Endothelin B receptor stimulation inhibits suicidal erythrocyte death

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ABSTRACT **Endothelins (ETs), potent endothelium-derived mediators, stimulate formation of nitric oxide, which, in turn, protects against suicidal erythrocyte death or eryptosis, characterized by phosphatidylserine exposure at the erythrocyte surface and triggered by increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$). The present study explored whether the ET1-receptor ETB influences suicidal erythrocyte death. To this end, $[Ca^{2+}]_i$ (Fluo3-fluorescence) and phosphatidylserine exposure (annexin V-binding) were determined utilizing FACS analysis. Energy depletion increased $[Ca^{2+}]_i$ and phosphatidylserine-exposure, effects significantly blunted by ET1 ($IC_{50} \approx 100$ nM) and the ETB receptor-agonist sarafotoxin 6c ($IC_{50} \approx 10$ nM) but not by ET2 and ET3. ET1 and sarafotoxin significantly delayed the kinetics of suicidal erythrocyte death following energy depletion. ETB stimulation did not blunt the effect of Ca^{2+}-ionophore ionomycin (1 μM) on phosphatidylserine exposure. The *in vivo* significance was tested using rescued ETB-knockout ($etb^{-/-}$) and wild-type ($etb^{+/+}$) mice. The number of phosphatidylserine-exposing erythrocytes, of reticulocytes and spleen size were significantly larger in $etb^{-/-}$ mice than in $etb^{+/+}$-mice. The $etb^{-/-}$ erythrocytes were more susceptible to the eryptotic effect of oxidative stress and more rapidly cleared from 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. The observations disclose a novel function of ET1, *i.e.*, protection from suicidal erythrocyte death.—Föller, M., Mahmud, H., Qadri, S. M., Gu, S., Braun, M., Bobbala, D., Hocher, B., Lang, F. Endothelin B receptor stimulation inhibits suicidal erythrocyte death. *FASEB J.* 24, 3351–3359 (2010). www.fasebj.org**

Key Words: anemia · cell membrane scrambling · splenomegaly · ETB receptor · Ca^{2+} · sarafotoxin 6c

The suicidal death of erythrocytes, or eryptosis (1), is characterized by cell shrinkage and cell membrane scrambling, leading to exposure of phosphatidylserine at the erythrocyte surface (2–5). The cell membrane scrambling is stimulated by cytosolic Ca^{2+} (2, 4), which may enter erythrocytes through Ca^{2+}-permeable cation channels (6–8). Ca^{2+} further activates Ca^{2+}-sensitive K^+ channels (9, 10), leading to the exit of KCl with osmotically obliged water and thus to cell shrinkage (11). Suicidal erythrocyte death or eryptosis has similarities to erythrocyte senescence, which involves binding of autologous IgG followed by recognition and removal through phagocytosis (12).

Several anemic conditions are paralleled by accelerated suicidal erythrocyte death, such as iron deficiency (13), phosphate depletion (14), hemolytic uremic syndrome (15), sepsis (16), malaria (17, 18), Wilson's disease (19), sickle cell disease (20–27), thalassemia (20, 24, 25, 28, 29), and glucose-phosphate dehydrogenase deficiency (25, 30). Moreover, suicidal erythrocyte death is stimulated by a wide variety of endogenous mediators and xenobiotics (31–37). Triggers of suicidal erythrocyte death further include energy depletion by withdrawal of glucose, which is known to increase the cytosolic Ca^{2+} activity (38).

Suicidal erythrocyte death is inhibited by nitric oxide (39, 40). Thus, NO donors are the most powerful inhibitors of suicidal erythrocyte death (39, 40). NO is partially effective through activation of the cGMP-dependent kinase (cGKI) (41). Accordingly, the cGKI-knockout mouse suffers from severe anemia and splenomegaly (41). Erythrocytes are not only targets of NO but participate in the regulation of nitric oxide formation (42–45). Deoxygenated hemoglobin functions as a nitrite reductase that produces NO by reacting with the nitrite normally present in circulation (46). Oxygenated hemoglobin binds and deoxygenated hemoglobin releases NO (43, 47–51). NO release from deoxygenated erythrocytes thus contributes to vasodilation in hypoxic tissue.

NO synthase activity could be stimulated by endothelin, an effect mediated by activation of the endothelin B (ETB) receptor (52). Stimulation of NO synthesis could, at least in theory, counteract suicidal erythrocyte

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death. Along those lines, endothelin has been shown to counteract apoptosis (53).

Both human and murine erythrocytes express the ETB receptor (54–56). Acute exposure to endothelin activates Ca^{2+}-sensitive K^+ channels (54–56), an effect expected to shrink erythrocytes, thus favoring cell membrane scrambling (57).

The present study explored the effect of endothelin 1 (ET1) on suicidal erythrocyte death. It is shown that endothelin is without appreciable effect on suicidal erythrocyte death under control conditions but inhibits cell membrane scrambling of human erythrocytes during sustained glucose depletion. Moreover, experiments in rescued ETB receptor-knockout mice disclose the *in vivo* significance of endothelin-sensitive suicidal erythrocyte death.

MATERIALS AND METHODS

Solutions and incubations

The experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, and 1 $CaCl_2$. Where indicated, glucose was removed or 10–3000 nM ET1, 500 nM ET2, 500 nM ET3, 0.3–100 nM sarafotoxin 6c, 1 μM ionomycin or 0.1 mM *tert*-butylhydroperoxide (all from Sigma, Schnelldorf, Germany) were added to the NaCl Ringer. For the *in vitro* experiments on suicidal death of erythrocytes, incubations were carried out at 37°C in Ringer solution at a hematocrit of 0.4% in a total volume of 200 μl.

Human erythrocytes

Leukocyte-depleted erythrocytes from concentrates provided by the blood bank of the University of Tübingen were used. The volunteers providing erythrocytes gave informed consent. The study has been approved by the Ethical Commission of the University of Tübingen.

Mice

Experiments were performed in 9- to 16-wk-old male and female rescued ETB-knockout ($etb^{-/-}$) mice and corresponding wild-type mice ($etb^{+/+}$). The $etb^{-/-}$ mice have been described previously (58). All animal experiments were conducted according to the guidelines of the American Physiological Society, as well as the German law for the welfare of animals and were approved by local authorities.

Blood chemistry, blood count, and isolation of murine erythrocytes

For all experiments except for the blood count, heparin blood was retrieved from the mice. The plasma concentration of erythropoietin was determined using an immunoassay kit, according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). Vitamin B_{12} and folate were determined by competitive immunoassays, according to clinical standards in the laboratory of the University Hospital Tübingen. For blood count, EDTA blood was analyzed using an electronic hematology particle counter (scil Vet abc, Weinheim, Germany).

Western blot analysis

To examine the expression of the ETA and ETB receptor protein in murine erythrocytes, 150-μl blood pellets were lysed in 50 ml of 20 mM HEPES/NaOH (pH 7.4). Ghost membranes were pelleted (15,000 *g* for 20 min at 4°C) and lysed in 100 μl lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.5% SDS; 1 mM NaF; 1 mM Na_3VO_4; and 0.4% β-mercaptoethanol) containing protease inhibitor cocktail (Roche, Mannheim, Germany). Eighty micrograms of protein was solubilized in Laemmli sample buffer at 95°C for 5 min and resolved by 10% SDS-PAGE. For immunoblotting, proteins were electrotransferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at room temperature for 1 h. Then, the membrane was incubated with ETA (1:2000) or ETB (1:4000) receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After washing (TBST) and subsequent blocking the blots were incubated with secondary anti-rabbit antibody (1:2000; Cell Signaling, Beverly, MA, USA) for 1 h at room temperature. After washing, antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany).

Confocal microscopy and immunofluorescence

For the detection of annexin V-binding and CFSE-dependent fluorescence of erythrocytes from murine spleens, the spleens of $etb^{-/-}$ and $etb^{+/+}$ mice were homogenized mechanically in 1 ml cold PBS. The suspension was then centrifuged at 500 *g* for 10 min at 4°C. The cell pellet was resuspended in 200 μl cold PBS. Five microliters of Annexin V-APC (Becton Dickinson, Heidelberg, Germany) was added, and incubation was carried out for 20 min at 37°C protected from light. Then, the suspension was transferred onto a glass slide and mounted with Prolong Gold antifade reagent (Invitrogen). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging, Jena, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Determination of the osmotic resistance

Two microliters of blood was added to 200 μl of PBS solutions of decreasing osmolarity. After centrifugation for 5 min at 500 *g*, the supernatant was transferred to a 96-well plate, and the absorption at 405 nm was determined as a measure of hemolysis. Absorption of the supernatant of erythrocytes lysed in pure distilled water was defined as 100% hemolysis.

Phosphatidylserine exposure

FACS analysis was performed as described earlier (59). After incubation, erythrocytes were washed once in Ringer solution containing 5 mM $CaCl_2$. The cells were then stained with Annexin V-Fluos (Roche) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACSCalibur; Becton Dickinson). Cells were analyzed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$.

The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Measurement of the *in vivo* clearance of fluorescence-labeled erythrocytes

The experiment was performed as described previously (60). Briefly, erythrocytes (obtained from 200 μl blood) were fluorescence-labeled by staining the cells with 5 μM carboxyfluorescein-diacetate-succinimidyl-ester (CFSE; Molecular Probes, Leiden, Netherlands) in PBS and incubated for 30 min at 37°C. After washing twice in PBS containing 1% FCS, the pellet was resuspended in Ringer solution (37°C), and 100 μl of the CFSE-labeled erythrocytes were injected into the tail vein of the recipient mouse. Every day, blood was retrieved from the tail veins of the mice, and CFSE-dependent fluorescence intensity of the erythrocytes was measured in FL-1, as described above. The percentage of CFSE-positive erythrocytes was calculated in % of the total labeled fraction determined 5 min after injection.

Statistics

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

RESULTS

Fluo3 fluorescence was employed to determine whether ET1 alters erythrocyte Ca^{2+} concentration following incubation in glucose-containing and glucose-free medium. As illustrated in **Fig. 1**, removal of glucose was followed by the expected significant increase in Fluo3 fluorescence. More important, both ET1 (500 nM; Fig. 1*A*, *B*) and the ETB agonist sarafotoxin 6c (10 nM; Fig. 1*C*, *D*) significantly blunted the increase in the cytosolic Ca^{2+} activity following a 48-h glucose depletion, whereas they had no influence on Fluo3 fluorescence in energy-replete erythrocytes.

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 (**Fig. 2*B***) nor 10 nM sarafotoxin 6c (Fig. 2*D*) significantly modified annexin V-binding in the presence of glucose. However, both 500 nM ET1 (Fig. 2*A*, *B*) and 10 nM sarafotoxin 6c (Fig. 2*C*, *D*) significantly blunted the

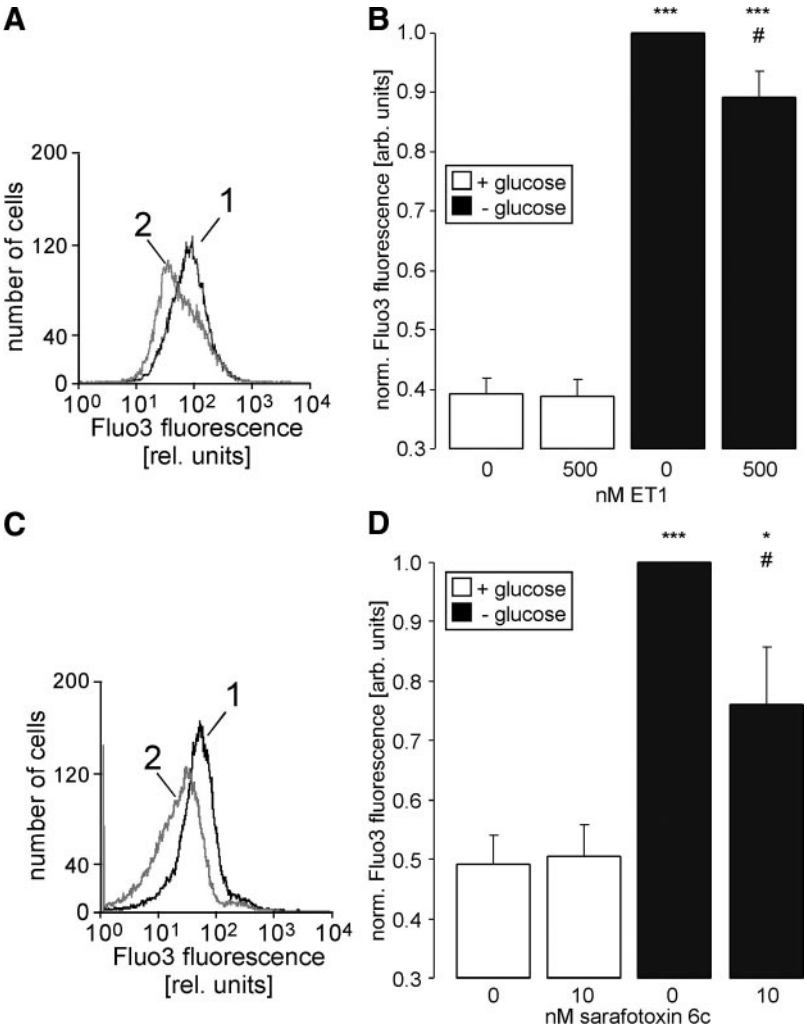

Figure 1. Cytosolic Ca^{2+} concentration in erythrocytes following energy depletion in the absence and presence of ET1 or sarafotoxin 6c. *A*) Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 h to glucose-depleted Ringer without (trace 1) or with 500 nM ET1 (trace 2). *B*) Arithmetic means ± SE (n=17) of the normalized Fluo3 fluorescence in erythrocytes exposed for 48 h to isotonic Ringer (left bars) or to glucose-depleted Ringer (right bars) without (0) or with 500 nM ET1. $^{***}P < 0.001$ *vs.* presence of glucose; $^{\#}P < 0.05$ *vs.* absence of ET1; ANOVA. *C*) Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 h to glucose-depleted Ringer without (trace 1) or with (trace 2) 10 nM sarafotoxin 6c. *D*) Arithmetic means ± SE (n=7) of the normalized Fluo3 fluorescence in erythrocytes exposed for 48 h to isotonic Ringer (left bars) or to glucose-depleted Ringer (right bars) without (0) or with 10 nM sarafotoxin 6c. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* presence of glucose; $^{\#}P < 0.05$ *vs.* absence of sarafotoxin 6c; ANOVA.

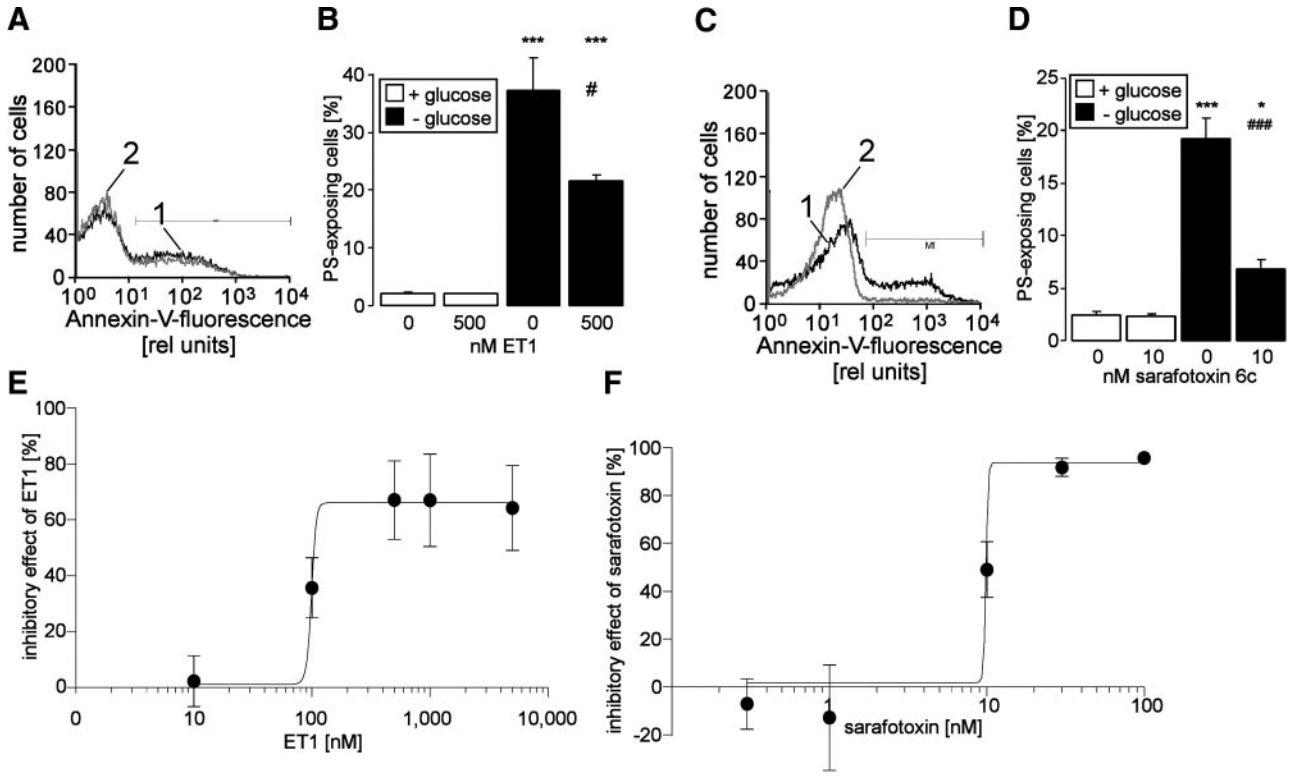

Figure 2. PS exposure of erythrocytes following energy depletion in the absence and presence of ET1 or sarafotoxin 6c. *A*) Histogram of annexin V-binding in a representative experiment of erythrocytes exposed for 48 h to glucose-depleted Ringer without (trace 1) or with 500 nM ET1 (trace 2). *B*) Arithmetic means ± SE (n=5 erythrocyte specimens studied in quadruplicates) of the percentage of annexin V-binding erythrocytes exposed for 48 h to isotonic Ringer (left bars) or to glucose-depleted Ringer (right bars) without (0) or with 500 nM ET1. ***$P < 0.001$ *vs.* presence of glucose; $^{\#}P < 0.05$ *vs.* absence of ET1; ANOVA. *C*) Histogram of annexin V-binding in a representative experiment of erythrocytes exposed for 48 h to glucose-depleted Ringer without (trace 1) or with (trace 2) 10 nM sarafotoxin 6c. *D*) Arithmetic means ± SE (n=5 erythrocyte specimens studied in quadruplicates) of the percentage of annexin V-binding erythrocytes exposed for 48 h to isotonic Ringer (left bars) or to glucose-depleted Ringer (right bars) without (0) or with 10 nM sarafotoxin 6c. *$P < 0.05$, ***$P < 0.001$ *vs.* presence of glucose; $^{\#\#\#}P < 0.001$ *vs.* absence of sarafotoxin 6c; ANOVA. *E*) Dose-response curve of ET1. Arithmetic means ± SE (n=12 erythrocyte specimens) of the relative inhibitory effect of ET1 on energy depletion-induced phosphatidylserine exposure. 100% inhibitory effect was defined as the maximal inhibition observed in the concentration range of ET1 from 10 to 5000 nM. *F*) Dose-response curve of sarafotoxin 6c. Arithmetic means ± SE (n=12 erythrocyte specimens) of the relative inhibitory effect of sarafotoxin 6c on energy depletion-induced phosphatidylserine exposure. 100% inhibitory effect was defined as the maximal inhibition observed in the concentration range of sarafotoxin 6c from 0.3 to 100 nM.

phosphatidylserine exposure following a 48-h depletion of glucose. The IC_{50} value for ET1 was 99 nM (Fig. 2*E*) and for sarafotoxin 6c 10 nM (Fig. 2*F*). A kinetics analysis revealed that the incubation period at 37°C 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 h 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 ± 0.08 to 25.14 ± 1.24% in the absence, to 27.25 ± 1.19% in the presence of 500 nM ET1 and to 24.70 ± 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-h depletion of glucose resulted in 20.6 ± 1.7% PS-exposing erythrocytes, an effect attenuated in the presence of 500 nM ET1 (15.9±1.1% PS-exposing erythrocytes) but not significantly influenced by 500 nM ET2 (20.8±1.7%) or ET3 (21.5±1.6%; all n=4 erythrocyte specimens).

The *in vivo* significance of the ETB receptor for erythrocyte survival was investigated by experiments in rescued $etb^{-/-}$ mice and in $etb^{+/+}$ mice. The expression of both the ETA and ETB receptor in membrane preparations of blood from $etb^{-/-}$ and $etb^{+/+}$ mice was studied by Western blot analysis. As shown in **Fig. 3*A***, bottom panel, the ETB receptor could, indeed, be detected in a membrane preparation of blood from $etb^{+/+}$ mice but not from $etb^{-/-}$ mice. The ETA

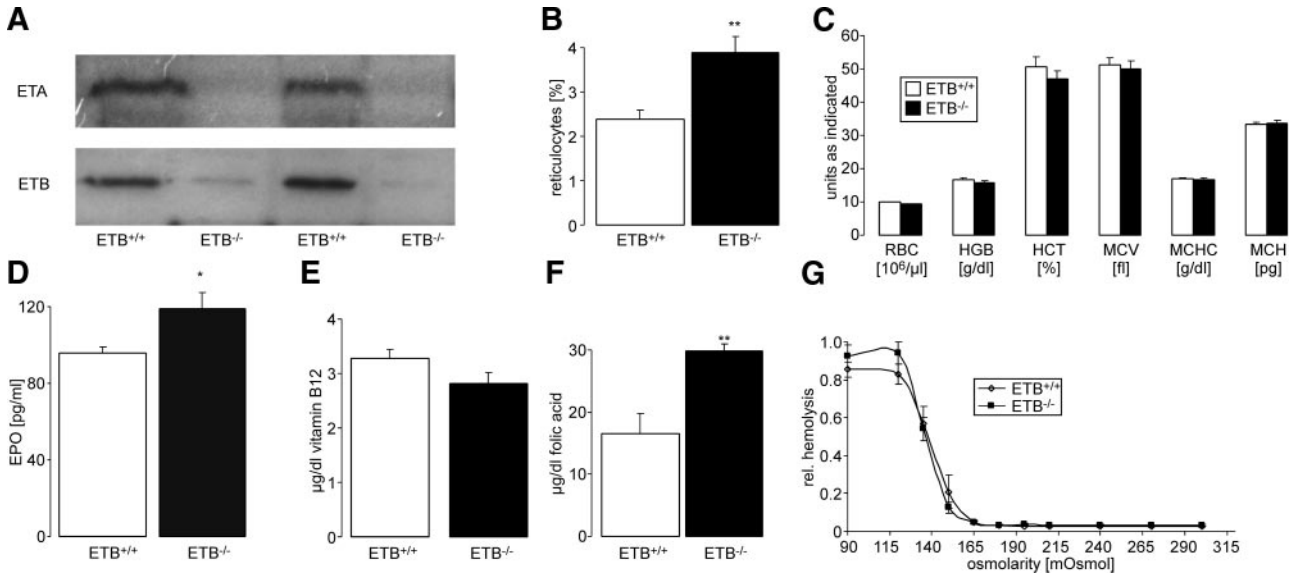

Figure 3. Erythrocyte parameters in $etb^{-/-}$ mice. *A*) Original Western blots demonstrating the expression of the ETA (top panel) and ETB (bottom panel) receptor protein in two different membrane preparations of blood from rescued $etb^{-/-}$ and $etb^{+/+}$ mice. *B*) Arithmetic means ± SE of the reticulocyte number (n=9–10) in blood from rescued $etb^{-/-}$ and $etb^{+/+}$ mice. *C*) Arithmetic means ± SE (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 $etb^{-/-}$ and $etb^{+/+}$ mice. *D*) Arithmetic means ± SE of the plasma erythropoietin concentration (n=8) of $etb^{-/-}$ and $etb^{+/+}$ mice. *E*) Arithmetic means ± SE of the plasma vitamin B_{12} concentration (n=4) of $etb^{-/-}$ and $etb^{+/+}$ mice. *F*) Arithmetic means ± SE of the plasma folic acid concentration (n=4) of $etb^{-/-}$ mice and $etb^{+/+}$ mice. *G*) Osmotic resistance of erythrocytes from $etb^{-/-}$ and $etb^{-/-}$ mice. Solid bars and symbols, $etb^{-/-}$ mice; open bars and symbols, $etb^{+/+}$ mice. *$P < 0.05$, **$P < 0.01$ *vs.* $etb^{+/+}$; *t* test.

receptor could be readily detected in a membrane preparation of blood from $etb^{+/+}$ mice (Fig. 3*A*, top panel) most likely due to its expression in leukocytes (61) 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 (62).

As shown in Fig. 3*B*, 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 (Fig. 3*C*). 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 B12 was not significantly different between genotypes (Fig. 3*D–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 (Fig. 3*G*).

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 **Fig. 4*A, B***, 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 Fig. 4*C, D*, erythrocytes from $etb^{-/-}$ mice were significantly more susceptible to the eryptotic effect of oxidative stress. On oxidative stress, the increase in intracellular calcium concentration was significantly more pronounced in $etb^{-/-}$ erythrocytes than in $etb^{+/+}$ erythrocytes (Fig. 4*E, F*).

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 labeled with the fluorescent dye CFSE and reinjected into the same mice. As shown in **Fig. 5**, 4 d after injection, the percentage of cleared erythrocytes in $etb^{-/-}$ mice was, indeed, more than twice as high as in $etb^{+/+}$ mice.

To investigate the fate of the cleared erythrocytes, the spleens of $etb^{+/+}$ mice and $etb^{-/-}$ mice were investigated. As shown in **Fig. 6*A, B***, $etb^{-/-}$ mice suffered from pronounced splenomegaly. Confocal microscopy was utilized to analyze the splenocytes from $etb^{-/-}$ mice and $etb^{+/+}$ mice. Figure 6*C* shows that 5 d after injection of the labeled erythrocytes, the spleens of the $etb^{-/-}$ mice contained significantly more CFSE-positive phosphatidylserine-exposing erythrocytes than the spleens of the $etb^{+/+}$ mice.

DISCUSSION

The present study discloses a hitherto unknown effect of endothelin, *i.e.*, the inhibitory effect on suicidal erythrocyte death. As shown earlier (6, 63), energy

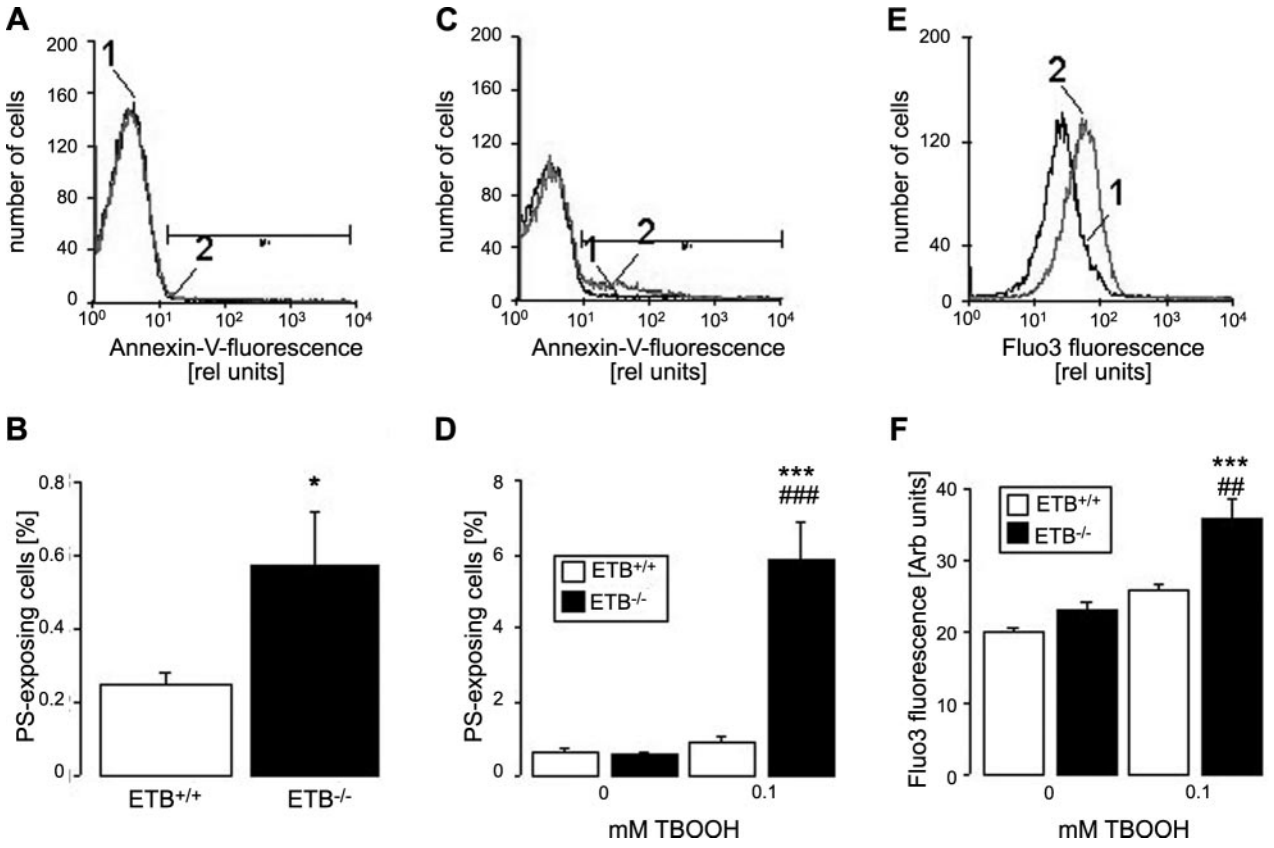

Figure 4. Suicidal erythrocyte death of etb$^{-/-}$ and wild-type mice. *A*) Histogram of annexin V-binding in a representative experiment of erythrocytes from *etb*$^{-/-}$ mice (trace 2) and *etb*$^{+/+}$ mice (trace 1) immediately stained after retrieval. *B*) Arithmetic means ± SE (n=9) of the percentage of annexin V-binding erythrocytes from *etb*$^{-/-}$ and *etb*$^{+/+}$ mice immediately stained after retrieval. *$P < 0.05$; *t* test. *C*) Histogram of annexin V-binding in a representative experiment of erythrocytes from *etb*$^{-/-}$ mice (trace 2) and *etb*$^{+/+}$ mice (trace 1) exposed for 30 min to 0.1 mM *tert*-butylhydroperoxide. *D*) Arithmetic means ± SE (n=9) of the percentage of annexin V-binding erythrocytes from from *etb*$^{-/-}$ and *etb*$^{+/+}$ mice exposed for 30 min to 0.1 mM *tert*-butylhydroperoxide. *E*) Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from *etb*$^{-/-}$ mice (trace 2) and *etb*$^{+/+}$ mice (trace 1) exposed for 30 min to 0.1 mM *tert*-butylhydroperoxide. *F*) Arithmetic means ± SE (n=9) of the Fluo3 fluorescence of erythrocytes from *etb*$^{-/-}$ and *etb*$^{+/+}$ mice exposed for 30 min to 0.1 mM *tert*-butylhydroperoxide. ***$P < 0.001$ *vs.* presence of glucose; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* *etb*$^{+/+}$; ANOVA. Solid bars, *etb*$^{-/-}$ mice; open bars, *etb*$^{+/+}$ mice.

depletion (glucose removal) increases cytosolic Ca^{2+} activity, which, in turn, stimulates scrambling of the cell membrane with subsequent phosphatidylserine exposure at the erythrocyte surface (2, 4, 5).

ET1 and sarafotoxin 6c did not significantly modify cytosolic Ca^{2+} activity or phosphatidylserine exposure in isotonic, glucose-containing, extracellular fluid. Both ET1 and sarafotoxin 6c, however, significantly blunted the effect of glucose withdrawal on phosphatidylserine exposure.

The ETB receptor has previously been described in murine erythrocytes (54). In the same study, the ETA receptor could not be detected in erythrocyte progenitor cells. The present study confirmed the expression of the ETB receptor in *etb*$^{+/+}$ erythrocytes but not in *etb*$^{-/-}$ erythrocytes. Furthermore, in blood preparations from *etb*$^{+/+}$ mice, the ETA receptor could be detected most likely because of leukocyte contaminations since leukocytes are known to express the ETA receptor (61). More important, ETB receptor deficiency is obviously not paralleled by up- but by down-regulation of ETA most likely due to high ET1 levels in *etb*$^{-/-}$ mice (58, 62).

The present observations suggest a role of endothelin receptor signaling in the protection against anemia. Phosphatidylserine-exposing erythrocytes are bound to phosphatidylserine receptors on macrophages (64), which engulf and degrade the phosphatidylserine exposing cells (65). Accordingly, phosphatidylserine-exposing erythrocytes are rapidly cleared from circulating blood (13). The antiscrambling effect of endothelin is thus expected to suppress the suicidal death and clearance of erythrocytes and thus, to counteract anemia. Along those lines, despite an increased percentage of reticulocytes, which points to enhanced formation of new erythrocytes, the erythrocyte number was rather decreased in *etb*$^{-/-}$ mice. The observation strongly suggests that the life span of circulating erythrocytes was decreased in *etb*$^{-/-}$ mice. Accordingly, the spleen of those mice was enlarged pointing to increased splenic erythrocyte sequestration.

Phosphatidylserine-exposing erythrocytes may further adhere to the vascular wall (66, 67), stimulate blood clotting (68, 69), and thus interfere with micro-

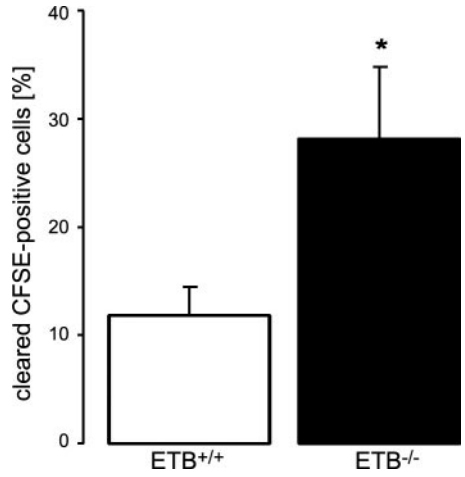

Figure 5. Accelerated erythrocyte clearance of erythrocytes in $etb^{-/-}$ mice. Percentage of cleared CFSE-labeled circulating erythrocytes drawn from $etb^{-/-}$ (solid bar) and $etb^{+/+}$ mice (open bar) 4 d after injection into the same mice. Values are normalized arithmetic means ± SE (n=7) of the percentages of CFSE-labeled erythrocytes. *$P < 0.05$ *vs.* $etb^{+/+}$; *t* test.

circulation. Conversely, through activation of ETB receptors, endothelin may counteract adhesion of erythrocytes to the vascular wall and thus contribute to the maintenance of microcirculation. The effects of endothelin on erythrocytes could add to the known effects of endothelin on microcirculation. ET1, considered the most powerful natural vasoconstrictor, acts mainly in the underlying layer of vascular smooth muscle cells (VSMCs) through ETA receptors, causing vasoconstriction and increased VSMC proliferation. Accordingly, ET1 receptor blockers have been shown to lower blood pressure (70). Although vasoconstriction is its prevalent action, ET1 can also act in ETB receptors present in endothelial cells and promote vasodilatation by fostering the production of NO (71).

Uptake of eryptotic cells by macrophages can, at least in theory, trigger the release of proinflammatory cytokines, which may, in turn, stimulate release of glucocorticoids and foster the development of metabolic syndrome associated with oxidative stress (69). Glucocorticoids have, in turn, been shown to lower the expression of ETA and ETB receptors in an animal model of glucocorticoid-induced hypertension (72).

Through activation of ETB receptors, endothelin might counteract systemic inflammation, adhesion of erythrocytes to the vascular wall, and might contribute to the maintenance of microcirculation. Chronic ET1 administration, however, can inhibit insulin-mediated glucose uptake (71) and thus favor the development of metabolic syndrome.

Endothelin may further influence suicidal erythrocyte death of stored erythroctyes. The life span of those erythrocytes is limited by oxidative stress and subsequent suicidal erythrocyte death (73, 74).

In summary, 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. FJ

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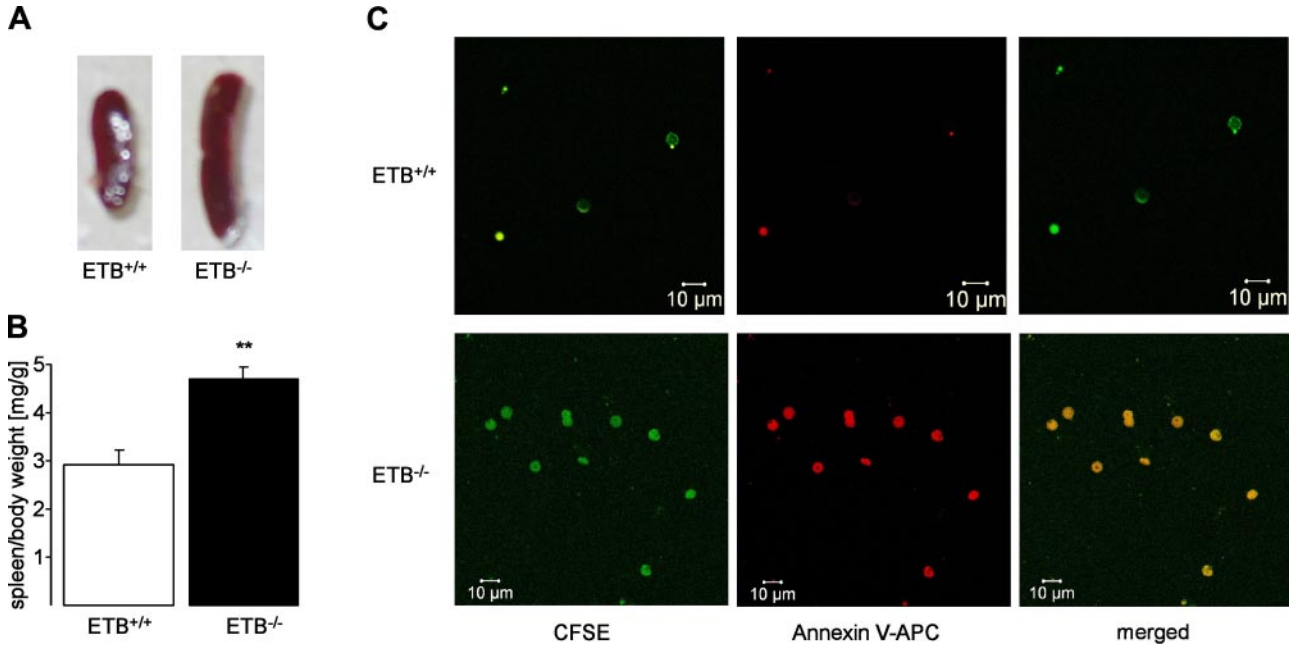

Figure 6. Splenomegaly associated with increased erythroid cell mass in $etb^{-/-}$ mice. *A*) Photograph of spleens from $etb^{-/-}$ (right panel) and $etb^{+/+}$ mice (left panel). *B*) Arithmetic means ± SE (n=5) of the spleen/body weight ratios of $etb^{-/-}$ (solid bar) and $etb^{+/+}$ mice (open bar). **$P < 0.01$ *vs.* $etb^{+/+}$; *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 $etb^{-/-}$ (bottom panels) and $etb^{+/+}$ mice (top panels).

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