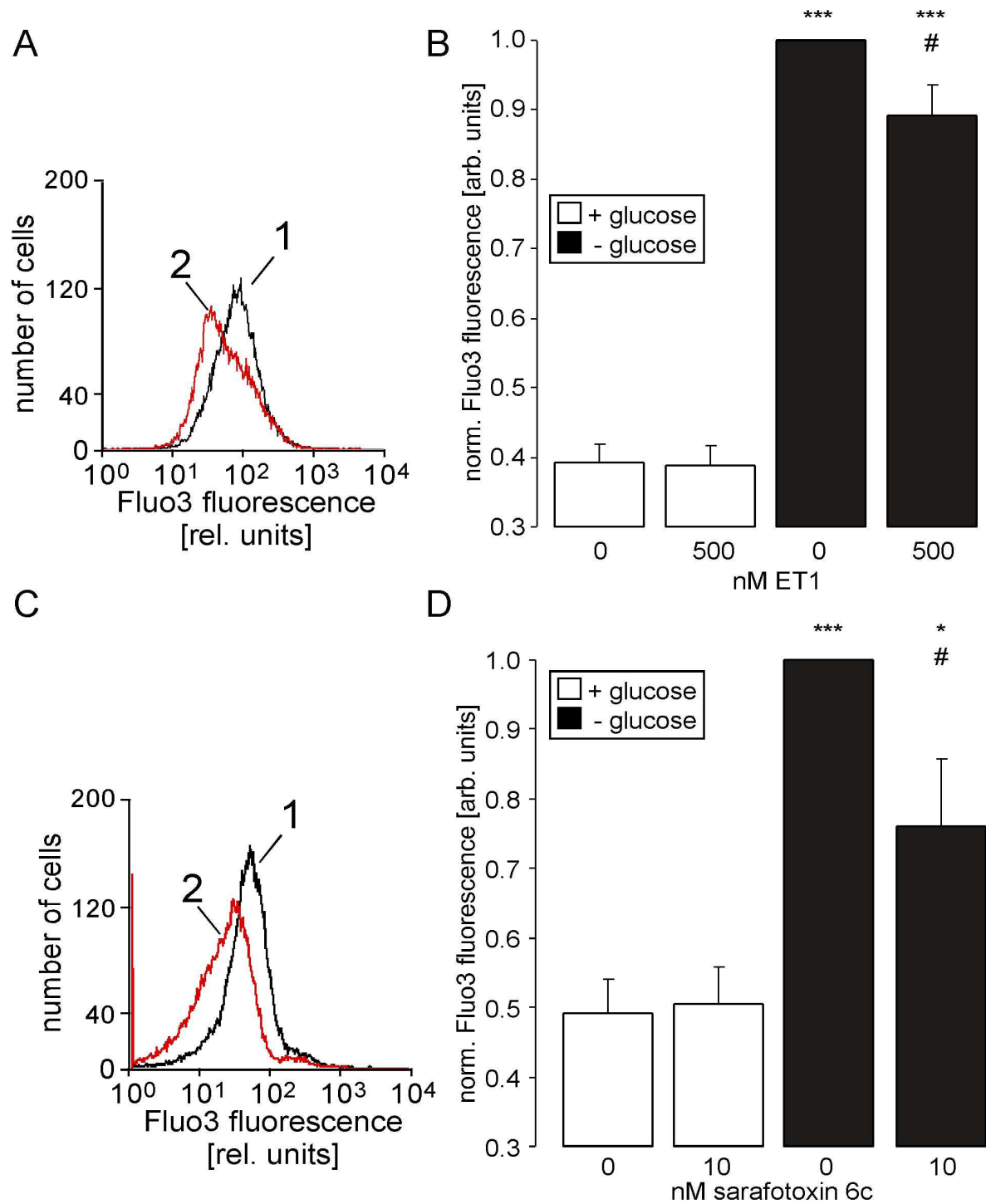
Figure 17. Cytosolic Ca^{2+} concentration in erythrocytes following energy depletion in the absence and presence of endothelin 1 or sarafotoxin 6c

A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 hours to glucose-depleted Ringer without (1) or with 500 nM endothelin 1 (2).

B. Arithmetic means ± SEM (n = 17) of the normalized Fluo3 fluorescence in erythrocytes exposed for 48 hours to isotonic Ringer (left bars) or to glucose-depleted Ringer (right bars) without (0) or with 500 nM endothelin 1. *** indicates significant difference from the presence of glucose (ANOVA, $p < 0.001$). # indicates significant difference from the absence of endothelin 1 (ANOVA, $p < 0.05$)

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C. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 hours to glucose-depleted Ringer without (1) or with (2) 10 nM sarafotoxin 6c.

D. Arithmetic means ± SEM (n = 7) of the normalized Fluo3 fluorescence in erythrocytes exposed for 48 hours to isotonic Ringer (left bars) or to glucose-depleted Ringer (right bars) without (0) or with 10 nM sarafotoxin 6c. *, *** indicate significant difference from the presence of glucose (ANOVA, $p < 0.05$, $p < 0.001$). # indicates significant difference from the absence of sarafotoxin 6c (ANOVA, $p < 0.05$)

An increase in the cytosolic Ca^{2+} activity triggers scrambling of the erythrocyte cell membrane leading to exposure of phosphatidylserine at the erythrocyte surface. Thus, annexin V-binding was employed to identify erythrocytes exposing phosphatidylserine at their surface. Energy depletion was indeed followed by a significant increase in the percentage of annexin V-binding erythrocytes. Neither 500 nM ET1 (Figure 18B) nor 10 nM sarafotoxin 6c (Figure 18D) significantly modified annexin V-binding in the presence of glucose. However, both, 500 nM ET1 (Figure 18A,B) and 10 nM sarafotoxin 6c (Figure 18C,D) significantly blunted the phosphatidylserine exposure following a 48 hours depletion of glucose. The IC_{50} value for ET1 was 99 nM (Figure 18E) and for sarafotoxin 6c 10 nM (Figure 18F).
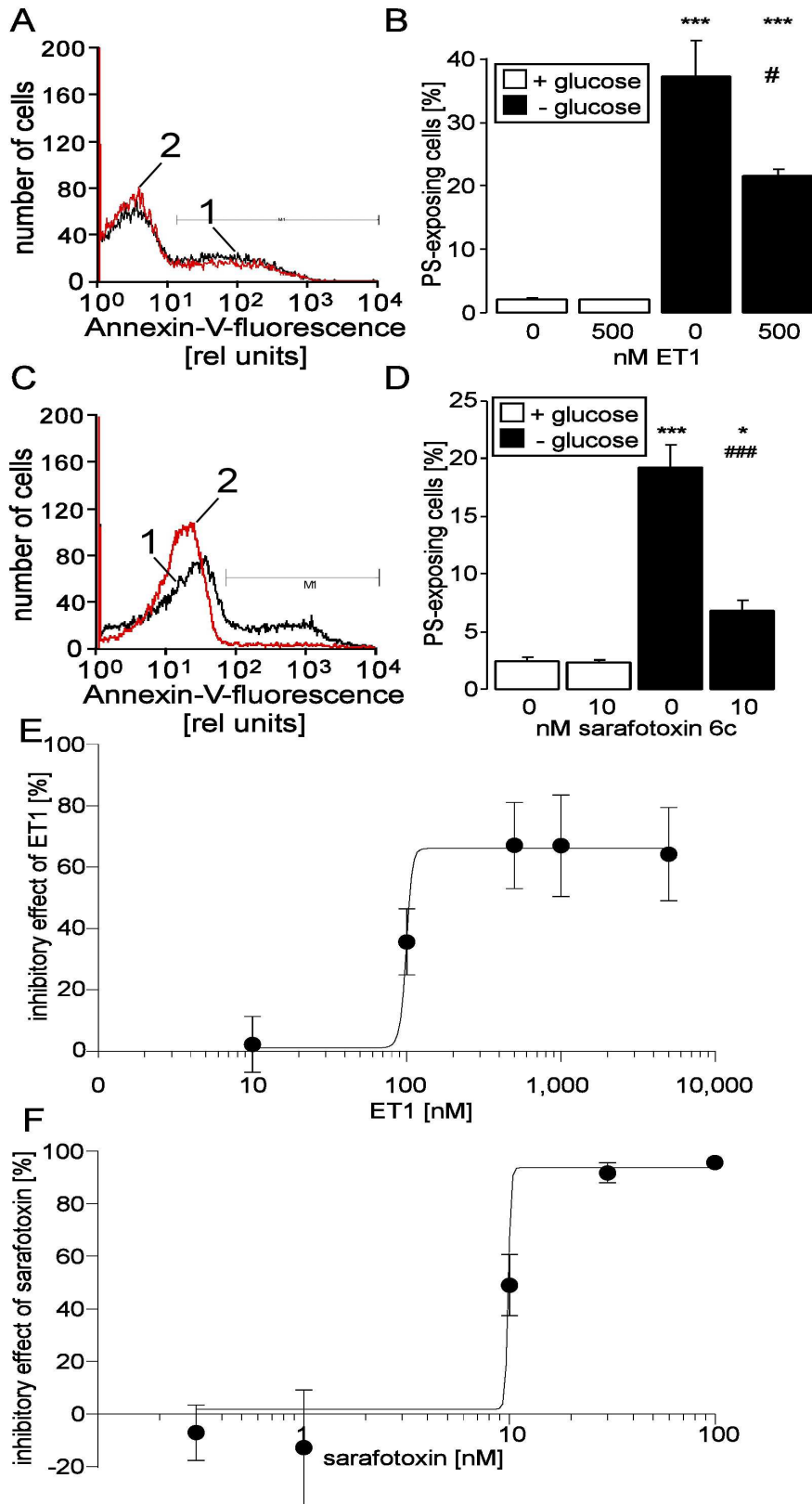
Figure 18. PS exposure of erythrocytes following energy depletion in the absence and presence of endothelin 1 or sarafotoxin 6c.

A. Histogram of annexin V-binding in a representative experiment of erythrocytes exposed for 48 hours to glucose-depleted Ringer without (1) or with 500 nM endothelin 1 (2).

B. Arithmetic means ± SEM (n = 5 erythrocyte specimens were studied in quadruplicates) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to isotonic Ringer (left bars) or to glucose-depleted Ringer (right bars) without (0) or with 500 nM endothelin 1. *** indicates significant difference from the presence of glucose (ANOVA, $p<0.001$). # indicates significant difference from the absence of endothelin 1 (ANOVA, $p<0.05$)

C. Histogram of annexin V-binding in a representative experiment of erythrocytes exposed for 48 hours to glucose-depleted Ringer without (1) or with (2) 10 nM sarafotoxin 6c.

D. Arithmetic means ± SEM (n = 5 erythrocyte specimens were studied in quadruplicates) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to isotonic Ringer (left bars) or to glucose-depleted Ringer (right bars) without (0) or with 10 nM sarafotoxin 6c. *, *** indicate significant difference from the presence of glucose (ANOVA, $p<0.05$, $p<0.001$). ### indicates significant difference from the absence of sarafotoxin 6c (ANOVA, $p<0.001$)

E. Dose-response curve of endothelin 1. Arithmetic means ± SEM (n = 12 erythrocyte specimens) of the relative inhibitory effect of endothelin 1 on energy depletion-induced phosphatidylserine exposure. 100% inhibitory effect were defined as the maximal inhibition observed in the concentration range of endothelin 1 from 10 – 5000 nM.

F. Dose-response curve of sarafotoxin 6c. Arithmetic means ± SEM (n = 12 erythrocyte specimens) of the relative inhibitory effect of sarafotoxin 6c on energy depletion-induced phosphatidylserine exposure. 100% inhibitory effect were defined as the maximal inhibition observed in the concentration range of sarafotoxin 6c from 0.3 – 100 nM.

The in vivo significance of the ETB receptor for erythrocyte survival was investigated by experiments in rescued ETB knockout (etb$^{-/-}$) mice and in wild type mice (etb$^{+/+}$). The expression of both, the ETA and ETB receptor, in membrane preparations of blood from etb$^{-/-}$ and etb$^{+/+}$-mice was studied by Western Blotting. As shown in figure 19A, lower panel, the ETB receptor could indeed be detected in a membrane preparation of blood from etb$^{+/+}$-mice but not from etb$^{-/-}$-mice. The ETA receptor could be readily detected in a membrane preparation of blood from etb$^{+/+}$-mice (Figure 19A, upper panel) most likely due to its expression in leukocytes (Sampaio et al., 2004) but not in a blood membrane preparation from etb$^{-/-}$-mice, a finding, in accordance with a previous study reporting the down-regulation of ETA receptors in ETB receptor-deficient mice (Davenport et al., 2004).

As shown in Fig. 3B, the reticulocyte number was significantly higher in etb-/--mice than in etb$^{+/+}$-mice. The etb$^{-/-}$-mice tended to have a lower erythrocyte count, a difference, however, not reaching statistical significance (Figure 19C). The reticulocytosis in the absence of an enhanced erythrocyte count is suggestive for an

increased erythrocyte turnover and was associated with an increased plasma concentration of erythropoietin (EPO) and of folic acid, whereas the plasma concentration of vitamin B_{12} was not significantly different between the genotypes (Figure 19D-F). The hemolysis at low extracellular osmolarity tended to be slightly more pronounced in etb$^{-/-}$-erythrocytes than in etb$^{+/+}$-erythrocytes, a difference, however, not reaching statistical significance (Figure 19G).
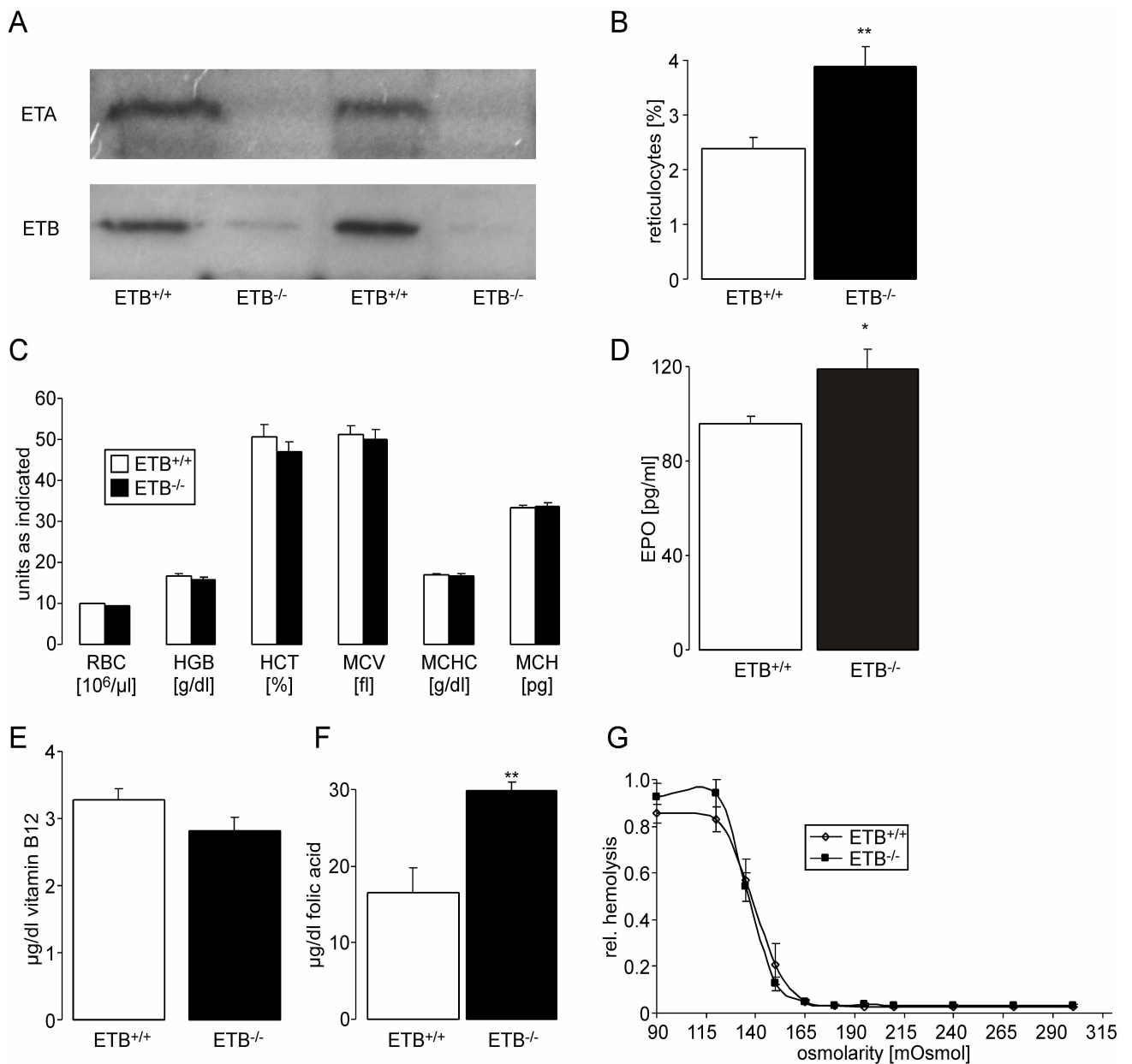

Figure 19. Erythrocyte parameters in etb$^{-/-}$-mice

A. Original Western blots demonstrating the expression of the ETA (upper panel) and ETB (lower panel) receptor protein in two different membrane preparations of blood from rescued ETB knockout (etb$^{-/-}$) and wild type mice (etb$^{+/+}$).

B. Arithmetic means ± SEM of the reticulocyte number (n = 9-10) in blood from rescued ETB knockout mice (etb^{-/-}, black bar) and wild type mice (etb^{+/+}, white bar) * significant difference between genotypes (p<0.05) (t-test).

C. Arithmetic means ± SEM (n = 10) of erythrocyte count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH) in blood from etb^{-/-}-mice (etb^{-/-} black bars) and wild type mice (etb^{+/+}, white bars).

D. Arithmetic means ± SEM of the plasma erythropoietin concentration (n = 8) of etb^{-/-}-mice (etb^{-/-}, black bar) and wild type mice (etb^{+/+}, white bar) * significant difference between genotypes (p<0.05) (t-test).

E. Arithmetic means ± SEM of the plasma vitamin B12 concentration (n = 4) of etb^{-/-}-mice (etb^{-/-}, black bar) and wild type mice (etb^{+/+}, white bar).

F. Arithmetic means ± SEM of the plasma folic acid concentration (n = 4) of etb^{-/-}-mice (etb^{-/-}, black bar) and wild type mice (etb^{+/+}, white bar) * significant difference between genotypes (p<0.05) (t-test).

G. Osmotic resistance of erythrocytes from etb^{-/-} (etb^{-/-}, closed symbols) and wild type etb^{-/-}-mice (etb^{+/+}, open symbols).

Further experiments were performed to explore whether the reticulocytosis and erythrocyte count of etb^{-/-}-mice could be explained by enhanced susceptibility to suicidal erythrocyte death. As shown in Figure 20AB, the percentage of phosphatidylserine-exposing freshly drawn erythrocytes from etb^{-/-}-mice was approximately twice that of erythrocytes from etb^{+/+}-mice.

Oxidative stress is further known to foster suicidal erythrocyte death. Therefore, erythrocytes from etb^{-/-}-mice and etb^{+/+}-mice were exposed to oxidative stress and suicidal erythrocyte death was determined. As shown in Figure 20CD, erythrocytes from etb^{-/-}-mice were significantly more susceptible to the eryptotic effect of oxidative stress. Upon oxidative stress, the increase in intracellular calcium concentration was significantly more pronounced in etb^{-/-}-erythrocytes than in etb^{+/+}-erythrocytes (Figure 20 EF).
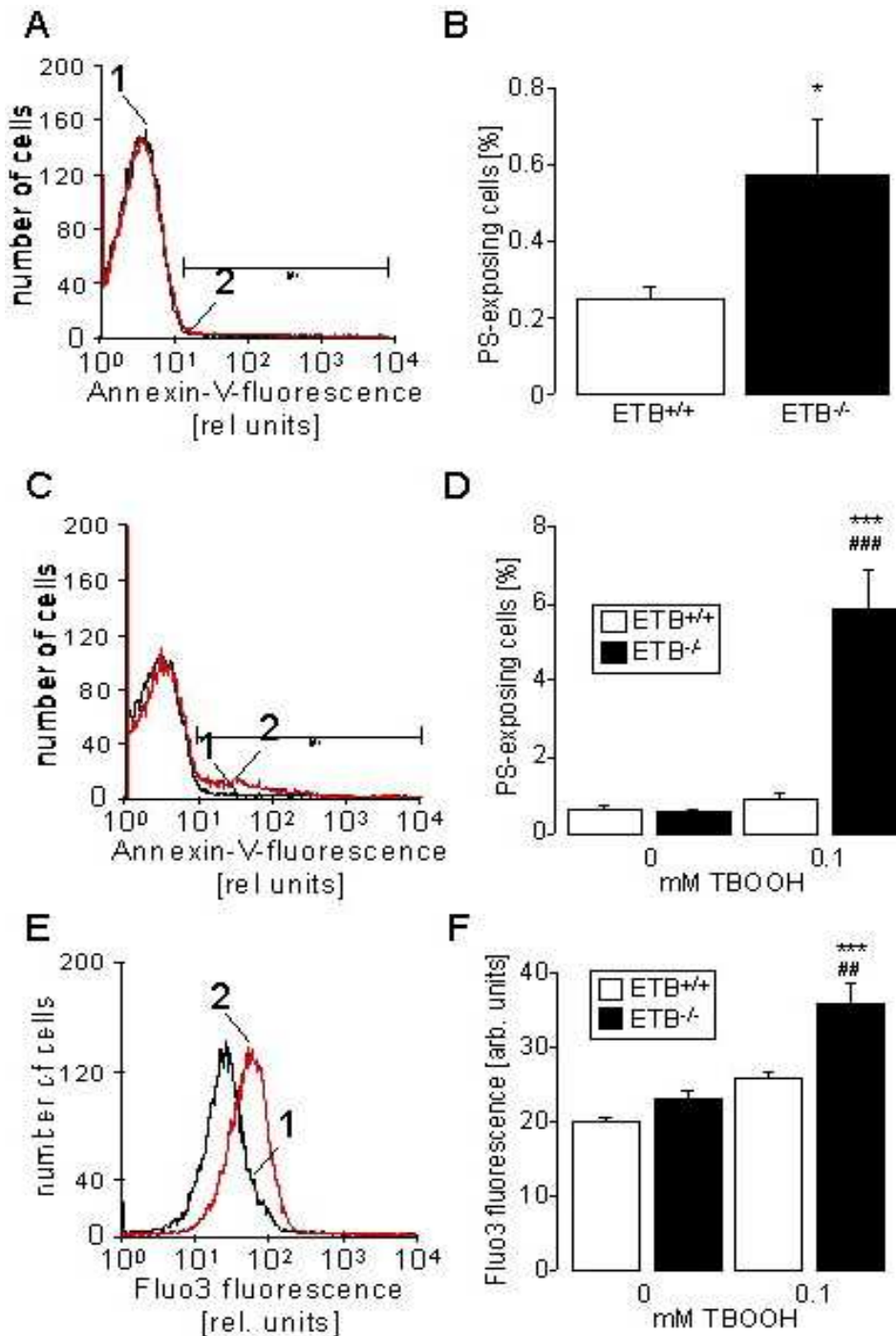
Figure 20. Suicidal erythrocyte death of etb$^{-/-}$ and wild type mice.

A. Histogram of annexin V-binding in a representative experiment of erythrocytes from etb$^{-/-}$-mice (2) and wild type mice (1) immediately stained after retrieval.

B. Arithmetic means ± SEM (n = 9) of the percentage of annexin V-binding erythrocytes from etb$^{-/-}$-mice (etb$^{-/-}$, black bar) and wild type mice (etb$^{+/+}$, white bar) immediately stained after retrieval. * indicates significant difference (p<0.05, t-test).

C. Histogram of annexin V-binding in a representative experiment of erythrocytes from etb$^{-/-}$-mice (2) and wild type mice (1) exposed for 30 min to 0.1 mM tert-butylhydroperoxide.

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D. Arithmetic means ± SEM (n = 9) of the percentage of annexin V-binding erythrocytes from etb^{-/-} mice (etb^{-/-}, black bars) and wild type mice (etb^{+/+}, white bars) exposed for 30 min to 0.1 mM tert-butylhydroperoxide.

E. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from etb^{-/-}-mice (2) and wild type mice (1) exposed for 30 min to 0.1 mM tert-butylhydroperoxide.

F. Arithmetic means ± SEM (n = 9) of the Fluo3 fluorescence of erythrocytes from etb^{-/-}-mice (etb^{-/-}, black bars) and wild type mice (etb^{+/+}, white bars) exposed for 30 min to 0.1 mM tert-butylhydroperoxide.

*** indicates significant difference from presence of glucose (p<0.001, ANOVA).

\#\#, \#\#\# indicate significant difference between genotypes (p<0.01, p<0.001, ANOVA).

The increased susceptibility of etb^{-/-}-mice to suicidal erythrocyte death may affect erythrocyte survival in vivo by enhanced clearance of eryptotic erythrocytes, since phosphatidylserine-exposing erythrocytes are rapidly engulfed and degraded by macrophages. Therefore, erythrocytes from mice of both genotypes were labelled with the fluorescent dye CFSE and reinjected into the same mice. As shown in Figure 21, four days after injection, the percentage of cleared erythrocytes in etb^{-/-}-mice was indeed more than twice as high as in etb^{+/+}-mice.
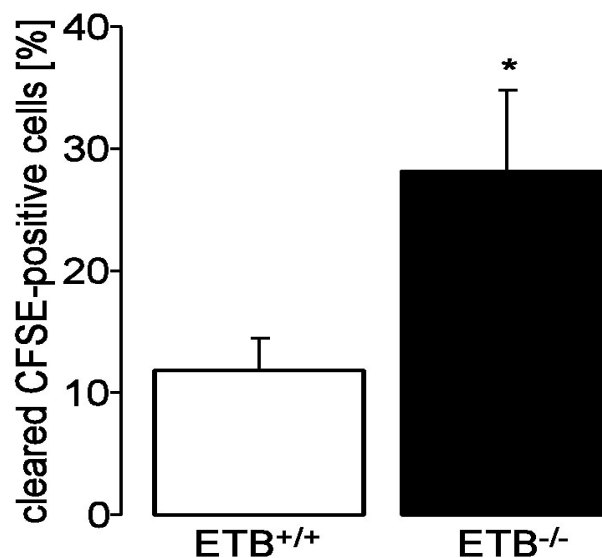

**Figure 21. Accelerated erythrocyte clearance of erythrocytes in etb^{-/-} mice.**

Percentage of cleared CFSE-labeled circulating erythrocytes drawn from etb-/--mice (black bar) and wild type mice (white bar) four days after injection into the same mice. Values are normalized arithmetic means ± SEM (n = 7) of the percentages of CFSE-labeled erythrocytes. * Significant difference between genotypes (p<0.05; t-test).

To investigate the fate of the cleared erythrocytes, the spleens of etb$^{+/+}$-mice and etb$^{-/-}$-mice were investigated. As shown in Figure 22AB, etb$^{-/-}$-mice suffered from pronounced splenomegaly.   Confocal microscopy was utilized to analyze the splenocytes from etb$^{-/-}$-mice and etb$^{+/+}$-mice. Figure 22C shows that five days after injection of the labelled erythrocytes the spleens of the etb$^{-/-}$-mice contained significantly more CFSE-positive phosphatidylserine-exposing erythrocytes than the spleens of the etb$^{+/+}$-mice.
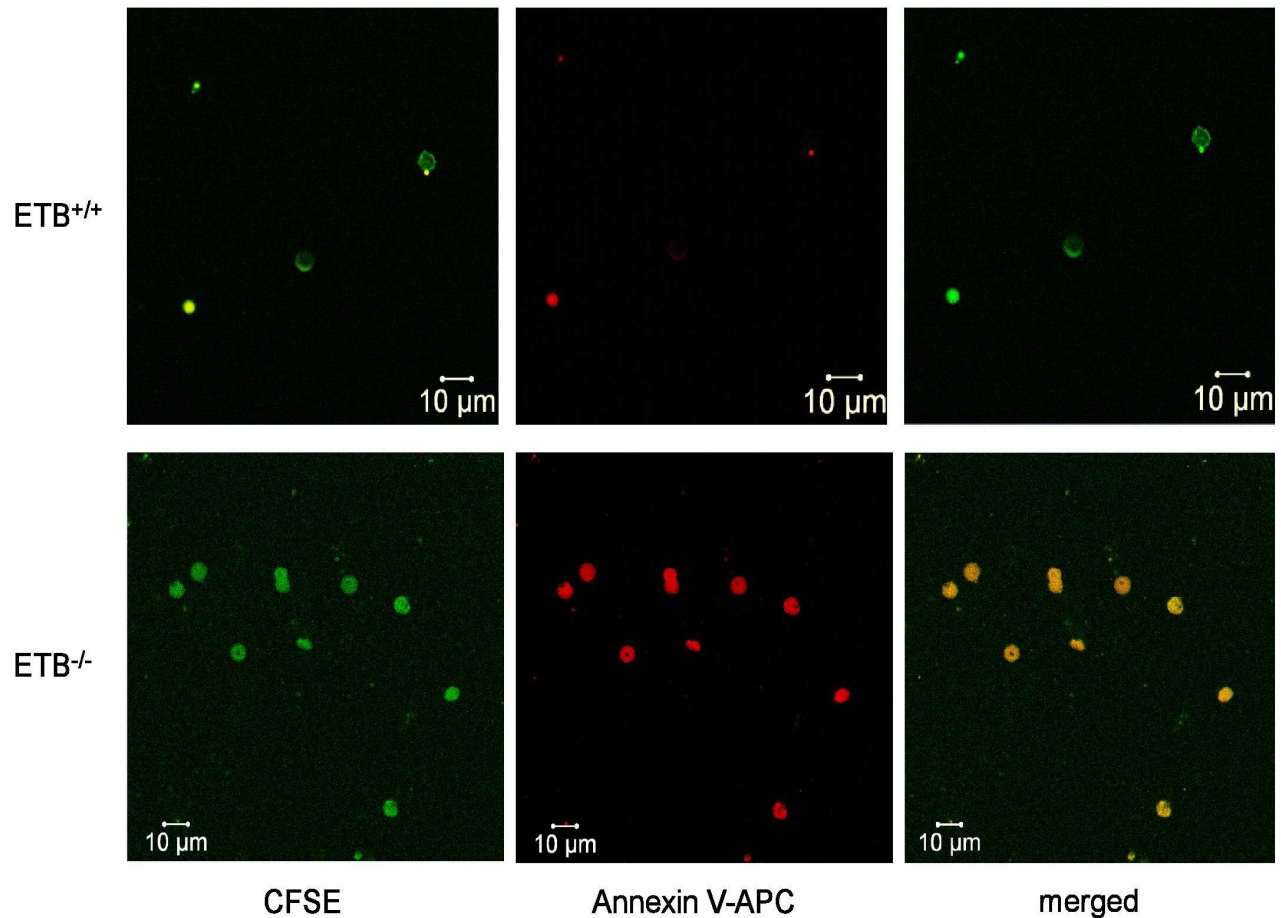
A

ETB$^{+/+}$ ETB$^{-/-}$

B

C

ETB⁺/⁺

ETB⁻/⁻

CFSE   Annexin V-APC   merged
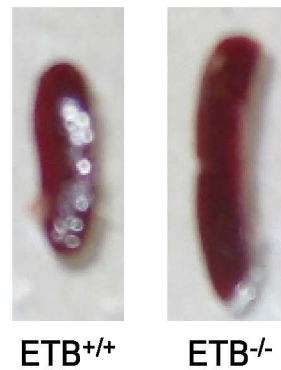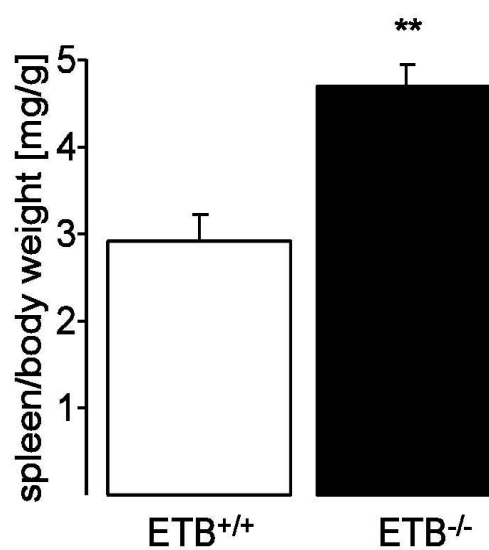
Figure 22. Splenomegaly associated with increased erythroid cell mass in etb$^{-/-}$-mice.

A. Photograph of spleens from etb$^{-/-}$-mice (etb$^{-/-}$, right) and wild type mice (etb$^{+/+}$, left).

B. Arithmetic means ± SEM (n = 5) of the spleen/body weight ratios of etb$^{-/-}$-mice (etb$^{-/-}$, black bar) and wild type mice (etb$^{+/+}$, white bar). ** significant differences between genotypes (p<0.01; t-test).

C. Confocal microscopy of CFSE-dependent (left panels), annexin V-APC (middle panels)-dependent and merged fluorescence (right panels) of erythrocytes from the spleens of etb$^{-/-}$-mice (etb$^{-/-}$, lower panels) and wild type mice (etb$^{+/+}$, upper panels).

A kinetics analysis in vitro revealed that the incubation period at 37℃ in glucose-free solution required to induce PS exposure of 50% of the erythrocytes was 55.4 ± 2.0 h in the absence and significantly increased to 62.5 ± 3.6 h in the presence of 100 nM sarafotoxin 6c (all n = 6; p<0.05). In another series of experiments, the time to induce PS exposure of 50% of the erythrocytes was 59.1 ± 1.2 h without and 64.2 ± 2.4 h in the presence of 500 nM ET1 (all n = 5; p<0.05).

To test, whether stimulation of ETB influences the membrane-scrambling effect of Ca^{2+}, erythrocytes were incubated in Ringer solution in the presence or absence of

500 nM ET1 or 5 nM sarafotoxin 6c for 2 hours and then further incubated for 30 min in the absence or presence of 1 µM Ca^{2+} ionophore ionomycin. As a result, exposure to ionomycin increased the percentage of PS-exposing erythrocytes from $0.64 \pm 0.08\%$ to $25.14 \pm 1.24\%$ in the absence, to $27.25 \pm 1.19\%$ in the presence of 500 nM ET1 and to $24.70 \pm 1.88\%$ in the presence of 5 nM sarafotoxin 6c (n = 3-5 erythrocyte specimens studied in quadruplicates). Another series of experiments aimed to compare the inhibitory potential of ET1 to that of ET2 and ET3. As a result, a 48 hours depletion of glucose resulted in $20.6 \pm 1.7\%$ PS-exposing erythrocytes, an effect attenuated in the presence of 500 nM ET1 ($15.9 \pm 1.1\%$ PS-exposing erythrocytes) but not significantly influenced by 500 nM ET2 ($20.8 \pm 1.7\%$) or ET3 ($21.5 \pm 1.6\%$; all n=4 erythrocyte specimens).

6. Discussion

6.1. Temperature sensitivity of suicidal erythrocyte death

The present study elucidates the effects of temperature on phosphatidylserine exposure and cell volume. These events are concomitantly associated with increased intacellular Ca^{2+} activity, increase in cellular ceramide abundance, and depletion of intracellular ATP, all hallmarks of suicidal erythrocyte death (Lang et al., 2008).

The augmentation of intracellular Ca^{2+} activity leads to cell shrinkage as a result of the activation of Gardos channels (Ca^{2+}-sensitive K^+ channels) (Brugnara et al., 1993) which results in subsequent intracellular K^+ loss, hyperpolarization and exit of Cl^- ions with associated loss of osmotically obliged water (Lang, Gulbins, Lerche, Huber, Kempe, and Foller, 2008). Phosphatidylserine exposure on the cell membrane arises from the ensuing Ca2+-sensitive scrambling of the outer leaflets which characterizes eryptosis (Bratosin et al., 2001). Furthermore, cell membrane scrambling could also be a consequence of cell shrinkage (Schneider et al., 2007).

Cell shrinkage of erythrocytes under hyperthermic conditions may be a consequence of the influence of temperature changes on the transport systems in the cells. However, cell shrinkage paralleled by exposure of phosphatidylserine could result from the eryptotic processes within the cell such as the activation of Ca^{2+}-sensitive K^+ channels. There could be a possible interplay of several factors within the cell that may lead to cell shrinkage. The possible role of Na^+/K^+ ATPase, in temperature-sensitive cell volume changes could give us an insight into mechanisms other than eryptosis (Dhaka et al., 2006; Lang et al., 1998; Liman, 2006; Stewart, 2004; Volkl et al., 1986; Yao et al., 2005). Transmembranal transport proteins and cell surface receptors of cell have an altered function under hyperthermia which affects the ionic gradient in cells, membrane potential and intracellular pH. Besides the compromised functioning of Na^+/K^+ ATPase under hyperthermia, there is also a functional loss of the $HCO3^-/Cl^-$ ATPase. Many of the changes in the transport mechanisms of the cell do not correlate with the rate of cell death in vitro in different cell lines and their effects on apoptosis is not completely understood (Hildebrandt et al., 2002). Furthermore, temperature sensitive reactions are required to sustain metabolism and cell signalling pathways. The complexity in the interplay of several temperature sensitive mechanisms may result in a non-linear relationship between eryptosis and temperature. Temperature-dependent mechanisms may antagonize one another and

this may cause a dissonance in the relationship of temperature and various cell parameters.

The role of temperature in survival of nucleated cells has been studied intensively and there is increasing evidence to speculate the role of temperature in therapeutic induction of apoptosis in cancer cells. Hypothermic conditions have been shown to enhance cell survival by counteracting apoptosis (Fillon et al., 2002; Jamieson et al., 2008). Hyperthermia has been shown to induce apoptosis in many different types of nucleated cells (Han et al., 2008; Yu et al., 2008). Some cell types show different susceptibility to apoptosis triggered by hyperthermia. Above a threshold temperature, they are likely to undergo necrosis instead of apoptosis (Hildebrandt et al., 2002).

Paradoxically, however, it is also possible that hyperthermia may not induce apoptosis in nucleated cells (Sharif-Khatibi et al., 2007). One of the factors that determine this phenomenon is the increased expression of anti-apoptotic, pro-survival proteins, such as heat shock proteins (Calderwood et al., 2008).

It is also known that Ca^{2+} signaling may stimulate cell proliferation thereby conferring cell survival (Berridge, 2005). On the other hand, it has also been shown that sustained increase in intracellular Ca^{2+} may trigger apoptosis in nucleated cells (Orrenius et al., 2003). This pattern is similar to observations made in suicidal death of erythrocytes. Moreover, erythrocytes are known to be devoid of mechanisms that upregulate the expression protective proteins that are exposed to a hypothermic environment. Therefore, it is likely that erythrocytes exhibit differences in their pattern to succumb to apoptosis than nucleated cells. They are uniformly stimulated by increased Ca^{2+} entry into the cell to undergo eryptosis (Lang et al., 2005).

From the present study, hemolysis is also stimulated by hyperthermia in addition to eryptosis. Hemolysis, unlike eryptosis, however, has been found to be triggered at higher temperatures (>41℃), and nearly no increase below. This is in contrast to hyperthermia-induced eryptosis which shows significant phosphatidylserine exposure at temperatures slightly higher than the physiological temperature. It may be worth mentioning that under conditions of excessive energy consumption and increased heat dissipation such as strenuous exercise, temperatures are likely to increase and trigger eryptosis although hemolysis may occur only beyond temperature 41 ℃. This is applicable to body tissues with high energy turnover where temperatures could exceed 37℃.

Besides stimulating cell membrane scrambling and decreasing cell volume, increase in intracellular Ca^{2+} activity influences the morphological basis of the cytoskeleton (Nunomura et al., 1997). Several enzymes are activated and these include transglutaminase (Anderson et al., 1977), phospholipases (Allan et al., 1976), calpain (Anderson, Davis, and Carraway, 1977), protein kinases, and phosphatases (Minetti et al., 1996). Calpain causes the degradation of proteins in the cell membrane and could lead to cell membrane blebbing which is an important characteristic of suicidal erythrocyte death (Bratosin, Estaquier, Petit, Arnoult, Quatannens, Tissier, Slomianny, Sartiaux, Alonso, Huart, Montreuil, and Ameisen, 2001).

Apoptotic erythrocytes are taken up by macrophages that express phosphatidylserine receptors (Boas et al., 1998) and subsequently undergo rapid elimination from the circulating blood (Kempe et al., 2006). It has been shown that excessive eryptosis may be involved in the pathogenesis of clinical anemia and therefore it is likely that hyperthermia *in vivo* may predispose the body to anemia.

A strong relationship between a compromised microcirculation and excessive eryptosis has been suggested in previous studies. Phosphatidylserine exposing erythrocytes are increasingly predisposed to adherence to the vascular wall and this mechanism may be implicated in the derangement of the microcirculation (Closse et al., 1999).

PGE_2 is believed to be a proximal mediator of hyperthermia in the body. PGE_2 has been shown to be a key player in eryptosis. PGE_2 is known to activate eryptosis-inducing cation channels and the Gardos channel responsible for cell shrinkage. Hyperosmotic shock and Cl^- removal trigger the release of PGE_2. Furthermore, eryptosis induced by Cl^- removal is blunted by COX inhibitors like acetyl salicyclic acid and diclophenac. All these evidence point to the important role of PGE_2 in eryptosis (Lang et al., 2005d; Lang et al., 2005e). It could thus be speculated, that the close interaction between PGE_2, hyperthermia and eryptosis may enable us to understand the mechanisms of anemia and excessive eryptosis in pyrexia-related disorders.

The O_2 affinity of haemoglobin is lowered by higher temperatures (Slonim et al., 1987) and this enhances CO_2 production which at least theoretically may predispose erythrocytes to oxidative stress and consequently eryptosis (Zappulla, 2008).

In the present study, it is shown that 1µM of the CysLT1 receptor antagonist, cinalukast was able to inhibit the phosphatylserine exposure induced by

hyperthermia. There is a striking similarity in the physiological inhibitory effect cinalukast to the anti-inflammatory steroid cortisol on prostaglandins and leukotriene formation (Ganong, 1995).

It is believed that increased eryptosis and enhanced phosphatidylserine exposure *in vivo* may activate macrophages to release pro-inflammatory cytokines such as Il-6 and TNF-α which, in turn, increase both prostaglandin formation (Ganong, 1995) and cortisol secretion (Zappulla, 2008). Cortisol may in turn down regulate prostaglandin formation (Ganong, 1995).

Hyperthermia-induced eryptosis may play a key role in the pathophysiology of pyrexic conditions like sepsis (Kempe et al., 2007) and malaria (Brand et al., 2008;Foller et al., 2009b;Koka et al., 2008b;Koka et al., 2008a;Koka et al., 2009;Lang et al., 2009). According to cross-sectional studies, there is a very important relationship between the prevalence of anemia and fever during malarial epidemics (Takem et al., 2010), a fact which strongly suggests the role of hyperthermia-induced eryptosis in anemia concomitant to clinical malaria.

The malarial parasite may destroy the host cell even when suicidal death of the host erythrocytes does not occur. Suicidal death of infected erythrocytes, however, could partially precede the haemolytic events induced by the malarial pathogen (Foller, Bobbala, Koka, Huber, Gulbins, and Lang, 2009b). The subsequent phagocytosis of the infected cells in malaria leads to the removal of the pathogen prior to its invasion of new erythrocytes thus preventing increased parasitemia in the circulation. It could be concluded that fever in malaria may serve to enhance clearance of infected erythrocytes and thus favourably influence parasitemia (Foller et al., 2009a).

Eryptosis may be a feature of several clinical disorders like iron-deficiency (Kempe, Lang, Duranton, Akel, Lang, Huber, Wieder, and Lang, 2006), Hemolytic Uremic Syndrome (Lang et al., 2006), Wilson's disease (Lang et al., 2007) and several hemoglobinopathies (Lang, Gulbins, Lerche, Huber, Kempe, and Föller, 2008; Kuypers, 2007). Moreover several xenobiotics and endogenous mediators stimulate eryptosis (Lang, Gulbins, Lerche, Huber, Kempe, and Foller, 2008;Lui et al., 2007;Mahmud et al., 2009;Niemoeller et al., 2008b;Niemoeller et al., 2008a;Sopjani et al., 2008a;Sopjani et al., 2008b;Wang et al., 2008). The effects of these xenobiotics on the suicidal erythrocyte death could be exacerbated in the presence of hyperthermia.

In conclusion, hyperthermia stimulates suicidal erythrocyte death or eryptosis, which in turn may lead to anemia and to derangements of microcirculation. The enhanced eryptosis may thus contribute to the pleotropic effects of hyperthermia.

6.2. Endothelin B receptor stimulation inhibits suicidal erythrocyte death

In the present study a completely novel function of endothelin is unravelled, that is, its role in the regulation of erythrocyte survival. The present results point to the amelioration of suicidal erythrocyte death in *in vitro* conditions under the influence of endothelin-1. *In vitro* findings are further confirmed by the enhanced susceptibility of erythrocytes from etb$^{-/-}$-mice compared to wild type etb$^{+/+}$-mice. Previous studies (Duranton et al., 2002; Klarl et al., 2006) have shown that energy depletion from the medium (removal of glucose from Ringer solution) triggers cell membrane scrambling which in turn stimulates phosphatidylserine exposure at the surface of the erythrocyte membrane, as a result of augmented intracellular Ca^{2+} activity (Berg et al., 2001;Bratosin, Estaquier, Petit, Arnoult, Quatannens, Tissier, Slomianny, Sartiaux, Alonso, Huart, Montreuil, and Ameisen, 2001;Lang et al., 2003). Under isotonic and glucose-replete extracellular environment, there was neither significant modification of cytosolic Ca^{2+} activity nor exposure of phosphatidylserine on the cell surface with the addition of endothelin 1 and sarafotoxin 6c. However, there was significant inhibition of both intracellular Ca^{2+} activity and phosphatidylserine exposure on the cell surface on energy depletion by withdrawal of glucose. This indicates the blunting effect of both endothelin and sarafotoxin 6c on suicidal erythrocyte death in vitro. (Rivera et al., 1999) previously described the presence of the ETB receptors in murine erythrocytes although they showed the absence of ETA receptors in erythrocyte progenitor cells. ETB receptors were absent in erythrocytes of etb$^{-/-}$-mice but were expressed in erythrocytes of etb$^{+/+}$-mice and this was substantiated in the present study using western blot analysis. However, ETA receptor expression was also observed in blood samples from etb$^{+/+}$-mice. The likely explanation for this would be the presence of leukocyte contaminations in the blood preparations from etb$^{+/+}$-mice. Furthermore, (Davenport et al., 2004) have shown the expression of ETA receptors in leukocytes. It may be worth mentioning that the deficiency in ETB receptors, at least in theory, does not cause an up-regulation of ETA receptors but a down-regulation in the expression of ETA receptor which could be explained by the high levels of endothelin 1 in etb$^{-/-}$-mice (Quaschning et al., 2005;Davenport and Kuc, 2004).

In previous studies, endothelin has been described to play an unfavourable role in cancer progression by the attenuation of apoptosis in different forms of cancer cell lines. It has been shown to modulate apoptosis in human smooth muscle cells antagonising chemically-induced apoptotic stimuli. It has been observed that endothelin 1 activates phosphatidylinositol 3-kinase and mitogen-activated protein kinase in human pericardial smooth muscle cells and both are shown to play a role in the modulation of apoptosis by insulin-like growth factor-1. Endothelin 1 may thus modulate apoptosis in nucleated cells through this signaling pathway (Wu-Wong et al., 1997). Endothelin 1 has been shown to be a survival factor for rat colon carcinoma cells against FasL-mediated apoptosis. The attenuation of apoptosis in colon cancer cells by endothelin 1 involves the protein kinase C signaling pathway but is independent of the ceramide pathway (Eberl et al., 2000).

Endothelin 1 acts as a survival factor from apoptosis through the ETB receptors in rat endothelial cells by an autocrine and paracrine mechanism. In endothelial cells, endothelin 1 fosters the phosphoinositide breakdown for the activation of protein kinase C resulting from the generation of diacylglycerol in endothelial cells suggesting a crucial role of protein kinase C in the antiapoptotic effects of endothelin 1 (Shichiri et al., 1997). The mitogenic effect of endothelin 1 is responsible for the growth of several human cancer cell lines *in vitro* like ovarian, prostate, Kaposi's sarcoma, and melanoma cells. These effects are modulated by either ETA or ETB receptors alone or together (Grant et al., 2003).

Suicidal erythrocyte death can be blunted by nitric oxide and nitric oxide donors are considered to be very potent inhibitors of suicidal erythrocyte death (Nicolay et al., 2007b; Koka et al., 2008b). Nitric oxide may exert its effectiveness through the activation of cGMP-dependent kinase. Mice lacking cGK1 have a severe phenotype of anemia and enlarged spleen (Foller et al., 2008a). Nitric oxide synthase activity could be enhanced by endothelin through the mediation of ETB receptors and this atleast in theory could modulate suicidal erythrocyte death.

The present study is strongly suggestive of the role of endothelin receptor signaling in the modulation of programmed cell death of erathrocytes, thus, playing a protective role against anemia. Apoptotic erythrocytes expose phosphatidylserine on their cell surfaces and consequently bind to phosphatidylserine receptors on macrophages (Fadok et al., 2000). These macrophages engulf the apoptotic erythrocytes and trigger their degradation (Boas, Forman, and Beutler, 1998). Furthermore, these

events lead to a rapid clearance of phosphatidylserine exposing erythrocytes from the circulation (Kempe, Lang, Duranton, Akel, Lang, Huber, Wieder, and Lang, 2006).

Theoretically, the suppression of suicidal erythrocyte death and the clearance of erythrocytes from the circulation may characterize the anti-apoptotic effect of endothelin. In the present study, suicidal erythrocyte death has been shown to be enhanced in etb$^{-/-}$-mice. The etb$^{-/-}$-mice, showed increased percentage of reticulocytes which indicates enhanced formation of new erythrocytes. But despite the increased percentage of reticuolcytes, the erythrocyte number was relatively lower in in etb$^{-/-}$-mice. These observations enable us to conclude that the lifespans of circulating erythrocytes was decreased in etb$^{-/-}$-mice. Spleen analysis of both etb$^{+/+}$ and etb$^{-/-}$-mice, revealed that the spleens of etb$^{-/-}$-mice were significantly larger than their wild type counterparts, strongly suggesting the decreased erythrocyte life span, enhanced clearance from the circulation and increased splenic erythrocyte sequestration. In the present study, *in vivo* clearance of labelled-erythrocytes from the circulation was enhanced in etb$^{-/-}$-mice than in etb$^{+/+}$-mice suggesting the presence of increased apoptotic erythrocytes in the circulation at a given time. These labelled- apoptotic-erythrocytes were indeed greater in the spleens of etb$^{-/-}$-mice than in etb$^{+/+}$-mice as indicated in confocal immunofluorescence microscopy of the spleen. The plasma concentrations of erythropoietin in etb$^{-/-}$-mice were higher than in etb$^{+/+}$-mice which suggest enhanced erythropoiesis to compensate for augmented eryptosis and increased clearance of erythrocytes from the circulation.

The pathophysiological role of eryptotic erythrocytes in the microcirculation is substantiated by the fact that phosphatidylserine-exposing erythrocytes have an increased propensity to adhere to the vascular wall (Andrews et al., 1999; Pandolfi et al., 2007). This may stimulate blood clotting and further compromise the microcirculation (Chung et al., 2007; Zappulla, 2008). In contrast, endothelin may counteract the adhesion of erythrocytes to the vascular wall by activating the ETB receptors and inhibiting eryptosis which may contribute to the impediment of the microcirculation. The effects of endothelin on erythrocytes by modulating eryptosis could thus contribute to the known array of effects on the microcirculation by endothelin.

Endothelin 1 is considered to be one the most potent endogenous vasoconstrictor in humans. The pharmacological effect of endothelin 1 on the blood vessel is through

its mediation on the underlying layer of vascular smooth muscle cells (VSMCs) through ETA receptors. These receptors cause potent vasoconstriction and additionally, they have shown to induce increased proliferation of VSMC. In these lines, ET1 receptor blockers have been shown to decrease blood pressure (Shaw et al., 2005). In contrast to the primary function of vasoconstriction, ET1 can also induce vasodilatation by acting on ETB receptors present on endothelial cells by enhancing the production of NO (Sarafidis et al., 2007). Therefore, two opposite pharmacological roles in vascular tone are mediated by different mechanisms of endothelin 1.

The release of pro-inflammatory cytokines is triggered by the uptake of phosphatidylserine exposing erythrocytes by macrophages. The pro-inflammatory cytokines stimulate the release of glucocorticoids and thus foster the development of metabolic syndrome and associated oxidative stress (Zappulla, 2008). The expression of ETA and ETB receptors is downregulated by glucocortioids which has been shown in an animal model of glucocorticoid-induced hypertension (Villeneuve et al., 2000).

Disturbances in microcirculation may be mediated by systemic inflammation and consequent adhesion of erythrocytes to the vascular wall. Endothelin may activate ETB receptors and counteract these changes in the microcirculation. Insulin-mediated glucose uptake can be inhibited by chronic administration of endothelin 1 and therefore may facilitate the development of metabolic syndrome (Sarafidis and Bakris, 2007).

Storage of erythrocytes is compromised by oxidative stress and the life span of stored erythrocytes is markedly decreased by the ensuing suicidal erythrocyte death. Endothelin may play an important role in the modulation of the survival of stored erythrocytes susceptible to enhanced suicidal erythrocyte death by influencing their lifespan (Kriebardis et al., 2007; Lion et al., 2010).

In conclusion, the present study provides *in vitro* and *in vivo* evidence for an inhibitory effect of endothelin on suicidal erythrocyte death and thus discloses a completely novel function of endothelin and its receptor ETB.

7. Summary

Suicidal erythrocyte death is regulated by a wide variety of factors such as systemic diseases, endogenous mediators, and xenobiotics. The hallmarks of eryptosis include cell shrinkage, cell membrane blebbing and phospholipid cell membrane scrambling with consequent phosphatidylserine exposure at the cell surface. It is triggered by increased cytosolic Ca^{2+} activity on one hand and ceramide formation on the other. Besides, the clinical relevance of eryptosis in anemia, it has been shown to be of benefit in limiting the plasmodial activity in malaria and a physiological means of averting hemolysis.

In the first section of the present study, the effect of temperature on erythrocyte survival *in vitro* was elucidated. Fever and hyperthermia are frequently associated with anemia. In most clinical conditions they are considered as two mutually independent clinical consequences of a common cause. The present study, aims to unravel the role of excessive suicidal erythrocyte death as a cause of anemia in pyrexia related disorders. To this end, annexin V-binding was used to determine PS-exposure, forward scatter to measure cell volume, Fluo3 fluoresence to estimate cytosolic Ca^{2+} activity, and binding of fluorescent antibodies to determine ceramide abundance using FACS analysis. A luciferin-luciferase based assay was used to measure the cytosolic ATP concentrations. Graded hyperthermic conditions from 37℃ to 41℃ decreased forward scatter, stimulated annexin V-binding of human erythrocytes which was accompanied by increased cytosolic Ca^{2+} activity, decreased cellular ATP content and a moderate increase in ceramide formation. Hyperthermia-triggered increased annexin V-binding was significantly abrogated by the leukotriene receptor CysLT1 antagonist, cinalukast (1 µM). This study reveals that hyperthermia potentiates suicidal erythrocyte death and theoretically be one of the causes of anemia in pyrexia related disorders.

In the second section, the role of endothelin B receptor stimulation in erythrocyte survival was studied. Endothelins are potent peptides with diverse physiological functions. They are known to stimulate nitric oxide formation which protects against suicidal erythrocyte death. The present study explores whether the ET1-receptor, ETB, influences suicidal erythrocyte death. To this end, cytosolic Ca^{2+} activity and phosphatidylserine exposure were determined using Fluo-3 fluorescence and annexin V-binding respectively, in FACS analysis. Energy depletion increased

cytosloic Ca^{2+} activity, and phosphatidylserine exposure, effects that were significantly blunted by ET-1 and the ETB receptor-agonist sarafotoxin 6c but not by ET-2 and ET-3. ET-1 and sarafotoxin 6c significantly delayed the kinetics of suicidal erythrocyte death following energy depletion. ETB stimulation did not blunt the effect of the Ca^{2+} ionophore ionomycin (1 µM) on phosphatidylserine-exposure. The *in vivo* significance was tested using rescued ETB-knockout (etb$^{-/-}$) and wildtype (etb$^{+/+}$) mice. The number of phosphatidylserine-exposing erythrocytes, reticulocytes and spleen size were significantly larger in etb$^{-/-}$-mice than in etb$^{+/+}$-mice. The etb$^{-/-}$-erythrocytes were more susceptible to the effect of oxidative stress and more rapidly cleared from the circulating blood than etb$^{+/+}$-erythrocytes. Finally, the spleens from etb$^{-/-}$-mice were enlarged and contained markedly more phosphatidylserine-exposing erythrocytes than spleens from etb$^{+/+}$-mice. These observations, thus disclose a novel anti-eryptotic function of ET1.

Zusammenfassung

Der suizidale Erythrozytentod wird durch eine große Vielfalt von Faktoren wie systemischen Erkrankungen, endogenen Botenstoffen und Xenobiotika reguliert. Kennzeichen der Eryptose sind u.a. Zellschwund, Ausknospung der Zellmembran („Blebbing") und als Folge der Umlagerung von Phospholipiden der Zellmembran („Scrambling") das Erscheinen von Phosphatidylserin an der Zelloberfläche. Dies wird zum einen durch erhöhte cytosolische Ca^{2+} Konzentration, zum anderen durch Bildung von Ceramid ausgelöst. Neben der klinischen Relevanz der Eryptose für Anämien konnten günstige Wirkungen derselben bei der Begrenzung der Auswirkung von Plasmodien bei Malaria und bei der Abwendung schädlicher Folgen der Hämolyse gezeigt werden.

Im ersten Abschnitt der vorliegenden Arbeit wurde der Einfluss der Temperatur auf das Überleben von Erythrozyten untersucht. Fieber und erhöhte Temperatur sind häufig mit Anämie verbunden. Im Rahmen der meisten klinischen Zustände werden sie als voneinander unabhängige klinische Folgen einer gemeinsamen Ursache betrachtet. Die vorliegende Arbeit zeigt übermäßigen suizidalen Erythrozytentod als eine Ursache von Anämie bei Erkrankungen, die mit erhöhter Körpertemperatur einhergehen. Zu diesem Zweck wurden die Annexin V- Bindung zur Bestimmung der Phosphatidylserin-Exposition, das Signal des Vorwärtsstreu-lichtes in der Durchflusszytometrie zur Messung des Zellvolumens, Fluo3-Fluoreszenz zur Einschätzung der cytosolischen Ca^{2+} Konzentration und die Bindung fluoreszie-render Antikörper zur Bestimmung von Ceramid mithilfe der Durchflusszytometrie eingesetzt. Mithilfe eines auf Luciferin-Luciferase basierenden Tests wurde die cytosolische ATP-Konzentration gemessen. Abgestufte Erhöhung der Temperatur auf Werte zwischen 37℃ und 41℃ führte zu einer Ve rminderung des Zellvolumens, zu einer Erhöhung der Annexin V-Bindung humaner Erythrozyten zusammen mit einem Anstieg der cytosolischen Ca^{2+}- und Abfall der ATP- Konzentration sowie einem leichten Anstieg der Bildung von Ceramid. Die durch erhöhte Temperatur ausgelöste Annexin V-Bindung wurde signifikant durch Cinalukast, einen Antagonisten zum Leukotrienrezeptor CysLT1, vermindert. Diese Arbeit zeigt, dass erhöhte Temperatur suizidalen Erythrozytentod begünstigt und als eine Ursache von Erkrankungen, die mit erhöhter Körpertemperatur einhergehen, in Betracht gezogen werden sollte.

Im zweiten Teil der Studie wurde untersucht, welchen Einfluss die Stimulation des Endothelin B-Rezeptors auf Erythrozyten nimmt. Endotheline sind wirkungsstarke vom Endothel freigesetzte Peptide mit zahlreichen physiologischen Funktionen. Es ist bekannt, dass sie die Bildung von Stickstoffmonoxid anregen, was wiederum vor suizidalem Erythrozytentod schützt.

Die vorliegende Arbeit untersuchte, ob der ET1-Rezeptor ETB den suizidalen Erythrozytentod beeinflusst. Zu diesem Zweck wurden die cytosolische Ca^{2+} Konzentration und Exposition von Phosphatidylserin mittels Durchflusszytometrie anhand von Fluo-3 Fluoreszenz bzw. Annexin V-Bindung bestimmt. Energieentzug führte zu erhöhter cytosolischer Ca^{2+} Konzentration und Expression von Phosphatidylserin. Beide Effekte wurden durch Endothelin-1 und den Endothelin B-Rezeptoragonisten Sarafatoxin 6c signifikant verringert, jedoch nicht durch ET-2 und ET-3. ET-1 und Sarafatoxin 6c verzögerten signifikant den zeitlichen Verlauf des auf Energieentzug folgenden suizidalen Erythrozytentodes. Stimulierung von ETB verminderte nicht die durch das Calciumionophor Ionomycin (1µM) ausgelöste Phosphatidylserin-Exposition. Die *in vivo* Bedeutung wurde an ETB-transgenen- (etb$^{-/-}$) und Wildtyp (etb$^{+/+}$)-Mäusen geprüft. Die Anzahl an Phosphatidylserin-exponierenden Erythrozyten, Retikulozyten sowie die Größe der Milz waren in etb$^{-/-}$-Mäusen signifikant höher als in etb$^{+/+}$-Mäusen. etb$^{-/-}$-Erythrozyten waren anfälliger für die Auswirkungen von oxidativem Stress und wurden schneller aus dem Blutkreislauf entfernt als etb$^{+/+}$-Erythrozyten. Die Milzen von etb$^{-/-}$-Mäusen waren vergrößert und enthielten signifikant mehr Phosphatisylserin exponierende Erythrozyten als die Milzen der etb$^{+/+}$-Mäuse. Diese Beobachtungen enthüllen eine neuartige antieryptotische Wirkung von Endothelin-1.

8. References

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9. Acknowledgements

10. Curriculum vitae

Personal information

Full name: Syed Minnatullah Qadri

Sex: Male

Nationality: Indian

Date of birth: 6th July, 1982

Place of birth: Hyderabad, India

Education

- ♦ May, 1998: Central board of secondary education examination, India

- ♦ March, 2000: State board of Intermediate Education examination Andhra Pradesh, India

- ♦ October, 2006: Diploma de licenta.
Facultatea de medicina si farmacie,
Universitatea din Oradea, Romania.

- ♦ January, 2007: Resident in cardiology and internal medicine,
Bihor county hospital, Romania

- ♦ June, 2008: Doctoral student, Institut für Physiologie,
Medizinische Fakultät, Eberhard Karls Universität
Tübingen. Germany

11. List of publications

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