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Topological transformation of liposomes by a membrane-affecting domain of recombinant human erythropoietin

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Abstract

Recombinant human erythropoietin (rh-Epo) is well accepted as a hematopoietic drug, but many other pleiotropic properties are currently under investigation. Rh-Epo-induced receptor-mediated signal transductions are accompanied with membrane dynamic processes, which facilitate the activation of individual pathways. However, its direct effect on membrane dynamics is still unknown. In the present study, we have proven the capability of rh-Epo to associate to and transform artificial lipid membranes. Association studies using neutral, negatively, and positively charged liposomes with the native as well as modified rh-Epo were performed and analyzed by transmission electron microscopy and differential scanning calorimetry. By these studies, we demonstrated that rh-Epo has the capability to transform negatively charged unilamellar vesicles into so-called disc-like micelles. Rh-Epo association to the negatively charged head groups via lysine and arginine initiates this transformation. At physiological temperatures, conformational changes within the rh-Epo structure expose a defined amino-acid sequence, which is able to induce the formation of discoid membrane structures. Enzymatic digestion, analysis, and isolation of related peptides by rp-HPLC and characterization by MS/MS enabled the identification of the membrane-affecting domain of rh-Epo (MAD-E) that represents the exposed helix B of rh-Epo. Finally, association studies performed with these peptides confirmed that the MAD-E is responsible for the formation of disc-like micelles. Since this helix B of rh-Epo has recently been supposed to be involved in the activation of neuroprotective pathways, we believe that the membrane-transforming capacity of rh-Epo participates in the proliferative activity of rh-Epo.

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Keywords

Disc-like micelles; erythropoietin; liposomes; membrane-protein interaction; topological transformation

Introduction

Recombinant human erythropoietin (rh-Epo) is a highly glycosylated protein hormone with a molecular weight of 30.4 kDa that induces the maturation of progenitor cells into mature erythrocytes (Carnot and DeFlandre, 1906; DePaolis et al., 1995; Fisher, 2003; Jelkmann, 2000, 2007). The erythropoietic effect of rh-Epo results from binding, reorientation, and activation of the Epo-receptor homodimer (Epo-R₂) (Fisher, 2003; Matthews et al., 1996; Seubert et al., 2003; Tilbrook and Klinken, 1999). Since the approval of rh-Epo in patients with chronic renal failure, a wide range of pleiotropic properties of rh-Epo, such as neuroprotective properties in ischemia and spinal cord injury, stimulation of angiogenesis and macrophages, as well as cardiovascular protective functions, have been investigated (Arcasoy, 2008; Brines et al., 2008; Fisher, 2003; Haroon et al., 2003; Jelkmann, 2005, 2007; Lykissas et al., 2007; Smith et al., 2003). Modification of the rh-Epo molecule through carbamylation inhibits the erythropoietic function but retains its neuroprotective activity. The carbamylation affects primarily the lysine residues, which were identified as Epo-R₂-binding sites (Brines et al., 2004; King et al., 2007; Leist et al., 2004). In neuroprotection, an alternative pathway is activated after the formation of a heterodimeric receptor complex different to Epo-R₂ (Leist et al., 2004; Sirén et al., 2001; Wang et al., 2007). In both cases, the membrane environment and dynamics play a crucial role (Constantinescu et al., 2001a, 2001b; Sengupta et al., 2007). However, no studies on the interaction potency of Epo with membranes are currently available.

Therefore, the present study was focused on the determination and elucidation of the membrane-modulating activity of rh-Epo. To mimic the electrostatic association of rh-Epo close to the membrane surface, we used egg phosphatidyl glycerol (EPG)-containing liposomes to generate negatively charged binding sites.

The evaluation of membrane transformation mechanisms, conformational changes of the rh-Epo structure, and binding modalities led to the identification of a membrane-perturbing protein domain. To get more insight into the complex membrane-protein interaction mechanisms, the membrane modulations induced by rh-Epo and modifications thereof were tested on liposomes with varying lipid compositions.

Materials and methods

Lipids

HSPC (fully hydrogenated soy phosphocholine), DSPE-PEG2000 (distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol 2000), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), and EPG (egg phosphatidyl glycerol) were purchased from Lipoid (Ludwigshafen, Germany). Cholesterol (CHOL) was obtained from Solvay (Weesp, The Netherlands). DOTAP (1,2-dioleoyl-3-

trimethylammonium-propane) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA).

rh-Epo and modifications

rh-Epo was provided by Polymun Scientific GmbH (Vienna, Austria). The purified protein displayed a negative net charge (pI 5.6) in PBS (phosphate-buffered saline: $\text{Na}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$: 1.44 g/L, KCl: 0.2 g/L, KH_2PO_4 : 0.2 g/L; NaCl: 8 g/L; pH 7.4).

Reduced Epo (red-rh-Epo) was generated by reduction through incubation with DTT (dithiothreitol; 4.8 mg/mL) in PBS for at least 150 minutes at room temperature.

Carbamylated Epo (c-rh-Epo) was produced by incubating rh-Epo with 49 mg of KOCN per mg rh-Epo in 0.3 M of sodium borate buffer (pH 8) for 48 hours at 37°C (Leist et al., 2004). Lysine-carbamylation was confirmed by Lys-C digestion, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of liposomes

Multilamellar vesicles (MLVs) were prepared by the classical film rehydration method with various lipid compositions (HSPC, DOTAP, EGP, and EPG_{LOW}), as shown in Table 1 (Kasi et al., 1984; Perez-Soler et al., 1985; Van Slooten et al., 2001; Walde and Ichikawa 2001). Lipids (8–12 mg/mL) were dissolved in 10–30 mL of chloroform, and the solvent was vacuum evaporated while rotating at 35°C in a water bath. These lipid films were rehydrated in PBS above their phase-transition temperature (T_m).

Unilamellar Vesicles (ULVs) were prepared by down-sizing the MLVs by extrusion through 400, 200, and finally 100 nm of polycarbonate Whatman membranes (GE Healthcare, Vienna, Austria) to a mean size of 80–110 nm (extruder: Avestin, Mannheim, Germany). Extrusion temperatures were selected above the individual T_m s. For 400- and 200-nm membranes, extrusion cycles were repeated 3 times and for 100 nm 7 times at a working pressure of 2–by 50 bar (Olson et al., 1979). The mean size of ULVs was determined using DLS (dynamic light scattering; Zetasizer Nano-ZS; Malvern Instruments, Malvern, UK). Lamellarity was determined by transmission electron microscopy (TEM).

Association of Epo and peptides onto ULVs

For interaction experiments, pure unilamellar vesicles (ULVs) were generated according to the lipid composition of each formulation given in Table 1. Afterward, rh-Epo, red-rh-Epo, c-rh-Epo, or isolated peptides after trypsin digestion of rh-Epo were diluted in PBS and added to various liposome formulations to reach a final lipid-drug ratio of 1:1.5 (w/w). To verify the influence of the negative liposomal surface net-charged, the molar ratio of EPG was used in two different concentrations. EPG-ULVs with a higher EPG-concentration resulted in an EPG-drug ratio of 0.2:1 (w/w). Otherwise, EPG_{LOW} -ULVs were used in an EPG-drug ratio of 0.04:1 (w/w). Samples were incubated for 35 minutes above the T_m and at room temperature (20°C) and analyzed by TEM.

TEM

Briefly, 300-mesh copper grids, coated with Pioloform film (Agar Scientific, Stansted, UK), stabilized by carbon evaporation, and freshly glow discharged, were floated for 30 seconds, face down, on the surface of approximately a 10- μ L sample. For negative staining, the samples were incubated on 1 drop of a 1% uranyl acetate solution in water for 1 minutes (Hayat and Miller, 1990). After removal of the excess stain, the grids were air-dried. The specimens were analyzed in a Philips CM12 electron microscope (Eindhoven, The Netherlands), operating at 80 keV.

Differential scanning calorimetry (DSC)

Thermodynamic measurements were performed by using a VP-DSC differential scanning microcalorimeter, and data were analyzed with the appropriate software (both MicroCal, Milton Keynes, UK). Samples were degassed for a minimum of 30 minutes. Measurements were performed under the following conditions: temperature range: 10–70°C, scan rate: 30°C/hour, prescan duration: 15 minutes, gain: high. The T_m was defined as the temperature at the peak maximum. Calorimetric enthalpies (ΔH) were calculated by integrating the peak areas after baseline adjustment and normalization to the mass of phospholipids.

Digestion of Epo-EPG-ULVs and peptide mapping

Rh-Epo and Epo-EPG-ULVs were digested with trypsin by adding 10 μ L of trypsin solution (1 mg/mL; Merck, Vienna, Austria) to each sample and incubated for 24 hours at 37°C. Liposomes were removed from the peptides by diafiltration at 4°C (molecular weight cut-off: 100 kDa; Microcon; Millipore, Vienna, Austria). The peptides in the diafiltrate were separated on a rp-C4 column (250 $\times$ 4.6 mm, 5 μ m; YMC-Pack-Pro C4; YMC-Europe, Dinslaken, Germany), using a high-performance liquid chromatography (HPLC)-HP1100 equipped with a quaternary pump module (Agilent, Vienna, Austria). The flow rates were 0.75 and 1.25 mL/minute, respectively, and the column temperature was adjusted to 20 $\pm$ 2°C. Next, 50 μ L of each sample were injected, separated, and detected at a wavelength of 214 nm. The mobile phase consisted of a gradient of solvent A (0.06%; v/v) trifluoroacetic acid (TFA in H₂O) and solvent B (90%; v/v) acetonitrile [10% H₂O, 0.06% (v/v) TFA]. The gradient was as follows: 0% B over 10 minutes, 0–61% B in 115 minutes, 61–83% over 10 minutes, and 83–100% B over the next 10 minutes, with 100–0% B in 1 minute and 0% B for the last 5 minutes. Operation, acquisition, and analysis were performed by using the ChemStation software package (Agilent). Peptide patterns of the digested rh-Epo and rh-Epo-EPG-ULVs were compared and peak-area ratios ($\text{area}_{\text{rh-Epo-EPG-ULVs}}/\text{area}_{\text{rh-Epo}}$) were calculated. Afterward, only peptides with an area ratio below 0.6, indicating significant differences in peptide concentration of the two preparations, were collected separately and lyophilized. These fractions were reconstituted and characterized by mass spectroscopy (Q-ToF Global Ultima instrument; Waters Micromass, Waters, Manchester, UK). Sequence determination was performed with MassLynx 4.0 SP4 software (Waters Micromass).

Results and discussion

It is generally accepted that Epo interacts with different receptors on hematopoietic and neuronal cells. During hematopoietic activation, Epo binds by positively charged lysine and

arginine residues to Epo-R₂ (Fisher, 2003; Leist et al., 2004; Matthews et al., 1996; Tilbrook and Klinken, 1999; Wang et al., 2007). In contrast to this mechanism, it was demonstrated that the neuroprotective pathway is activated not only by rh-Epo, but also by carbamylation modified rh-Epo, indicating that, in this case, alternative amino-acid residues cause binding (Brines et al., 2004; Leist et al., 2004; Wang et al., 2007). To prove different association capabilities of rh-Epo, we generated neutral (HSPC), positively (DOTAP), and negatively (EPG) charged liposomes and confirmed the quality of pure liposomes in DLS and TEM (Figure 1A). All preparations showed unilamellar homogenous liposomes with a mean size of 80–110 nm. After the association of rh-Epo to various liposomal preparations, TEM revealed that rh-Epo transformed negatively charged spherical EPG-ULVs (Figure 1A) into disc-like micelles (Figure 1B) when they were incubated above their T_m . Disc-like micelles appeared as rice grain-shaped structures with a diameter of about 30 nm (Figure 1B). This appearance is typical for discoid structures, because they are mainly adsorbed at their hydrophobic edges onto the copper grid (Gursky, 2002; Jayaraman et al., 2005; Mehta et al., 2003; Sevcsik et al., 2007, 2008). Though rh-Epo containing vesicles were described as drug-delivery systems before, our observations showed, for the first time, that rh-Epo enables the transformation of liposomal membranes (Maitani et al., 1996, 1999; Moriya et al., 1997). However, those drug-delivery experiments were performed with liposomes consisting of DPPC and various sterols. Those vesicles did not contain negatively charged phospholipids at all, which are essential for the disc-like micelle formation, as demonstrated by our TEM studies. Only negatively charged EPG-ULVs were transformed, while positively charged and neutral ULVs remained unaffected. In the case of anionic EPG, it is indicated that the topological transformation by rh-Epo is thermodynamically driven and initiated by protein association. Previously, severe membrane remodeling was demonstrated for small antimicrobial peptides (AMPs), lysozyme, apolipoproteins, toxins, and surfactants (Gursky, 2002; Jayaraman et al., 2005; Kuboi et al., 2000; Mehta et al., 2003; Sevcsik et al., 2007, 2008; Zschörnig et al., 2005). Additionally, tumor necrosis factor and interleukin-1 alpha were reported to disintegrate liposomal membranes (Oku et al., 1987, 1995). Calorimetric measurements revealed rh-Epo-induced thermodynamic changes only in EPG-containing liposomes, which are detected as a significant peak shift. In the presence of rh-Epo, the T_m increased from 30.2 to 32.7°C, indicating that bilayers are interdigitated by the protein and, therefore, stabilized in the gel phase (Figure 2). In parallel, the enthalpy (ΔH) decreased, which is typical for systems including very small structures, such as disc-like micelles. These findings are similar to those observed in interdigitation models with small AMPs and apolipoproteins (Gursky, 2002; Sevcsik et al., 2007, 2008). Further, rh-Epo-containing liposomal suspensions were measured with DLS to identify discoid subpopulation. By this method, the mean size of the vesicles is measured in batch mode. Although pure liposomes were characterized successfully, the identification of discoid micelles failed. It is commonly accepted that polydisperse samples are more difficult to handle in DLS, because calculation of average size is biased toward larger vesicles. This bias debases the resolution to about 1:4, which is not sufficient for our approach (Kaszuba et al., 2007). Therefore, TEM was chosen as a more suitable method to identify discoid structures in heterogeneous suspensions.

Through our findings, we conclude that both the head-group charge and the temperature are prerequisites for disc formation. In contrast to interleukin-2 and interferon-gamma, which associate to liposomes by simple electrostatic, nontransforming interactions, rh-Epo binds with defined positively charged amino acids, as demonstrated by experiments with carbamylated rh-Epo (Kopenhagen et al., 1998a, 1998b; Kuboi et al., 2000; Van Slooten et al., 2001; Zschörnig et al., 2005). Within these examinations, the positively charged lysines and arginines were neutralized by carbamylation, which results in the deprivation of the membrane-modulating activity of rh-Epo (Arcasoy, 2008; Brines et al., 2004; Leist et al., 2004; Tilbrook and Klinken, 1999; Wang et al., 2007). By additionally performed studies with rh-Epo and EPG_{LOW}-ULVs, the disc-like micelle formation was drastically reduced. Therefore, we can conclude that the membrane remodeling process is concentration dependent.

However, the electrostatic attraction alone cannot explain the formation of discoid structures, because simple adsorption is insufficient to interrupt membrane structures, as reported for interleukin-2 and interferon-gamma (Kopenhagen et al., 1998b; Van Slooten et al., 2001; Kuboi et al., 2000; Nomura et al., 2001; Sevcsik et al., 2007, 2008; Takiguchi et al., 2002). Thus, the effect of the protein structure was examined by applying DTT-treated rh-Epo to EPG-ULVs. The reduced rh-Epo exhibited an enhanced potency to perturb EPG-ULVs already at room temperature (20°C), in contrast to native rh-Epo, which required temperatures above the T_m of the lipid composition. This result reveals that an increased temperature is rather important to induce protein conformational changes than to increase membrane fluidity.

In order to test the membrane-perturbing potency of rh-Epo, EPG-bilayers were stabilized by the addition of cholesterol (CHOL) to the lipid composition. However, CHOL did not diminish the formation of disc-like micelles, leading to the assumption that the modulating efficiency of rh-Epo was not inhibited by cholesterol (Sulkowskia et al., 2005).

To localize the membrane-affecting domain within the protein sequence, rh-Epo and rh-Epo associated to EPG-ULVs were trypsin digested and the resulting peptides were separated by rp-HPLC. Calculation of the area ratios between both digests revealed ratios between 0.9 and 1.1 for all but two peptides, which had a ratio lower than 0.6, demonstrating that both peptides were still associated to the membranes (Figure 3).

These two protein fragments were collected and analyzed by tandem mass spectrometry (MS/MS) for sequence determination. The peak marked with a transparent arrow in Figure 3 corresponds to sequence Met-54 to Arg-76 and the one with a filled arrow to the sequence Gly-77 to Lys-97. Therefore, we call the amino-acid sequence spanning Met-54 to Lys-97 the membrane-affecting domain of rh-Epo (MAD-E), located in helix B and partially in helix C (Figure 4) (Elliott et al., 1997; Leist et al., 2004).

To confirm our hypothesis that these two peptides resemble the MAD-E of rh-Epo, we tested them in the association model. The peptides were added separately or together to EPG-ULVs. The TEM pictures revealed that only the combination of both peptides was able to interrupt the membrane to such an extent that lipid disc-like micelles could be stabilized.

Conclusion

The transformation of liposomes is reported for molecules such as lysozyme and apolipoproteins, small AMPs, toxins, and surfactants leading to lipid structures different- to round-shaped vesicles (Gursky, 2002; Jayaraman et al., 2005; Kuboi et al., 2000; Mehta et al., 2003; Sevcsik et al., 2007, 2008; Zschörnig et al., 2005). Even cytokines such as interleukin-1 alpha and tumor necrosis factor are described as membrane-destabilizing proteins inducing leakage in liposomes (Oku et al., 1987, 1995). In the case of membrane-transforming proteins and peptides, hydrophobic sequences at the terminal end of the molecule with alpha-helical structures are proposed to be predestinated for pore formation and, finally, the formation of disc-like micelles (Gursky, 2002; Jayaraman et al., 2005; Mehta et al., 2003; Sevcsik et al., 2007, 2008). However, our studies demonstrated, for the first time, that exposed amino-acid residues of helix B of rh-Epo are capable of perturbing lipid bilayers and hence transforming them into discoid structures.

In general, it is well accepted that proteins associate with charged lipid head groups of membrane surfaces by electrostatic attraction. This association is predominantly reversible without the destruction of proteins or membranes (Kopenhagen et al., 1998b; Van Slooten et al., 2001). Membrane transformation is only induced if additional conformational changes of the protein occur and a penetrating sequence is interacting with the membrane. In the case of lysozyme, penetration into artificial membranes causes enhanced permeability of the lipid bilayer and induces conformational changes of the protein (Kuboi et al., 2000; Zschörnig et al., 2005). Nevertheless, rh-Epo and lysozyme attract membranes with, in principal, different mechanisms. Specific protein binding of rh-Epo occurs toward negatively charged EPG, although the protein net charge is negative. A series of certain amino acids located within the protein penetrates into the membrane and stabilizes the membrane in the gel phase (Sevcsik et al., 2007, 2008). In the case of rh-Epo, we identified and isolated the so-called membrane-affecting domain of rh-Epo (MAD-E) spanning Met 54 to Lys 97 of the helix B of the rh-Epo molecule. For a long time, the biological function of this helix was unexplained. Just recently, it was identified to be involved in the neuroprotective signal transduction (Arcasoy, 2008; Brines et al., 2008). If, and in which form, this membrane-affecting property is of biological relevance is one of the exciting questions for future studies.

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Figure 1. Transmission electron micrographs of (A) ULVs incubated above T_m without rh-Epo and (B) disc-like micelles in liposomal suspension appeared after the addition of rh-Epo to unilamellar EPG-vesicles (EPG-ULVs) and incubation above T_m . The black arrows mark disc-like micelles of about 30 nm in diameter.

Figure 2. Thermograms of empty (black) and rh-Epo-containing (grey) EPG-liposomes are recorded at a scan rate of 30°C/hour. T_m , transition temperature [°C]; H , enthalpy [kcal]; c_p , heat capacity.

Figure 3.

HPLC-chromatograms of pure digested rh-Epo and digested Epo-EPG-ULVs. Area ratios (ARs) are calculated by $\text{area}_{\text{rh-Epo-EPG-ULVs}} / \text{area}_{\text{rh-Epo}}$. The black arrow marks the peak corresponding to the amino-acid sequence, Gly-77-Lys-97, and the transparent arrow marks Met-54-Arg-76 of rh-Epo.

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Table 1

List of unilamellar liposomes used for association studies, including their lipid compositions and molar ratios.

Sample code	Lipid composition	Mol%	Charge
HSPC-ULV	HSPC:DSPE-PEG2000	96:4	Neutral
DOTAP-ULV	DPPC:DOTAP	88:12	Positively charged
EPG-ULV	DPPC:DMPC:EPG	40:17:43	Negatively charged
EPG _{LOW} -ULV	DPPC:DMPC:EPG	62:26:12	Negatively charged
EPG+CHOL-ULV	DPPC:DMPC:EPG:CHOL	37:16:40:7	Negatively charged