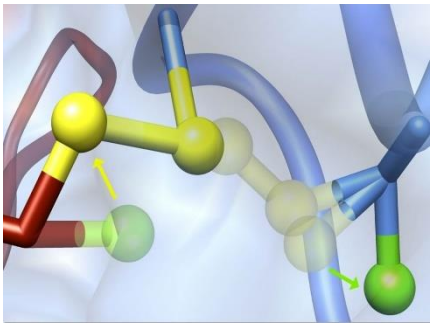
SPP 1710/2

Dynamics of Thiol-based Redox Switches in Cellular Physiology



Targeting spectrin redox switches to regulate the mechanoproperties of red blood cells

Cortese-Krott, Miriam M. & Gohlke, Holger
Heinrich Heine University
Düsseldorf

Project Description

1 State of the art and preliminary work

1.1 State of the art

RBC function, structural characteristics and mechanoproperties. Red blood cells (RBCs) are anucleated, biconcave shaped cells specialized in compartmentalization and transport of oxygen (O_2) from the lungs to peripheral tissues, and bring metabolic waist from the tissues in form of carbon dioxide (CO_2) to the lungs to be exhaled[1].The biochemical, biological, and biophysical characteristics of RBC are highly specialized for this purpose[2]. Loss of RBC integrity (or hemolysis) and release of Hb into the circulation induce toxic effects, including systemic nitric oxide (NO) scavenging and oxidation reactions[2]. The mechanical and biophysical properties of RBC are particularly important for RBC physiology[2]. RBC need to be elastic and flexible to survive the dramatic changes in flow conditions along the vascular tree, and to be able to change their shape in the narrower vessels of the microcirculation[1]. Interestingly, pathological conditions leading to changes in redox state and/or oxidative modifications of RBC proteins via reactive species, including diabetes or genetic defects of antioxidant enzymes, e.g. glutathione peroxidase (GP_x), are known to affect cell shape, RBC deformability, membrane stability and resistance to osmotic stress, as well as half-life of RBC in the circulation and/or induce hemolytic anemia[2]; these effects are also induced by treating RBC *in vitro* with reagents targeting thiol groups[3-6], including *N*-ethylmaleimide (NEM) and diamide[3, 5, 7, 8]. However, the specific redox switches regulating the stability of the RBC membrane and RBC mechanoproperties are not known.

Structural and functional characteristics of spectrin. RBC integrity, biconcave shape and mechanical properties of RBC are mainly defined by the very peculiar structural dynamics of their cytoskeleton[9], and by the presence of the erythrocyte-specific isoform of spectrin (Sp1). Spectrin is a very large (220 kDa) fibril-shaped protein, composed of antiparallel α and β subunits, which consist of 20 and 16, respectively, full three-helix bundle structures called spectrin repeats (SR), twisted around each other and associated head-to-head to form dimers and tetramers[10]. Spectrin fibrils are arranged to form the sides and the diagonals of a hexameric unit, and are connected to each other via nodes of short actin polymers, stabilized by adducin and band 4.1. The spectrin hexameric units form a flexible network under the lipid bilayer[9], and are connected to the transmembrane protein band 3[9, 11] via ankyrin. The biconcave shape of the RBC is due to the constant tension of the spectrin fibrils[12]. There is strong evidence that chemical modification of thiols in spectrin affects the structure and stability of the RBC cytoskeleton, membrane symmetry, and RBC shape[3-5, 13]. The localization of cysteine redox switches (CRS) on spectrin are unknown, also because of the lack of a structural spectrin model with sufficient resolution. The nature/reversibility and the conditions leading to spectrin modifications have remained elusive either.

1.2 Preliminary work

Enzymatic and non-enzymatic production of reactive species in RBCs. The chemistry and biochemistry of reactive species in RBCs is very complex. Reactions leading to heme degradation lead to the production of reactive oxygen species (ROS) including $O_2^{-\bullet}$, $OH^{\bullet}$, and

H_2O_2[2], which are normally rapidly detoxified by a battery of antioxidant systems comprising SOD, catalase, GP_x and the GSH recycling system, as well as thioredoxin and glutaredoxin. RBC are know for scavenging NO (via reaction with oxyhemoglobin), but also to transport NO / NO metabolites, and to produce NO and reactive nitrogen species (RNS), e.g. N_2O_3 and other nitrosating species, from nitrite under hypoxic conditions. In addition, the **Cortese-Krott lab** demonstrated that RBC carry a catalytically active endothelial NO synthase (eNOS) and produce RNS under normoxic conditions[i, ii]. Moreover, blood cell eNOS participates in the control of RBC velocity in the microcirculation[iii], and *in vivo* in regulation of circulating nitrite levels and blood pressure[iv]. Interestingly, RBC were shown to metabolize and produce also sulfide and reactive sulfur species [14]. Recently, the **Cortese-Krott lab** found that the chemical interaction between NO or nitrosothiols with sulfide leads to formation of S/N hybrid species, which may contribute to the bioactivity of both sulfide and NO, including modulation of blood pressure and heart contractility[v], as well as redox control, likely via post-translational modifications in CRS in Keap-1[vi]. The outcome of these chemical interactions or their effects on redox state and CRS in RBCs is not known.

Putative cysteine redox switches in spectrin. There is preliminary evidence that spectrin carries CRS. Treating isolated spectrin with monovalent or divalent thiol group-targeting molecules such as *N*-ethylmalemide (NEM) or diamide leads to formation of cysteine disulfide and/or destabilization of its interaction with band 4.1 and actin[3, 4]. As pointed out by Jones *et al*.[15], regulatory cysteines in proteins are often highly conserved among vertebrates, and spectrin is a highly conserved protein. Goodman found conserved cysteines in the C-terminus region of both α chains of all erythrocytic and non-erythrocytic isoforms of spectrin (Sp1 and Sp2), including Cys 2071 and 2100, which are targeted by ubiqitinylation after cysteine oxidation. Preliminary analysis carried out by us comparing α and β-Sp1 sequences of humans, rodents and fish by using the alignment tool of uniprot.org revealed a few highly conserved cysteines in both chains, e.g. Cys 74, 11,604, 619, 961, 964, 1284 and 1891 in the β-chain. Shear stress induces exposure of Cys 1877 and 595, respectively of mouse α and β-Sp1, which are conserved in the human sequences[16]. Further analysis by comparing the α and β chains with each other, and erythrocytic and non-erythrocytic isoforms, as well as their localization on the tertiary and quaternary structure, and their chemical accessibility and chemical properties (p*K*a, redox potential) will reveal, which of these cysteines are good candidates for redox switches.

Targeting spectrin cysteine redox switches with NO. Several studies indicate that intracellularly produced NO and RNS in RBCs may affect RBC deformability[17-19] and RBC velocity[iii] but the molecular mechanisms are unknown. One work proposed that nitrosation of spectrin may play a role in the effects of NO[19]. However, the effects of extracellular NO are not without controversy[18]. As a proof of concept, we aimed to determine whether human spectrin thiols can be nitrosated. Purified spectrin or RBC ghosts (i.e., RBC depleted of hemoglobin and other cytoplasmatic proteins) were treated with the nitrosothiol *S*-nitroso cysteine (SNOC); nitrosation was then analyzed by (a) reduction of *S*-nitrosospectrin by using acetic triiodide solution and detection of released NO by chemiluminescence (by applying a method established for albumin[20]), and (b) biotin switch assay, followed by western blotting. As a positive control, human serum albumin was analyzed in parallel. We found that treatment with SNOC resulted in nitrosation of both of purified spectrin, as well as spectrin included in the intact cytoskeleton of RBC ghosts. Localization and effects of nitrosation of spectrin and/or cytoskeletal proteins on

their structural and functional properties are not known.

Development of molecular modeling and simulation methods and application studies. Research in the **Gohlke lab** focuses on investigating, predicting, and modulating molecular interactions of biomacromolecules and on analyzing structure, energetics, and dynamics of biomolecules. The **Gohlke lab** develops and applies approaches from the fields of computational biophysics and molecular bioinformatics, including MD simulations,[21] free energy calculations,[vii] and techniques for protein structure[viii, xi] and complex[x, 22] prediction, and is part of the developers team of the AMBER package of molecular simulation programs[21, 23, 24].

Structural model of spectrin at the atomistic level. The molecular origins of spectrin flexibility and its elastic properties have been investigated by atomistic and coarse-grained molecular simulations[25], based on crystal structures of up to four SRs[26] and the tetramerization domain complex[27]. Likely, the most advanced quaternary structure of the full-length spectrin heterotetramer is the *chinese finger trap* model by Brown *et al*[28]. For investigating the location of cysteine switches and their role on spectrin properties at an atomistic level, a structural model with sufficient resolution to determine the accurate interacting surfaces between each complimentary pair of repeats is required. However, such a model has not yet been presented. For generating a structural model of the α/β-β/α-complex of human SpI at the atomistic level, the **Gohlke lab** performed all-atom MD simulations of 500 ns length with the Amber ff14SB force field for three SR of β spectrin (PDB ID: 3EDV[29]), which revealed that the SR are structurally stable, whereas pronounced relative motions between the repeats occur,[28] in accordance with electron microscopy results that showed marked conformational changes of spectrin length and diameter[30]. This information was used to generate a structural model of the α/β-β/α-complex of SpI, together with (a) distance information derived from cross-linking experiments between the α3 and α4 units of SpI[28, 31], (b) the length of the spectrin α/β-β/α-complex derived from the protein composition of the erythrocytes[28] and (c) in-house cross-linking experiments on the full α/β-β/α-complex of SpI, in collaboration with Dr. G. Poshmann and Prof. K. Stühler (Proteomics Core Facility, BMFZ, HHU/UKD) using cross-links of varying length (DSS, EDC, BS3, EGS) among 21 inter- and intramolecular cross-links, one connecting two SR (K1201 with K1381) confers long-range information. The angles between two SR of PDB ID: 3EDV were adapted according to distance restraints of Brown *et al*.[28] and the new cross-link between K1201 and K1381, considering the variability in distances displayed by cross-links[32]. The helical structure of one spectrin α/β chain[30] was generated by repeatedly superimpositioning the copies of that SR, yielding a structural model of 38 SR[29] of SpI. The sequence of human α/β spectrin is 23% identical (53% similar) to the sequence of this structural model. Thus, the 38-SR model was used as a template for comparative modeling with the in-house tool TopModel[viii, ix]. Finally, two α/β-chains were manually arranged such that they fulfil cross-links information [31], resulting in the tetrameric α/β-β/α-complex. Our and the *chinese finger trap* model[28] have an overall length, SR number per turn, handedness, hollowness, and intra-subunit and inter-subunit distances in agreement with experiment[28]. However, both models vary in the roundness of the helix due to different inter-SR angles. The difference might arise because former studies used distance information from cross-link experiments of shorter spectrin fragments[28, 33], rather than full-length SpI as in our case. Hence, additional inter-SR and inter-subunit cross-links, providing distance

information on sequentially distant amino acids or amino acids on the two subunits, are required to more accurately define the pitch and helix twist of SpI.

Modulation of RBC deformability and blood viscosity by NO and oxidants. The effects of oxidative modifications of the spectrin cytoskeleton on RBC rheology were assessed by using ectacytometry in a laser rotational cell analyses (LORCA, Mechatronics, NL) and a low shear rotational viscometer (LS300, ProRheo, GE)[34]. Oxidation of spectrin with *tert*-buthylhydroperoxide (*t*-BuOOH) was shown to affect structure and electrophoretic properties of spectrin[6]. We found *t*-BuOOH induces a concentration-dependent decreased deformability and increased blood viscosity. Although erythrocyte deformability and blood viscosity were not affected by treatment with increasing concentrations of SNOC, pretreatment with SNOC protected RBCs from *t*BuOOH-induced decrease of deformability, indicating that SNOC may protect cytoskeletal proteins from oxidation. Since treatments were not added at the same time, this indicates that the antioxidant effects of SNOC were not due to direct reactions with *t*-BuOOH, but rather may be dependent on protection of defined CRS. The targeted proteins and CRS, which may be responsible for these effects, need to be identified.

1.3 Project-related publications

1.3.1 Articles published by outlets with scientific quality assurance

i. **Cortese-Krott MM**, Rodriguez-Mateos A, Sansone R, Kuhnle GG, Thasian-Sivarajah S, Krenz T, Horn P, Krisp C, Wolters D, Heiß C, Kröncke KD, Hogg N, Feelisch M, Kelm M. Human red blood cells at work: identification and visualization of erythrocytic eNOS activity in health and disease. *Blood.* 2012 Nov15;120(20):4229-37.

ii. **Cortese-Krott MM,** Rodriguez-Mateos A, Kuhnle GG, Brown G, Feelisch M, Kelm M. A multilevel analytical approach for detection and visualization of intracellular NO production and nitrosation events using diaminofluoresceins. *Free Radic Biol Med.* 2012 Dec 1;53(11):2146-58.

iii. Horn P*, **Cortese-Krott MM***, Keymel S, Kumara I, Burghoff S, Schrader J, Kelm M, Kleinbongard P. Nitric oxide influences red blood cell velocity independently of changes in the vascular tone. *Free Radic Res.* 2011 Jun;45(6):653-61.

iv. Wood KC*, **Cortese-Krott MM**,* Kovacic JC, Noguchi A, Liu VB, Wang X, Raghavachari N, Boehm M, Kato GJ, Kelm M, Gladwin MT. Circulating blood endothelial nitric oxide synthase contributes to the regulation of systemic blood pressure and nitrite homeostasis. *Arterioscler Thromb Vasc Biol*. 2013 Aug;33(8):1861-1871

v. **Cortese-Krott MM**, Kuhnle GG, Dyson A, Fernandez BO, Grman M, DuMond JF, Barrow MP, McLeod G, Nakagawa H, Ondrias K, Nagy P, King SB, Saavedra JE, Keefer LK, Singer M, Kelm M, Butler AR, Feelisch M. Key bioactive reaction products of the NO/H_2S *interaction are S*/N-hybrid species, polysulfides, and nitroxyl. *Proc Natl Acad Sci USA.* 2015 Aug 25;112:E4651-60.

vi. **Cortese-Krott MM**, Pullmann D, Feelisch M. Nitrosopersulfide (SSNO(-)) targets the Keap-1/Nrf2 redox system. Pharmacol Res. 2016 Sep 20;113(Pt A):490-499.

vii. Homeyer N, Stoll F, Hillisch A, **Gohlke H.** Binding free energy calculations for lead optimization: Assessment of their accuracy in an industrial drug design context*. J Chem Theory Comput.* 2014;10:3331−3344.

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x. Greife, A., Felekyan, S., Ma, Q., Gertzen, C.G.W., Spomer, L., Dimura, M., Peulen, T.O., Wöhler, C., Häussinger, D., **Gohlke, H.**, Keitel, V., Seidel, C.A.M. Structural assemblies of the di- and oligomeric G-protein coupled receptor TGR5 in live cells: an MFIS-FRET and integrative modeling study. *Sci Rep.* 2016, 6, 36792.

1.3.2 Patents (*pending*)

Metz A, Gohlke H, Schanda J, Wichmann C, Grez M. Inhibitors of NHR2 and/or RUNX1/ETO-tetramerization. EP/30.04.13/EPA 13165993, 2014

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2.1 Composition of the project group

In the **Cortese-Krott lab** will be directly involved in the project but will not be paid out of the project funds: Lukas Diederich, MSci, Sivatharsini Thasian-Sivarajah, BSci, research assistant, will assist with the training of the PhD student enrolled for this project; Stefanie Becher, VMTA, will help with animal experiments including treatments, blood and organ sampling. In the **Gohlke lab,** Dr. Markus Dick, Postdoc, has generated the computational results and will support the project and assist in guiding the PhD student in terms of molecular modeling and simulation work; Birte Schmitz, PTA, will perform and analyse molecular simulations..

2.2 Cooperation with other researchers

2.2.1 *Researchers with whom you have agreed to cooperate on this project*

We will continue long-term collaborations with Prof. Dr. Martin Feelisch, University of Southampton, UK, for measurements of NO and sulfide metabolism in cardiovascular physiology and Prof. Dr. Brant Isakson, for the analysis of microcirculation. We will collaborate with Dr. G.Poshmann and Prof. K. Stühler (HHU), Prof. Dr. Hermann Gaub (LMU), for atomic force microscopy analysis, and Prof. Dr. Gerhard Gompper (FZ Jülich), for mesoscale simulations.

2.2.2 *Researchers with whom you have collaborated scientifically within the past three years*

The **Cortese-Krott lab** collaborated on related projects with the **Gohlke lab**, and with M. Feelisch and B. Isakson.