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Mini review

POTENTIAL ROLES OF THE NFκB AND GLUTATHIONE PATHWAYS IN MATURE HUMAN ERYTHROCYTES

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Abstract: Anucleated erythrocytes were long considered as oxygen-transporting cells with limited regulatory functions. Components of different nuclear signaling pathways have not been investigated in those cells, yet. Surprisingly, we repeatedly found significant amounts of transcription factors in purified erythrocyte preparations, i.e. nuclear factor κB (NFκB), and major components of the canonical NFκB signaling pathway. To investigate the functional role of NFκB signaling, the effects of the preclinical compounds Bay 11-7082 and parthenolide on the survival of highly purified erythrocytes were investigated. Interestingly, both inhibitors of the NFκB pathway triggered erythrocyte programmed cell death as demonstrated by enhanced phospholipid scrambling

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Abbreviations used: Ca^{2+} – calcium; ERK2 – extracellular signal-regulated kinase 2; GPIIb/IIIa – glycoprotein IIb/IIIa; GSH – reduced glutathione; ICAM-1 – inter-cellular adhesion molecule; IFN-γ – interferon-γ; IκB-α – inhibitor of kappaB; IKK-α – IκB kinase-α; IL-6 – interleukin-6; IL-8 – interleukin-8; JNK1 – c-Jun N-terminal kinase 1; NFκB – nuclear factor κB; PS – phosphatidylserine; R – putative phosphatidylserine receptor

(phosphatidylserine exposure) and cell shrinkage. Anucleated erythrocytes are an ideal cellular model allowing the study of nongenomic mechanisms contributing to suicidal cell death. As NFκB inhibitors might also interfere with the anti-oxidative defense systems of the cell, we measured the levels of reduced glutathione (GSH) after challenge with the inhibitors. Indeed, incubation of erythrocytes with Bay 11-7082 clearly decreased erythrocyte GSH levels. In conclusion, the pharmacological inhibitors of the NFκB pathway Bay 11-7082 and parthenolide interfere with the survival of erythrocytes involving mechanisms other than disruption of NFκB-dependent gene expression. Besides affecting erythrocyte survival, NFκB inhibition and induction of erythrocyte phosphatidylserine exposure may influence blood clotting. Future studies will be aimed at discriminating between NFκB-dependent and NFκB-independent GSH-mediated effects of Bay 11-7082 and parthenolide on erythrocyte death.

Key words: Eryptosis, Nuclear factor kappa B, NFκB, IKK-α, IκB-α, Bay 11-7082, Parthenolide

EXPRESSION OF DIFFERENT COMPONENTS OF THE CANONICAL NFκB PATHWAY IN MATURE HUMAN ERYTHROCYTES

NFκB was first discovered and characterized 25 years ago in immune cells [1]. The predominant inactive form of cytoplasmic NFκB is a heterotrimeric protein consisting of RelA (p65), p50 and the inhibitor of κ B (IκB)-α subunit. Upon cellular stimulation via different surface receptors of the innate immune system as well as receptors for cytokines, e.g. Toll-like-receptors (TLRs), B cell receptor (BCR), T cell receptor (TCR) or tumor necrosis factor receptor (TNFR), IκB-α is phosphorylated on serine 32 and 36 by IκB kinase (IKK), followed by polyubiquitination and 26S proteasomal degradation. Activated NFκB then translocates to the nucleus as a p65-p50 heterodimer, where it acts as a transcription factor, thereby enhancing the expression of a variety of genes involved in the regulation of immune responses. NFκB signaling thus leads to the induction of various cytokines (IL-6, IL-8, IFN-γ), adhesion molecules (ICAM1), acute phase response proteins (complement factor B) and regulator proteins of cell survival. Taken together, NFκB plays an important role in a great variety of immune reactions [2-5].

Mature human erythrocytes are devoid of nuclei, and nuclear signaling should not play a functional role in this specialized cell type. However, we could repeatedly demonstrate by Western blotting that purified human erythrocyte concentrates from various volunteers expressed both subunits of active NFκB, namely RelA and p50 (Fig. 1). Further purification and dilution experiments clearly showed that the immuno-reactive bands did not originate from contaminating white blood cells or thrombocytes [6]. Mature erythrocytes expressed not only active NFκB but also the regulatory upstream elements of the NFκB signaling pathway IκB-α kinase (IKK) and IκB-α (Fig. 1). Taking into

account that the canonical pathway of NFκB activation, including the expression levels of its components, is tightly regulated, these results were quite astonishing. Recently, experimental evidence was provided that mature erythrocytes contain functional 20S proteasomes [7]. Due to proteasomal degradation, the halflife of IκB-α should thus be relatively short. However, the finding that IκB-α is expressed in mature human erythrocytes, which have an average life span of approx. 120 days, points to special mechanisms of protein stabilization.

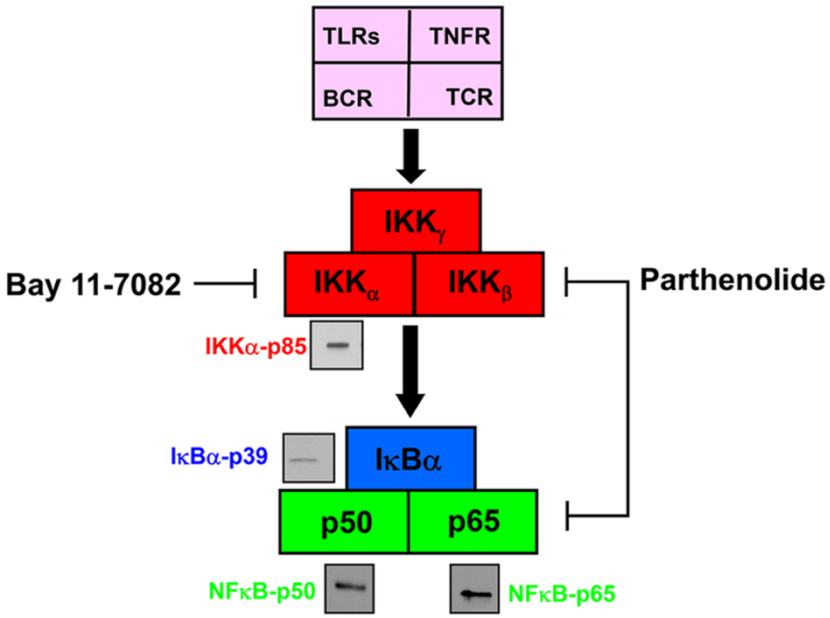

Fig. 1. Expression of components of the canonical NFκB signaling pathway in mature human erythrocytes. The scheme depicts the elements of the canonical NFκB signaling pathway including the upstream regulatory components. In addition, the expression of both subunits of active NFκB, namely RelA and p50, as well as IKK and IκB-α in mature human erythrocytes is shown. For this, erythrocyte extracts were subjected to Western blotting, and IKK1-α (85 kD), IκB-α (39 kD), RelA (65 kD) and the NFκB subunit p50 (50 kD) were detected using specific antibodies. Abbreviations: BCR – B cell receptor; IκBα – inhibitor of kappa B; IKK – IκBα kinase; p50 – 50 kD subunit of NFκB; RelA – 65 kD subunit of NFκB; TCR – T cell receptor, TLR – toll-like receptor; TNFR – tumor necrosis factor receptor.

The fact that mature erythrocytes still contain detectable amounts of the components of the NFκB pathway does not imply that the pathway is still functional. These “leftover” proteins might also represent functionally irrelevant remnants. To investigate whether NFκB plays a functional role in mature erythrocytes, we therefore tested different NFκB inhibitors in our experimental system.

TARGETING OF THE NFκB AND GLUTATHIONE PATHWAYS BY BAY 11-7082 AND PARTHENOLIDE

Since NFκB signaling is crucial for the activation and regulation of various immune responses, the different subunits of the protein and the other regulators of the NFκB signaling pathway were immediately recognized as important drug targets. Indeed, there are hundreds of inhibitors available to block NFκB activity [8]. From these inhibitors, we chose two chemically unrelated candidates that selectively block at different sites of the NFκB pathway (Fig. 1 and Tab. 1). The synthetic and lipophilic (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile (Bay 11-7082) blocks the kinase activity of IKK, thereby preventing IκB-α phosphorylation and degradation [9], and the sesquiterpene lactone (SL) and active component of the medical herb feverfew (*Tanacetum parthenium*) parthenolide blocks IKKα [10] and the NFκB subunit p65 (RelA) [11, 12]. In addition, it could be shown that a methylene-carbonyl substructure is crucial for SL-based inhibition of RelA at cysteine 38 [13].

Tab. 1. Targeting of the NFκB pathway in erythrocytes by specific inhibitors

Inhibitor	Target(s)	EC_{50} PS exposure	EC_{50} Cell shrinkage	Intracellular Ca^{2+} level[d]
Bay 11-7082[a]	IKK	11 μM[c]	11 μM[c]	28%[c,d]
Parthenolide[b]	IKK and RelA	30 μM[c]	35 μM[c]	19%[c,d]
Bay 11-7082[c]	GSH	EC_{50} GSH depletion 2 μM[c]	EC_{100} GSH depletion ≥ 7.5 μM[c]	

[a][9], [b][10, 11], [c][6], [d]Ca^{2+} level is expressed as the percentage of maximum ionomycin-induced Ca^{2+} increase

We treated erythrocytes with pharmacological doses of these inhibitors and found that both inhibitors were very effective inducers of programmed erythrocyte death, as characterized by phosphatidylserine (PS) exposure, cell shrinkage and elevation of intracellular Ca^{2+} (Tab. 1). PS exposure and cell shrinkage are the hallmarks of eryptosis, the suicidal death of erythrocytes [14-16]. In control experiments, we excluded a non-specific lytic effect of the NFκB inhibitors at the concentrations used [6]. Thus, these results argued in favor of a functional role of NFκB signaling in erythrocyte survival.

The effects of Bay 11-7082 and parthenolide on erythrocyte programmed cell death described in the previous paragraph were induced by pharmacological concentrations of the inhibitors. Thus, it seems reasonable that the inhibitors exert their effects on erythrocytes by inhibition of a currently unknown function of NFκB. Nevertheless, at least for parthenolide, it is already known that this drug might also influence the anti-oxidative defense of cells by lowering the level of reduced glutathione (GSH) [17-19]. In a chemical reaction, GSH is added to the double bond of parthenolide, resulting in a parthenolide-GSH-adduct (Fig. 2A). GSH depletion has previously been shown to trigger eryptosis [6].

Theoretical considerations and the comparison of the chemical structure of Bay 11-7082 with the structure of parthenolide led us to the speculation that Bay 11-7082 might also react with GSH to form Bay 11-7082-GSH adducts (Fig. 2B). As in the case of parthenolide, this reaction should decrease the level of GSH in erythrocytes. Indeed, treatment of erythrocytes with Bay 11-7082 completely depleted GSH from the cells with an IC_{50} of approximately 2 μM [6]. Taken together, the NFκB pathway inhibitors Bay 11-7082 and parthenolide induce erythrocyte cell death by simultaneously targeting NFκB signaling and the anti-oxidative defense system of the cells (Fig. 2C). Thus, the link between inhibitor-induced eryptosis and the presence of the NFκB pathway in mature human erythrocytes is elusive and needs further investigations. To clearly demonstrate an unconventional nongenomic role of NFκB in erythrocytes, future studies using more direct genetic approaches with targeted modifications of individual components of the NFκB pathway are needed. For instance, the effects of Bay 11-7082 and parthenolide on erythrocytes from IKK- [20] or p50/NFκB-deficient mice [21] could be analyzed.

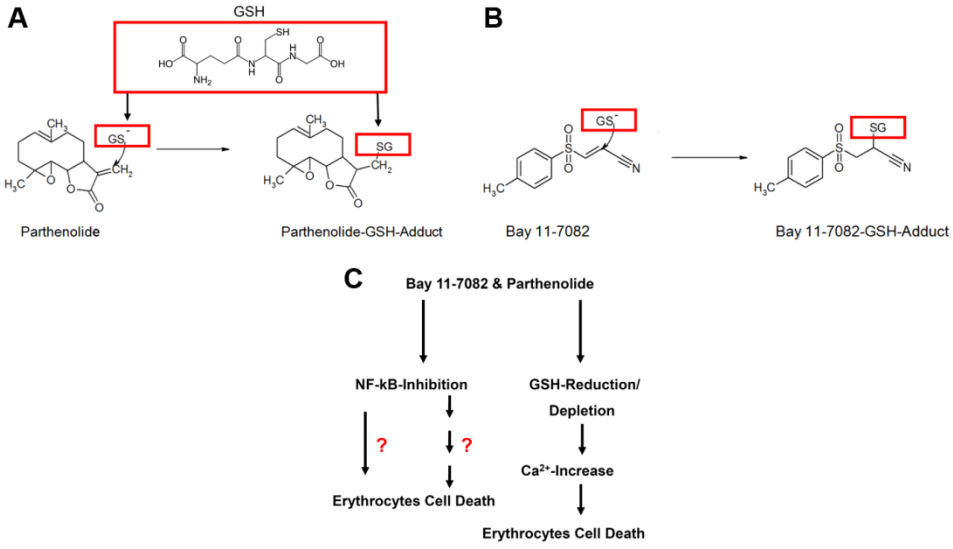

Fig. 2. Targeting of glutathione-dependent anti-oxidative defense by NFκB inhibitors. A – Reduced glutathione might chemically react with parthenolide (nucleophilic addition to a double bond) to form parthenolide-GSH adducts, thereby lowering the GSH level of cells [17]. B – Bay 11-7082 also contains a double bond which might be susceptible to a nucleophilic attack. This reaction would likewise lead to the formation of Bay 11-7082-GSH adducts and depletion of GSH in the cell. C – The scheme depicts NFκB signaling and GSH depletion as the main targets of Bay 11-7082 and parthenolide in erythrocytes. Interference with both pathways finally increases Ca^{2+} and leads to programmed erythrocyte death. GSH, reduced glutathione; NFκB, nuclear factor κB.

It should be kept in mind that there may be a direct interaction between the oxidative status of the erythrocytes and the expression levels of the members of the NFκB pathway. Recent results from our laboratory showing a correlation between GSH and NFκB expression levels in erythrocytes with different age support this assumption (data not shown).

POSSIBLE ROLE OF THE NFκB AND THE GLUTATHIONE PATHWAY IN BLOOD CLOTTING

NFκB is expressed in nucleus-free erythrocytes [6] and thrombocytes [22]. Thrombocytes play an essential role in hemostasis (blood clotting in response to injury) and in pathological conditions such as inflammation, immunity and tumor progression [23]. Recent observations revealed that the pharmacological drugs Bay 11-7082 and parthenolide also affect the function of thrombocytes [24].

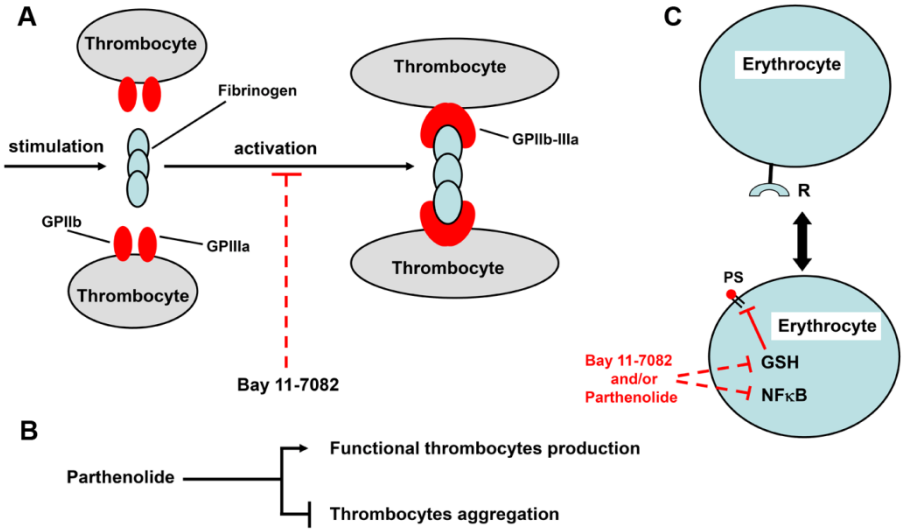

Fig. 3. Effects of NFκB inhibitors on adhesion of thrombocytes and erythrocytes. A – After stimulation and activation, thrombocytes aggregate via fibrinogen-mediated glycoprotein IIb/IIIa interaction (modified according to Dangel, [35]). Anti-thrombocyte agents, such as Bay 11-7082, inhibit this activation, thereby preventing thrombocyte aggregation [24]. B – Parthenolide is able to stimulate functional platelet production by inhibition of NFκB signaling [25] and to reduce thrombocyte aggregation [26]. C – Bay 11-7082 and parthenolide also inhibit the anti-oxidative defense and/or NFκB signaling in erythrocytes and concentration-dependently induce phosphatidylserine (PS) exposure on the erythrocyte surface. PS on the cellular surface then interacts with its putative receptor on neighboring erythrocytes (see double back arrow), and leads to intercellular adhesion of erythrocytes. Abbreviations: GPIIb-IIIa – glycoprotein IIb/IIIa; GSH – reduced glutathione; NFκB – nuclear factor κB; PS – phosphatidylserine; R – putative phosphatidylserine receptor.

As illustrated in Fig. 3A, Bay 11-7082 is able to reduce fibrinogen binding to glycoprotein GPIIb-IIIa (also named αIIbβ3 according to integrin nomenclature), thereby preventing thrombocyte aggregation. In addition, Bay 11-7082 inhibits thrombocyte activation and aggregation, and forces them to retain their granular contents, effects that are mediated by inhibition of JNK1 and ERK2 phosphorylation [25]. The fact that fibrinogen positively regulates NFκB activation and expression of inflammatory chemokines in endothelial cells [26] shows the multifunctional importance and applicability of such anti-inflammatory drugs as Bay 11-7082. Recently, it was shown that the electrophilic and anti-oxidant compound parthenolide stimulates functional thrombocyte production by inhibition of NFκB signaling in megakaryocytes [27]. Parthenolide is also able to inhibit thrombocyte aggregation [28] (Fig. 3B), and it was reported more than three decades ago that depletion of reduced glutathione (GSH) by the thiol oxidizing agent "diamide" resulted in immediate inhibition of thrombocyte aggregation and clot retraction [29]. These observations point to an important role of GSH in thrombocyte function. In line with this, Bay 11-7082 and parthenolide, with their capabilities to deplete reduced glutathione (GSH) and to inhibit NFκB activity [6], could control thrombocyte functions and blood clotting, and inhibit the risk of thrombosis. On the other hand, Bay 11-7082 and parthenolide also target NFκB and GSH in erythrocytes, and the challenge of erythrocytes with the inhibitors leads to increased PS exposure on the outer leaflet of the plasma membrane. PS then binds to its receptor [30] expressed on adjacent erythrocytes or on other cell types, such as macrophages or endothelial cells (Fig. 3C). Thus, increased adherence of erythrocytes might enhance the stabilization of an already formed blood clot [31, 32] or even result in blood clotting in some special circumstances, for example during sickle cell crisis [33], infection with plasmodia [34] or other pathological conditions. It is therefore crucial to analyze the concentration-dependent effects of Bay 11-7082 and parthenolide on thrombocyte aggregation to assess the minimal effective dose at which thrombocytes aggregation is inhibited by reduction of their adhesion molecules. As previously shown, low dose inhibitor concentrations ($\leq$ 5 μM for Bay 11-7082 and $\leq$ 10 μM for parthenolide) do not lead to erythrocyte cell death [6]. Due to the very effective depletion of GSH by Bay 11-7082 (IC_{50} = 2 μM; see also Tab. 1), it is tempting to speculate that low concentrations of the substance may reduce the expression levels of adhesion molecules on the thrombocyte surface without leading to cell death.

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