Review Article

Redox Status of Erythrocytes as an Important Factor in Eryptosis and Erythronecroptosis

(erythrocytes / regulated cell death / eryptosis / necroptosis / erythronecroptosis / apoptosis / reactive oxygen species / damage-associated molecular patterns / hydrogen peroxide / RCD / ROS / DAMPs)

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Abstract. Overall, reactive oxygen species (ROS) signalling significantly contributes to initiation and modulation of multiple regulated cell death (RCD) pathways. Lately, more information has become available about RCD modalities of erythrocytes, including the role of ROS. ROS accumulation has therefore been increasingly recognized as a critical factor involved in eryptosis (apoptosis of erythrocytes) and erythronecroptosis (necroptosis of erythrocytes). Eryptosis is a Ca^{2+}-dependent apoptosis-like RCD of erythrocytes that occurs in response to oxidative stress, hyperosmolarity, ATP depletion, and a wide range of xenobiotics. Moreover, eryptosis seems to be involved in the pathogenesis of multiple human diseases and pathological processes. Several studies have reported that erythrocytes can also undergo necroptosis, a lytic RIPK1/RIPK3/MLKL-mediated RCD. As an example, erythronecroptosis can occur in response to CD59-specific pore-forming toxins. We have systematically summarized available studies regarding the involvement of ROS and oxidative stress in these two distinct RCDs of erythrocytes. We have focused specifically on cellular signalling pathways involved in ROS-mediated cell death decisions in erythrocytes. Furthermore, we have summarized dysregulation of related erythrocytic antioxidant defence systems. The general concept of the ROS role in eryptotic and necroptotic cell death pathways in erythrocytes seems to be established. However, further studies are required to uncover the complex role of ROS in the crosstalk and interplay between the survival and RCDs of erythrocytes.

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Abbreviations: 4-HNE – 4-hydroxynonenal, 7-KC – 7-ketocholesterol, ACD – accidental cell death, AGEs – advanced glycation end products, CDK4 – cyclin-dependent kinase 4, CK1α – casein kinase 1α, DAMPs – damage-associated molecular patterns, GPx – glutathione peroxidase, GR – glutathione reductase, GSH – reduced glutathione, GSSG – oxidized glutathione, H2DCFDA – 2′,7′-dichlorodihydrofluorescein diacetate, Hb – haemoglobin, HFE – homeostatic iron regulator, JAK3 – Janus kinase 3, LPO – lipid peroxidation, LPS – lipopolysaccharide, MDA – malondialdehyde, metHb – methaemoglobin, MLKL – mixed lineage kinase domain-like protein, NAC – N-acetylcysteine, NCCD – Nomenclature Committee on Cell Death, NOS – NO synthase, NOX – NADPH oxidase, p38 MAPK – p38 mitogen-activated protein kinase, PDK1 – 3-phosphoinositide-dependent kinase 1, PKC – protein kinase C, PS – phosphatidylserine, RBCs – red blood cells, RCD – regulated cell death, RIPK1 – receptor-interacting protein kinase 1, RIPK3 – receptor-interacting protein kinase 3, RNS – reactive nitrogen species, ROS – reactive oxygen species, SOD – superoxide dismutase, TBARS – thiobarbituric acid reactive substances, TRIOL – cholestan-3β,5α,6β-triol, XOR – xanthine oxidoreductase.

Introduction

The Great Oxygenation Event (characterized by a dramatic rise in the atmospheric concentration of oxygen and triggered by oxygen-producing cyanobacteria) occurred approximately 2.3 billion years ago. It significantly contributed to the evolution of complex life on Earth, including development of multicellular organisms, and mediated emergence of more energetically efficient aerobic organisms (Schirrmeister et al., 2013; Zimorski et al., 2019). Since oxygen is toxic to anaerobic species, the oxygenated atmosphere led to the evolution of organisms that could detoxify oxygen and take advantage of it. Evolutionary development of mitochondrial respiration improved the efficiency of energy metabolism and shaped the complexity of eukaryotic life

(Lane, 2020). However, this change was not without certain disadvantages. Consumption of oxygen and its utilization for energetic purposes results in the formation of reactive oxygen species (ROS). ROS are highly reactive molecules such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen, all capable of oxidizing macromolecules and hence causing by-product damage to the cells and tissues (Thannickal, 2009).

Mitochondrial respiration (namely, complexes I and III of the electron transport chain) is considered to be the major source of cellular ROS (Mailloux, 2020). Mitochondrial dysfunction with ROS overproduction has been associated with eukaryotic aging, age-related disorders, and eventual death (Giorgi et al., 2018; Santos et al., 2018). On the other hand, advances in our understanding of ROS biology and their physiological role in cellular signalling have caused a paradigm shift. ROS are nowadays considered dual-function molecules, having detrimental as well as beneficial properties (Checa and Aran, 2020; Lennicke and Cochemé, 2021; Shields et al., 2021). It has become widely accepted that ROS-mediated redox signalling contributes to the regulation of multiple cellular processes and coordinates the activity of many transcription factors (Milkovic et al., 2019).

ROS-mediated regulation of cell survival and cell death is of particular interest since ROS facilitate the cell response under various stress conditions. ROS act as a bridge between survival and cell demise contributing to cell fate-determining decisions (survival, cell death, RCD type) in a concentration-dependent manner (Vardar Acar and Özgül, 2023). Cell death occurs when cellular basic and vital functions cannot be further maintained. According to the Nomenclature Committee on Cell Death (NCCD), cell death could be generally divided into accidental cell death (ACD) and regulated cell death (RCD) (Galluzzi et al., 2018). Multiple RCD modalities such as apoptosis, autophagy-related cell death, necroptosis, pyroptosis, ferroptosis, cuproptosis, lysosome-dependent cell death, NETotic cell death, parthanatos, and mitotic catastrophe have been described. Individual RCD modalities were earlier considered as non-overlapping cell death events associated with separate signalling pathways. However, it is now believed that individual RCDs are coordinated and interconnected to form an extensive signalling network governing cell fate with various mechanisms of its regulation in healthy cells and within certain pathologies (Zeng et al., 2020; Liu et al., 2022; Shen et al., 2023). Interestingly, ROS were suggested to act as a rheostat, providing critical crosstalk between different RCDs to determine the way a cell dies (Villalpando-Rodriguez and Gibson, 2021).

Recent studies caused a paradigm shift also in red blood cell (RBC) biology. Erythrocytes are no longer considered only immunologically inert passive oxygen carriers. It has been shown that erythrocytes significantly contribute to the innate immunity as immune sentinels and chemokine scavengers (Anderson et al., 2018; Lam et al., 2021; Minton, 2021). Furthermore, specific erythrocyte cell death modalities may affect the innate immune response if connected with release of damage-associated molecular patterns (DAMPs) (Galluzzi et al., 2023). For example, haemolysis is associated with the release of pro-inflammatory haemoglobin (Hb), haem derivatives, IL-33, HSP70, or ATP (Mendonça et al., 2016; Jeney, 2018). Since erythrocytes are the most abundant cells in the circulation, their lytic cell death associated with DAMPs release can be highly immunogenic. Therefore, the type of erythrocyte death can have a profound effect on immune response modulation. Experimental evidence suggests that erythrocytes can undergo two RCDs: apoptosis (eryptosis) (Lang and Lang, 2015b; Qadri et al., 2017; Föller and Lang, 2020; Alghareeb et al., 2023; Tkachenko, 2023; Tkachenko and Onishchenko, 2023) and necroptosis (LaRocca et al., 2014, 2015, 2016; McCaig et al., 2019; Seo et al., 2023). Apoptosis is relatively immunologically inert due to its non-lytic nature and lack of DAMPs release. Cell lysis-associated necroptosis engages the immune system in a DAMPs-mediated fashion (Nakano et al., 2022). On the other hand, alternative mechanisms of DAMP release have also been suggested for apoptotic cells, indicating that apoptosis likely contributes to the immune response regulation as well (Murao et al., 2021). Moreover, phosphatidylserine (PS)-mediated uptake of apoptotic cells by phagocytes in general promotes the anti-inflammatory phenotype of apoptosis (Szondy et al., 2017).

This all clearly indicates that the signalling molecules involved in RCD type determination (like ROS) can affect inflammatory processes far beyond the cellular level. In this review, we have summarized the role of ROS and oxidative stress in eryptosis and necroptosis of RBCs to highlight the critical involvement of redox homeostasis dysregulation in erythrocytic cell death type determination.

Reactive oxygen species sources and functions in erythrocytes

Similarly to nucleated cells, ROS are produced and regulate cellular physiology in erythrocytes. However, the sources of ROS in RBCs are substantially different from those in nucleated cells. Erythrocytes are devoid of organelles (to provide more space for oxygen-carrying Hb); therefore, the main source of ROS in RBCs is Hb autooxidation (Çimen, 2008). As a result, Hb containing Fe^{2+} is converted to Fe^{3+}-containing methaemoglobin. Additionally, switching of haem ferrous and ferric iron back and forth leads to formation of hydroxyl radicals due to the Fenton reaction (Tsamesidis et al., 2020).

Experiments also suggested that ROS could be generated in RBCs by NADPH oxidase (NOX), a multi-subunit protein complex transferring electrons to oxygen to obtain superoxide radicals (George et al., 2013). Xanthine oxidoreductase (XOR) was also reported to contribute to ROS generation in erythrocytes (Webb et al., 2008; Attanzio et al., 2019; Gajecki et al., 2022). Peroxynitrite ($ONOO^-$) can be formed as a result of endog-

enously produced nitric oxide (NO) (Cortese-Krott, 2023). Apart from endogenous sources, erythrocytes are exposed to exogenously generated ROS. These could be released from neutrophils, macrophages, or endothelial cells (Mohanty et al., 2014; Möller et al., 2023). Additionally, RBCs can act as NO scavengers (Azarov et al., 2005; Liu et al., 2013), which could lead to excessive peroxynitrite formation.

It has been well established that redox signalling plays an important role in erythrocytic cellular physiology. Redox signalling regulates survival and clearance of erythrocytes, e.g., by promoting cell membrane scrambling. Within this process, PS is translocated to the outer leaflet of the cell membrane with subsequent efferocytosis (phagocytosis of apoptotic cells) of erythrocytes by phagocytic cells (Föller and Lang, 2020). Another erythrocytic cellular process regulated by ROS is deformability (required to pass through narrow capillaries and maintain gas exchange) (Diederich et al., 2018). ROS-dependent modulation of erythrocyte flexibility and response to shear stress seem to be mediated by oxidation of spectrin and band 3 proteins (Çimen, 2008; Lux, 2016). As an ultimate consequence, excessive ROS load might negatively affect RBC-mediated oxygen tissue delivery. Erythrocytes are also important regulators of the redox equilibrium in general due to their ability to sequester exogenous ROS and NO (Mahdi et al., 2021). Importantly, several links between erythrocyte-derived ROS and thrombosis have been reported (Bettiol et al., 2022).

Taken together, ROS are important signalling molecules in the physiology of erythrocytes. They affect multiple basic RBC functions and mediate the interplay between erythrocytes and other blood cells to maintain homeostasis.

Antioxidant system of RBCs

Excessive ROS production triggers lipid peroxidation and promotes protein oxidative modifications. This impairs the integrity of cell membranes and results in formation of dysfunctional proteins, respectively (Pandey and Rizvi, 2010). To counteract the detrimental effect of ROS over-generation, RBCs have evolved a sophisticated multicomponent antioxidant defence system composed of enzymatic and non-enzymatic components (Franco et al., 2019; Möller et al., 2023). The sources of ROS and individual components of the RBC antioxidant system are summarized in Fig. 1.

Glutathione (tripeptide γ-glutamyl-cysteinyl-glycine) plays one of the key roles in the antioxidant defence of RBCs. Intracellularly, it is present in a reduced (GSH) or oxidized (GSSG) form. Importantly, erythrocytes are able to maintain high intracellular levels of GSH; therefore, their GSH-to-GSSG ratio is over 10 under normal conditions (Amen et al., 2017). This high ratio is maintained by glutathione reductase, which uses NADPH generated by the pentose phosphate pathway of glucose oxidation as a source of reducing power. Another important component of this thiol-based link of the RBC antioxidant defence system is glutathione peroxidase. It is a selenium-containing enzyme that catalyses GSH-dependent decomposition of peroxides (Franco et al., 2019; Cortese-Krott, 2023; Möller et al., 2023). In RBCs, hydrogen peroxide is also decomposed by catalases (Cortese-Krott, 2023), the thioredoxin system (made up of peroxiredoxins and thioredoxins, both of which contain the functional cysteine thiol groups), as well as by the NADPH-dependent thioredoxin reductase (Cortese-Krott, 2023; Möller et al., 2023). Hydrogen peroxide is primarily produced from superoxide ions via dismutation reaction catalysed by superoxide dismutase (SOD) (Möller et al., 2023).

Non-enzymatic components of the RBC antioxidant system include ascorbic acid (vitamin C), tocopherols (vitamin E), and uric acid (Cortese-Krott, 2023; Möller et al., 2023). Erythrocytes do not possess ascorbic acid transporters or other mechanisms of its active transmembrane transport; therefore, intraerythrocytic concentrations of ascorbic acid reflect its blood concentrations. These levels are considered to be too low for direct ascorbic acid-mediated ROS scavenging (Li et al., 2012). This suggests that vitamin C is rather used to keep vitamin E in a reduced state in erythrocytes (Möller et al., 2023). Due to their lipid-soluble nature, tocopherols are accumulated mainly in the phospholipid bilayer of the cell membranes and prevent ROS-induced lipid peroxidation (to preserve the integrity of the cell membranes) (Niki, 2021).

Excessive exposure to exogenous ROS or their overproduction by erythrocytes themselves can be counteracted by protective activation of the antioxidant machinery. However, the lack of protein-synthesizing organelles does not allow up-regulation of antioxidant enzymes in RBCs. Continuous exposure to oxidants, therefore, depletes the antioxidant system and results in the imbalance between pro-oxidants and anti-oxidants, causing "oxidative stress" (Pandey and Rizvi, 2010; Maurya et al., 2015; Orrico et al., 2023). Oxidative stress in erythrocytes ultimately results in haemolysis with loss of membrane integrity and uncontrolled cell lysis (Orrico et al., 2023). To prevent that, it has been shown that ROS accumulation at haemolysis subthreshold levels could trigger RCDs of erythrocytes, namely eryptosis and necroptosis. In the next two sections, we have summarized the available evidence on how the ROS signalling contributes to these two distinct RCDs of erythrocytes.

ROS Signalling in Eryptosis

Brief overview of eryptosis

Eryptosis has been described as an apoptosis-like protective process to eliminate abnormal and/or dysfunctional erythrocytes negatively affected, e.g., by oxidative stress, hyperosmotic conditions, ATP shortage, or exposure to toxic compounds and xenobiotics (Pretorius et al., 2016; Alghareeb et al., 2023). Rapid removal of eryptotic erythrocytes is carried out by macrophages

through PS-mediated phagocytosis (Chang et al., 2018; Turpin et al., 2022).

Eryptosis characteristics include sustained membrane integrity, calpain-mediated membrane blebbing, K^+ efflux-dependent cell shrinkage, and the above-mentioned membrane asymmetry with PS externalization (Lang and Lang, 2015a; Scovino et al., 2022; Tkachenko, 2023; Tkachenko and Onishchenko, 2023; Tkachenko et al., 2023). Eryptotic signalling relies on several interconnected mediators. Multiple studies have suggested a critical role of Ca^{2+} signalling. Within eryptosis, it has been linked to cell shrinkage via Gardos channels (Ca^{2+}-sensitive potassium selective channels in RBCs), membrane lipid scrambling, and calpain activation (Repsold and Joubert, 2018; Föller and Lang, 2020; Dreischer et al., 2022; Tkachenko, 2023). Ca^{2+} inflow can be stimulated by prostaglandin E2 or shear stress (with participation of PIEZO1 channels) (Föller and Lang, 2020). It has been demonstrated that sphingomyelin-derived ceramide acts as another important second messenger in RBC cell death signalling, also promoting PS externalization (Lang et al., 2015). Additionally, eryptosis is upregulated by p38 mitogen-activated protein kinase (p38 MAPK), protein kinase C (PKC), casein kinase 1α (CK1α),

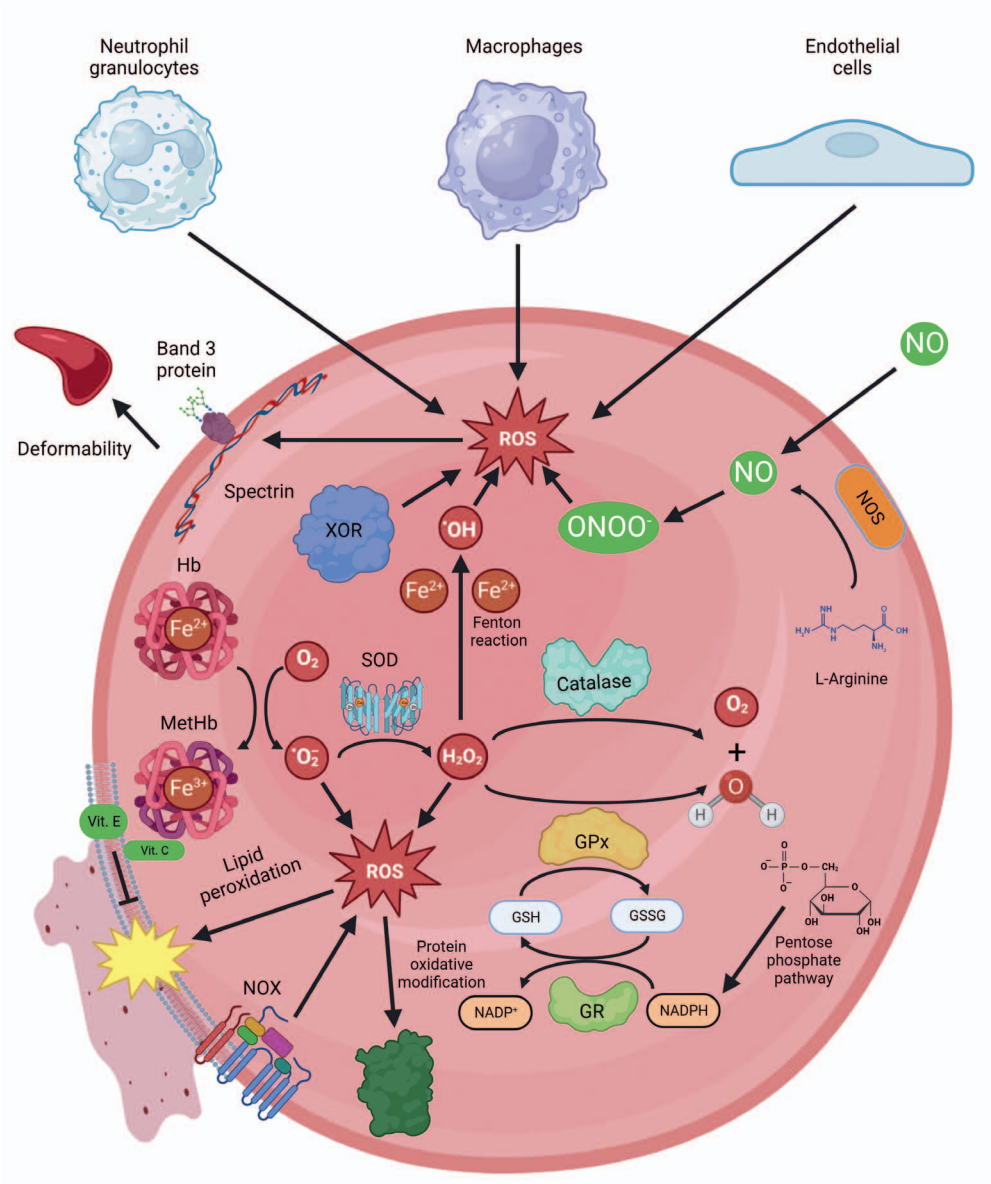

Fig. 1. ROS biology in erythrocytes. In RBCs, ROS are generated endogenously due to Hb autooxidation, Fenton chemistry, or NOX activity. Additionally, erythrocytes are exposed to exogenous ROS. NOS-derived or scavenged NO can be converted to peroxynitrite in RBCs, contributing to the pool of oxidants. ROS detoxification occurs by antioxidant enzymes, including catalase, SOD, or GPx, as well as non-enzymatic antioxidants such as tocopherols or ascorbic acid. This figure was created with biorender.com.

GPx – glutathione peroxidase, GR – glutathione reductase, GSH – reduced glutathione, GSSG – oxidized glutathione, Hb – haemoglobin, metHb – methaemoglobin, NOS – NO synthase, NOX – NADPH oxidase, ROS – reactive oxygen species, SOD – superoxide dismutase, XOR – xanthine oxidoreductase.

Janus kinase 3 (JAK3), cyclin-dependent kinase 4 (CDK4), or 3-phosphoinositide-dependent kinase 1 (PDK1) signalling (Tkachenko and Onishchenko, 2023).

ROS as inducers of eryptosis

We have systematically analysed all PubMed-indexed articles related to eryptosis since 2006 to summarize the contribution of impaired redox homeostasis to eryptosis initiation and regulation. As summarized in Table S1, oxidants are frequently accumulated within eryptosis triggered by xenobiotics. Moreover, free radicals and oxidants seem to be involved in accelerated eryptosis in elderly people (Lupescu et al., 2015), patients with diabetes mellitus type 2 (Calderón-Salinas et al., 2011; Kempe-Teufel et al., 2018), hypertension and dyslipidaemia (Pinzón-Díaz et al., 2018), acute cardiac failure (Attanasio et al., 2015), end-stage renal disease (Abed et al., 2014), uraemia associated with haemodialysis or peritoneal dialysis (Bissinger et al., 2016a), hepatitis B-related acute-on-chronic liver failure (Mei et al., 2022), arteritis (Bissinger et al., 2016b), systemic lupus erythematosus (Jiang et al., 2016), sickle cell anaemia (Nader et al., 2020), or in individuals exposed to lead (Aguilar-Dorado et al., 2014; Hernández et al., 2019). As a consequence, this accelerated rate of eryptosis can cause or exacerbate the above-mentioned condition-related anaemia. In these studies, ROS accumulation was detected either directly using ROS-sensitive probes such as 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), or indirectly, analysing the intensity of protein oxidative modifications and lipid peroxidation.

It is worthwhile to mention that the summary of available studies (Table S1) also documents the use of malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides or 4-hydroxynonenal (4-HNE), and protein carbonylation products as markers of ROS accumulation. Several studies also used C11-BODIPY$^{581/591}$, a lipid peroxidation sensor (Table S1). Furthermore, the ability of ROS-scavenging agents or antioxidants (including N-acetylcysteine, ascorbic acid, tocopherols, GSH, or tiron) to inhibit eryptosis could be assessed to evaluate the contribution of ROS signalling to eryptosis (Tkachenko and Onishchenko, 2023). Table S1 clearly illustrates that ROS are important messengers in eryptosis. However, the sources of ROS in eryptotic erythrocytes are poorly understood. On the other hand, it is important to emphasize that all major erythrocytic ROS generation pathways were shown to contribute to eryptosis-regulating ROS synthesis.

Sources of ROS in eryptosis

Attanzio et al. (2019) studied how Hb autooxidation contributes to ROS production in eryptosis. Although ROS generation occurred in response to Hb autooxidation, Hb autooxidation was not indispensable for it. The Fenton reaction seems to contribute to eryptosis-associated ROS overproduction in HFE haemochromatosis, an iron-storage disease associated with mutations of the *HFE* (homeostatic iron regulator) gene and consequent excessive accumulation of iron (du Plooy et al., 2018). Additionally, Perrone et al. (2023) linked Fenton chemistry-derived ROS to eryptosis in neonatal RBCs. Sickle cell Hb (HbS)-mediated Fenton chemistry-related ROS generation also promotes eryptosis in sickle cell anaemia (Lang et al., 2002; Bissinger et al., 2019). NOX-derived ROS seem to be crucial for eryptosis triggered by 7-ketocholesterol (7-KC), as this process was inhibited by specific NOX inhibitor diphenyleneiodonium (Attanzio et al., 2019). Interestingly, another oxysterol called cholestan-3β,5α,6β-triol (TRIOL) promoted eryptosis in a NOX-independent fashion (Attanzio et al., 2019). Indoxyl sulphate, which is an uremic toxin, also triggered ROS-dependent eryptosis through NOX activation (Dias et al., 2018). In addition, XOR-derived ROS were also linked to eryptosis induction (Attanzio et al., 2019). NO generally promotes survival of erythrocytes. It protects them from eryptosis through modulation of protein nitrosylation, thioredoxin, or protein kinase G activity (Nicolay et al., 2008; Lang et al., 2012; Alghareeb et al., 2023). On the other hand, NO overproduction stimulates reactive nitrogen species (RNS) accumulation. RNS, like peroxynitrite, can consequently trigger cell death of erythrocytes (Matarrese et al., 2005). Not much is known about the role of RNS in eryptosis, but TRIOL-induced eryptosis was shown to associate with NOS over-activation and RNS production (Attanzio et al., 2019). Of note, excessive NO promotes Hb autooxidation (Attanzio et al., 2019), which further contributes to the overall oxidative stress.

The above-outlined studies show that the major sources of eryptosis-related ROS are not uniform. It is possible to speculate that ROS sources likely depend on the nature of a particular xenobiotic and/or other eryptosis induction factors. More studies are needed to show details and various alternative sources of ROS generation in eryptotic erythrocytes.

Antioxidant system of erythrocytes in eryptosis

As in other cell types, ROS over-generation is counterbalanced by the compensatory activation of the RBC antioxidant system. Since erythrocytes cannot up-regulate antioxidant enzymes at the transcriptional level, ROS overproduction can rapidly result in depletion of their antioxidant capacity. As shown in Table S1, GSH depletion and down-regulation of catalase, glutathione reductase, glutathione peroxidase, and SOD are commonly observed in response to accumulation of oxidants during eryptosis. Such imbalance reflects acquired physiological dysfunction of compromised erythrocytes leading to their elimination by PS-mediated clearance. On the other hand, it is important to emphasize that oxidative stress is not a mandatory determinant of eryptosis. Eryptosis might be triggered by multiple other signalling pathways and their combinations.

ROS-mediated signals in eryptosis

The pro-eryptotic effect of ROS could be primarily attributed to the enhancement of Ca^{2+} influx via Ca^{2+}-

permeable channels (Föller and Lang, 2020; Alghareeb et al., 2023). Ca^{2+} consequently coordinates the eryptotic machinery to trigger Gardos channel-mediated cell shrinkage or membrane phospholipid scrambling. Simultaneous oxidative stress and intracellular Ca^{2+} accumulation in eryptosis was repeatedly shown in the majority of analysed studies (Table S1). However, as also illustrated in Table S1, ROS over-generation and oxidative stress do not always correlate with intracellular Ca^{2+} elevation in erythrocytes. This suggests that ROS might trigger alternative pathways to initiate or reinforce eryptotic signalling. Indeed, it was shown that ROS could activate caspase-3 in erythrocytes, promoting PS externalization (Dreischer et al., 2022). At the same time, our analysis reveals that multiple studies indicate that ROS-mediated eryptosis can be caspase-3-independent (Table S1). Regarding a possible crosstalk between various eryptotic signals, an interesting interplay between ROS signalling and ceramide pathway was extensively demonstrated in nucleated cells. It leads to the activation of ROS-generating enzymes such as NOX or XOR (Li and Zhang, 2013). Conversely, ROS can activate ceramide-generating acid sphingomyelinase to produce ceramide (Dumitru et al., 2007). A similar interaction was not studied in erythrocytes. However, it might be similarly important. ROS may serve as a bridge between ceramide and Ca^{2+} signalling also in eryptosis.

Redox-sensitive kinases in eryptosis

In general, multiple signalling pathways are redox-sensitive. It was well documented, e.g., for p38 MAPK, PKC, and JAK3 signalling pathways (Cosentino-Gomes

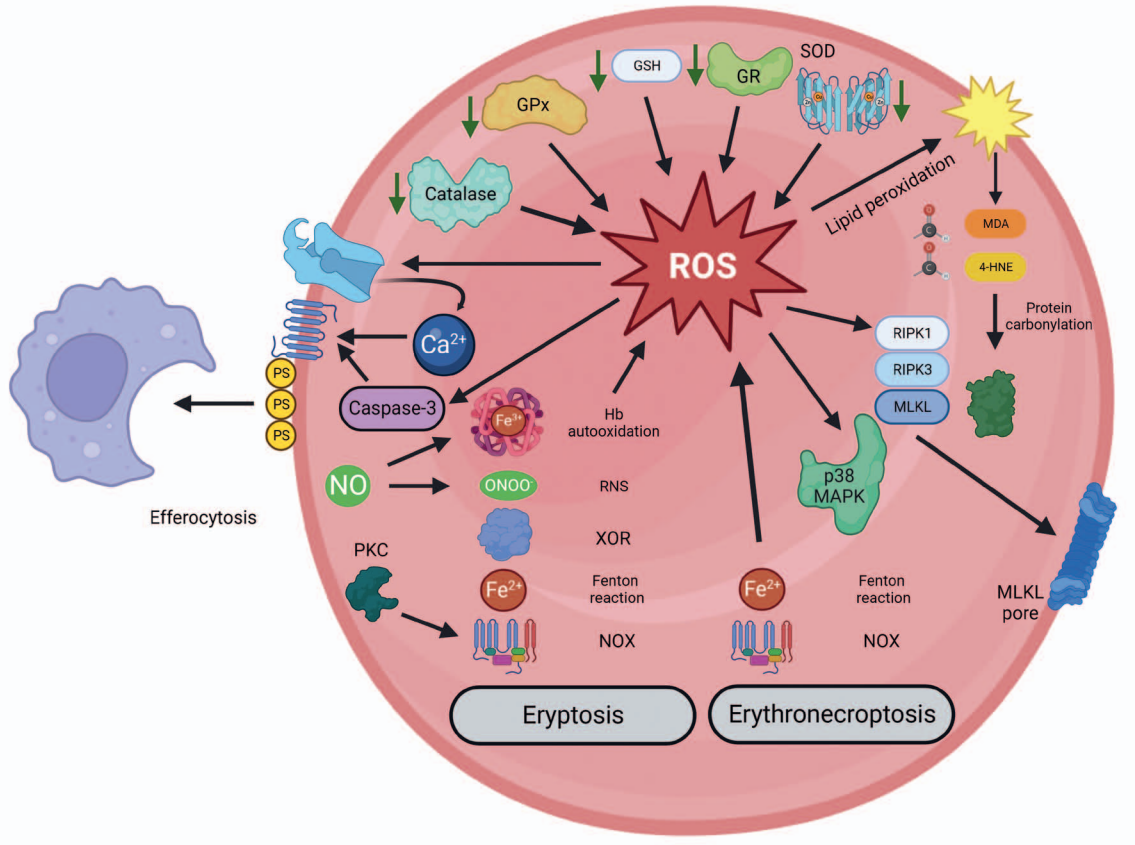

Fig. 2. ROS signalling in eryptosis and erythronecroptosis. ROS signalling is clearly implicated in the regulation of survival and determination of cell death mode in erythrocytes. Eryptosis-regulating ROS are produced as a result of haemoglobin autooxidation, Fenton chemistry, activation of NADPH oxidase and xanthine oxidoreductase or NO-derived pathways. ROS overproduction is frequently accompanied by depletion of the antioxidant system. In eryptosis, ROS-mediated Ca^{2+} influx and caspase-3 activation promote membrane phospholipid scrambling and hence phosphatidylserine-mediated clearance of eryptotic cells by macrophages. In erythronecroptosis, NADPH oxidase- and iron-derived ROS recruit RIPK1 to modulate the necrosome assembly. Thus, ROS signalling is one of the crucial regulators determining the cell death mode of erythrocytes. This figure was created with biorender.com.

4-HNE – 4-hydroxynonenal, GPx – glutathione peroxidase, GR – glutathione reductase, GSH – reduced glutathione, GSSG – oxidized glutathione, Hb – haemoglobin, MDA – malondialdehyde, metHb – methaemoglobin, MLKL – mixed lineage kinase domain-like protein, NOS – NO synthase, NOX – NADPH oxidase, p38 MAPK – p38 mitogen-activated protein kinase, PKC – protein kinase C, PS – phosphatidylserine, RIPK1 – receptor-interacting protein kinase 1, RIPK3 – receptor-interacting protein kinase 3, ROS – reactive oxygen species, SOD – superoxide dismutase, XOR – xanthine oxidoreductase.

et al., 2012; Corcoran and Cotter, 2013; Duhé, 2013; Truong and Carroll, 2013; Steinberg, 2015). At the same time, cellular signalling might support ROS generation. PKC promotes ROS synthesis via NOX and mitochondria (Cosentino-Gomes et al., 2012). Likewise, ROS generation occurs in response to p38 MAPK activation with at least partial contribution of mitochondrial ROS-generating pathways (Heusch et al., 2010; Ashraf et al., 2014). Little is known about the redox regulation of eryptosis-associated kinases in RBCs. PKC and JAK3 signalling seems to act differently in various cell types (apoptosis inhibition vs eryptosis stimulation) (Tkachenko, 2023); therefore, it could be hypothesized that also their redox regulation might be different between erythrocytes and nucleated cells. In erythrocytes, it was reported that p38 MAPK could be activated by oxidative stress to promote haemolysis (Hazegh et al., 2022). Importantly, PKCζ was shown to activate NOX-derived ROS production in eryptosis (Attanzio et al., 2019). Of note, PKC-triggered NOX-mediated ROS production was also demonstrated in eryptosis-prone sickle cells (George et al., 2013; Moumni et al., 2020).

Taken together, it could be suggested that ROS signalling in eryptosis includes several interchangeable alternative pathways (Fig. 2). However, further studies of oxidative stress in the eryptotic signalling transduction network are highly needed and should be encouraged.

ROS Signalling in Erythronecroptosis

Brief overview of necroptosis in erythrocytes

Necroptosis is a regulated receptor-interacting protein kinase 1/receptor-interacting protein kinase 3/mixed lineage kinase domain-like protein (RIPK1/RIPK3/MLKL)-dependent necrosis. It is involved in the regulation of the host immune response due to its highly pro-inflammatory consequences and plays a significant role in pathogenesis of many diseases (Ye et al., 2023). Accumulating evidence suggests that necroptosis can also be executed in enucleated mature erythrocytes (Table S2). Despite a growing interest regarding necroptosis of erythrocytes, our knowledge of its triggers is limited. Several studies show that RBC necroptosis could be triggered by bacterial CD59-dependent pore-forming toxins (LaRocca et al., 2014, 2015), exposure to other pore-forming toxins under high glucose concentrations (LaRocca et al., 2016), or lipopolysaccharide (LPS) (Seo et al., 2023). Additionally, necroptosis is primed by RBC storage (McCaig et al., 2019). Pore formation and necrosome assembly are reported as prerequisites for erythronecroptosis but are not required for necroptosis of nucleated cells (LaRocca et al., 2015; McCaig et al., 2019). Similarly to nucleated cells, RIPK1 is a crucial component of necroptosis signalling in erythrocytes. Erythronecroptosis is associated with necrosome assembly (LaRocca et al., 2014, 2015), RIPK3-mediated MLKL phosphorylation, subsequent MLKL dissociation from the necrosome complex, and initiation of MLKL-dependent membrane permeabilization (LaRocca et al., 2016; McCaig et al., 2019; Seo et al., 2023). Necroptosis is mediated by extrinsic FAS signalling (LaRocca et al., 2014, 2015). Unexpectedly, TNF-α and TRAIL do not trigger erythronecroptosis despite the fact that they are well-established inducers of this cell death pathway in nucleated cells (LaRocca et al., 2015). Syk-dependent echinocyte (abnormal spiculated RBCs with relatively uniform distribution of surface spikes) formation has been reported to facilitate necroptosis in stored erythrocytes following exposure to pore-forming toxins (McCaig et al., 2019). Syk-mediated echinocyte formation occurs in response to CD59 ligation and is FasL-mediated (LaRocca et al., 2015). It is worthwhile to mention that glucose-derived advanced glycation end products (AGEs) were also reported as downstream effectors of necroptosis in RBCs. Enhancement of AGEs production was associated with accelerated glycolysis (LaRocca et al., 2014, 2016). Additionally, acid sphingomyelinase-derived ceramide seems to be required for erythronecroptosis signalling (LaRocca et al., 2014, 2015).

Contribution of ROS to necroptosis of erythrocytes

ROS signalling has been widely associated with necroptosis of nucleated cells (Hsu et al., 2020). Our summary of studies in enucleated mature erythrocytes suggests that ROS may be critically important in erythronecroptosis as well (Table S2). Indeed, LaRocca et al. (2014) documented ROS over-generation in necroptotic erythrocytes exposed to CD59-dependent pore-forming toxins. Moreover, this ROS overproduction seemed to be NOX-dependent. Consistently with this study, selective inhibition of NOX by VAS2870 and application of antioxidant N-acetylcysteine (NAC) reduced storage-primed pore-forming toxin-mediated erythronecroptosis (McCaig et al., 2019). McCaig et al. (2019) reported that NOX-derived ROS trigger phosphorylation and hence recruitment of RIPK1 and associated necrosome assembly. At the same time, ROS generated in an iron-dependent way through Fenton chemistry contributed little to pore-forming toxin-induced hyperglycaemia-primed erythronecroptosis (LaRocca et al., 2016). On the other hand, iron- and Fenton reaction-driven ROS overproduction mediated pneumolysin (a human CD59-independent pore forming toxin)-triggered necroptosis of erythrocytes (LaRocca et al., 2014, 2015).

Sources of ROS in erythronecroptosis

The ROS-producing enzyme NOX and ferrous iron seem to be the major sources of ROS during RBC necroptosis (Fig. 2). There are no data available regarding the possible role of Hb autooxidation-derived ROS and exogenous ROS. Furthermore, given the role of erythrocytes in NO metabolism (and the fact that NO is considered to be a third gas transported by RBCs) (Premont et

al., 2020), it seems important to assess the contribution of NO-derived RNS such as peroxynitrite to necroptosis of erythrocytes. To our knowledge, there are no data regarding depletion of RBC antioxidant components caused by necroptosis-associated ROS accumulation. It could be assumed that erythronecroptosis-associated ROS accumulation develops as a consequence of antioxidant system components depletion. More studies are needed to determine whether necroptosis of erythrocytes associates with alterations of the balance between ROS and antioxidants.

Conclusion and future perspectives

In this review, we have systematically summarized available data regarding the important role of ROS as a regulator of survival and cell death of erythrocytes (both eryptosis and erythronecroptosis). ROS signalling and its RBC death regulatory function are just being discovered with multiple unknown issues yet to be clarified. In particular, it is not yet clear how ROS signalling coordinates the crosstalk between eryptosis and erythronecroptosis or how it ultimately contributes to the RCD mode decision. It is well documented that ROS promote Ca^{2+} influx and caspase-3 activation in eryptosis, while ROS aid to activate RIPK1 in erythronecroptosis. What are the alternative redox-sensitive targets downstream of ROS signalling? Eryptosis could be triggered by ROS produced by multiple different pathways. However, less data is available on the sources of ROS in erythronecroptosis. Is there any direct link between the ROS source and the cell death modality of erythrocytes? Can localized ROS elevation induce cell death? Does Hb autooxidation- and XOR-derived ROS or RNS contribute to necroptosis of RBCs? Given that erythrocytes are constantly exposed to exogenous oxidants (Gwozdzinski et al., 2021), it is of interest to assess their possible involvement in the regulation of erythrocyte cell death. This issue becomes even more important with our better understanding of immunogenic consequences of apoptosis and necroptosis. While the impact of antioxidant depletion (with the consequent imbalance between oxidants and antioxidants) is clearly established for eryptosis, it has not yet been studied in erythronecroptosis.

Notably, accelerated eryptosis has been linked to anaemia in the end-stage renal disease, hypertension, and diabetes mellitus type 2 (Tkachenko and Onishchenko, 2023). Therefore, reduction of RBC clearance by pharmacological eryptosis inhibition might be therapeutically utilized for anaemia prevention and/or treatment in these conditions. Taken together, our review provides novel insights into the redox biology of erythrocytes and emphasizes that pharmacological stabilization of the redox homeostasis in erythrocytes might be a promising therapeutic strategy to prevent eryptosis and erythronecroptosis-mediated clearance of RBCs.

Conflict of interest

Authors have no conflict of interest to disclose.

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