Interaction of albumin with the endothelial cell surface

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1Department of Physiology, Freie Universität Berlin, 14195 Berlin; 2Magnettech, 12489 Berlin; 3Bundesanstalt für Materialforschung und Prüfung, 12205 Berlin; and 4Institute of Anesthesiology, Deutsches Herzzentrum Berlin, 13353 Berlin, Germany

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Osterloh, Kurt, Uwe Ewert, and Axel R. Pries. Interaction of albumin with the endothelial cell surface. Am J Physiol Heart Circ Physiol 283: H398–H405, 2002. First published March 7, 2002; 10.1152/ajpheart.00558.2001.—Endothelial cells (EC) are covered with cell-borne proteoglycans and glycoproteins. Blood plasma proteins (e.g., albumin) adsorb to this glycocalyx forming a complex endothelial surface layer (ESL). We determined the molecular mobility of albumin by electron spin resonance (ESR) in the presence and absence of ECs to analyze interactions with the ESL. Albumin was spin labeled with 5- or 12-4,4-dimethyloxazolidine-N-oxyl (DOXYL)-stearic acid yielding information on the mobility of the molecular surface (5-DOXYL) or the entire protein (12-DOXYL). EC cultures grown on glass coverslips were immersed in labeled albumin and placed in the temperature-regulated cavity of an ESR spectrometer. Alternatively, ECs were labeled and then exposed to native albumin. At 37°C, rotational correlation times determined by modified saturation transfer ESR (ST-ESR) were 26 and 48 ns for 5-DOXYL- and 12-DOXYL-labeled albumin, respectively. Presence of ECs increased rotational correlation time values for 5-DOXYL-stearic acid to 37 ns but not for 12-DOXYL-stearic acid. Albumin was able to completely take up the label from labeled EC within 2 min. The present study shows that modified ST-ESR can be used to determine the mobility of biological macromolecules interacting with cellular surfaces. Reduction in albumin surface mobility in the presence of EC at unchanged mobility of protein proper and fast removal of labeled fatty acids from EC membranes indicate rapid transient interactions between albumin surface and ESL but no rigid incorporation of albumin into a macromolecular network that would interfere with its transport function for poorly water-soluble substances.

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label shows restricted mobility depending on its position along the backbone of the fatty acid (26).

Spectroscopical techniques encompass a vast range of molecular mobility determined as rotational correlation times ranging from $10^{-11}$ to $10^{-3}$ s mainly employing two different experimental setups: conventional ESR for rotational correlation time ranging between $10^{-11}$ and $10^{-5}$ s and saturation transfer ESR (ST-ESR) between $10^{-7}$ and $10^{-3}$ s (11). However, the usual approach of interpreting only out-of-phase ST-ESR spectra is not suited to reliably determine rotational correlation time values in the intermediate range between $10^{-5}$ and $10^{-7}$ s, i.e., typical values for plasma proteins in aqueous solutions at body or ambient temperature. In the present study, this problem has been addressed by developing an extended approach analyzing both the in-phase and out-of-phase spectral information. The extended ST-ESR technique was employed to assess changes of albumin mobility in the presence of endothelial cells and thus obtain information on the interaction of a typical plasma protein with the endothelial surface as a critical step for the generation of the ESL.

**MATERIALS AND METHODS**

**Cell preparation.** Cultured umbilical cord endothelial cells (ECV304; American Type Culture Collection) were grown in medium 199 with Earle’s salts (Biochrom, Berlin, Germany) containing 10% FCS, 1% L-glutamine, and 0.1% penicillin-streptomycin (10,000 E/10 mg/ml) on glass coverslips cut to the size of 6 × 60 mm and placed in petri dishes. Confluent cultures were transferred into protein-free Earle’s medium 199 buffered with 20 mM HEPES (without L-glutamine and NaHCO₃) for transportation. Whole blood samples (5 ml) were obtained from a healthy volunteer by puncture of an antecubital vein; heparin (50 international units) was added to avoid clotting. Procedures were performed according to the principles outlined in the Declaration of Helsinki. Plasma and blood cells were separated by 15-min centrifugation at 1,500 g, and the plasma fraction was subsequently cleared by 5-min centrifugation at 20,000 g. Influence of whole blood plasma on the cell surface layer was studied with cultures incubated in freshly prepared human plasma for 1 h. Incubation was performed at room temperature to minimize pinocytosis and possible metabolic effects.

**Spin labeling.** Stearic acids labeled with 4,4-dimethylazolidine-N-oxyl (DOXYL; Sigma, Deisenhofen, Germany) at the positions C5 or C12 were used as spin probes. All labeling procedures started with transferring the nitroxide-labeled stearic acid to a glass surface. This was achieved by dissolving 5- or 12-DOXYL-stearic acid in ethanol (1 mg/300 μl) in a glass test tube. Ethanol was subsequently evaporated with a stream of dry and cold nitrogen, leaving labeled stearic acid as a coat on the glass surface. Coated glass tubes were stored at −20°C.

Labeling of albumin was achieved by taking advantage of the fact that this protein has a natural affinity to bind poorly water-soluble substances, particularly long-chain fatty acids. Essentially fatty acid free albumin (0.005% fatty acids or 1 fatty acid molecule in 100 albumin molecules; Sigma, Deisenhofen, Germany), dissolved in the protein-free cell culture transport medium (40 mg/ml), was used to ensure effective labeling. Four milliliters of this solution were transferred into the glass test tubes coated with the respective spin probe and left incubating for at least 1 h at room temperature. To load cell membranes with the spin probes, coated test tubes were filled with 5 ml of protein-free HEPES buffered medium 199. Cell cultures on the glass coverslips were incubated in these tubes for 1 h at room temperature to restrict labeling to the outer cell membrane and to minimize distribution within the cell (e.g., by pinocytosis).

**ESR spectroscopy.** Coverslips with labeled cell cultures were attached to a spatula-shaped quartz holder for ESR measurements (Fig. 1) with a rim (height 0.3 mm) to allow tight adhesion of a 6 × 60-mm coverslip without further mechanical support. This arrangement allowed an aqueous layer of only 300 μm on top of the cells suitable for ESR-spectroscopy in the X-band (9.4 GHz). The cuvette with cultured cells was then placed in the center of a H₁₀₂ resonator (rectangular cavity) with parallel orientation to the Zeeman field of an X-band ESR spectrometer (model ESR 231; Academy of Science, Berlin, Germany). The same cuvette with a blank coverslip was used for reference measurements with solutions of albumin and experiments with whole blood preparations. Instrument settings common to all experiments were 9.4 GHz microwave frequency, 336 mT magnetic field center, 17 mT field sweep, 1.7 min scanning time, and 0.1 s time constant. For conventional ESR, microwave power was adjusted to 20 mW to achieve maximum sensitivity without spin saturation, and the magnetic field was modulated with 100 kHz and an amplitude of 0.1 mT.

Temperature control within the resonator cavity was achieved with a nitrogen temperature controller STT3 (Galileo, Berlin, Germany). Temperature settings were calibrated with a digital thermometer at the site of the sample in
the cavity and controlled electronically during the experiments (precision ± 0.5°C). Temperature in the cavity was set to 37°C before introducing the sample and adjusting the microwave bridge, and the first spectral scan was run at this temperature.

For the purpose of ST-ESR spectroscopy, the microwave power was increased to 100 mW, and the $B_1$ field strength at the sample position was 38 μT as estimated from the line broadening of the spectral lines of Fremy’s salt (37). Field modulation was 50 kHz with an amplitude of 0.76 mT checked with a nonsaturating standard substance 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma, Deisenhofen, Germany), while the phase-sensitive signal detection remained at 100 kHz, resulting in second harmonic spectra. The 90° out-of-phase (quadrature) and the in-phase spectra (Fig. 2) were collected either in two consecutive scans or simultaneously with a second phase-sensitive amplifier (Magnettech, Berlin, Germany). Phase adjustment was achieved with a precision of <1° using a strong external standard (DPPH) not showing saturation effects at the measurement conditions used, which was temporarily positioned adjacent to the sample.

In some experiments, spin-labeled cell cultures were removed from the cavity after completing the first recording of a spectral scan, flushed with unlabeled albumin or ascorbic acid (5 mM in protein-free Earle’s medium 199) and reinserted into the spectrometer. Changes in the amount of spin label were determined by analyzing conventional ESR-signal intensities obtained from double integration of the baseline corrected spectra. In all other experiments with cell cultures, the unlabeled cultures were inserted into medium containing labeled albumin before placing them in the resonator cavity. Spectra were collected at 37°C and at temperature levels stepwise lowered down to 3°C. Temperature equilibration on each level was reached within half a minute as tested with a temperature-sensitive probe in calibration measurements.

**Data processing.** Recorded spectra were interactively processed with a program written in Pascal (Delphi, Borland Software). Conventional ESR spectra were smoothed by a rectangular filter that best conserves peak heights. Only in cases of a smooth transition from signal peaks to noise was a Gauss filter preferred, which tends to damp peak heights but avoids oscillation artefacts typical for rectangular curve filtering in such cases. The filter parameter was adjusted according to the transition between the slope of the signal power and the noise plateau in the power spectrum. Smoothing of spectra was essential to precisely determine the location of maxima and minima indicating parallel and perpendicular tensors (T and $T_\perp$) and to allow quantification of the degree of anisotropy. From the distances in the spectrum corresponding to these tensors, the order parameter ($S$) was automatically derived using values of $A = 0.61, 0.61, or 3.24$ mT for nitrogen hyperfine splitting (14, 35). Order parameter values have been converted into rotational correlation time ($\tau$) by an empirical relationship (38). The dimensionless order parameter

![Fig. 2. Complex saturation transfer electron spin resonance (ESR) spectra.](http://prod.sagepub.com/doi/abs/10.1152/ajpheart.00180.2001)

A: baseline corrected and smoothed in-phase (solid lines) and out-of-phase spectra (dashed lines) of 12-4,4-dimethyl-oxazolidine-N-oxyl (DOXYL)-stearic acid bound to albumin at 3°C ($\tau = 98$ ns) and at 37°C ($\tau = 48$ ns). B: phase diagrams (out-of-phase spectra plotted against corresponding in-phase values) of the same spectra. Aspect ratio of the enclosing box with minimal area in the phase diagrams (arrows, phase ratio) was used to estimate the rotational correlation time ($\tau$) in addition to the amplitude ratio.
parameter $S$ (35), with theoretical values between 0 and 1, indicates the degree of mobility of the spin probe between total immobilization ($S = 1$, glass configuration, no motion) and maximal freedom of rotation ($S = 0$, ideal isotropic spectrum). However, the order parameter $S$ is only applicable in a range of rotational correlation time between $\sim 10^{-10}$ and $10^{-8}$ s. This is a typical range for spin probes incorporated into biomembranes, and respective studies often express molecular mobility in terms of the order parameter. However, especially for high values of the order parameter, these conversions bear a large degree of uncertainty. Therefore, the present study uses rotational correlation time instead of the order parameter $S$ wherever possible.

In ST-ESR, it was necessary to perform a baseline correction because of changes in offset at different amplifications used for recording in-phase and out-of-phase spectra and occasional baseline drifts occurring mostly at high amplifications. Both, the out-of-phase (or quadrature, $Q_2$) and the in-phase ($I_2$) spectra were rescaled with respect to signal amplification. Two sets of processed sample spectra are shown in Fig. 2, A and B, each consisting of an in-phase and an out-of-phase recording. Subsequently, the two single-phase spectra were combined into one complex spectrum for further determination of spectral parameters. Two approaches to represent such complex spectra have been introduced in the literature and are used here. One method is based on amplitude values of the two single spectra. The rotational correlation time is derived from the amplitudes of the maximum and the first adjacent minimum to the left (amplitude ratio) (40). The second approach uses a phase diagram ($P_2$, the index 2 indicating second mode spectra) (amplitude ratio) (40). The second approach uses a phase diagram ($P_2$, the index 2 indicating second mode spectra) (amplitude ratio) (40). The second approach uses a phase diagram ($P_2$, the index 2 indicating second mode spectra) (amplitude ratio) (40). The second approach uses a phase diagram ($P_2$, the index 2 indicating second mode spectra) (amplitude ratio) (40).

Differences between cell-free albumin solution and cell preparations with and without preincubation with albumin were tested by comparing results of the regression analysis employing the F-test based on the average vertical distance between regression lines and the sum of squared differences from the respective regression line at each temperature. The existence of differences was additionally tested for three temperature levels (37°C, 20°C, and 3°C) separately using the F-test and the t-test in cases where the F-test indicated nonhomogeneity. Significance was assumed at $P > 0.05$.

RESULTS

Conventional ESR of labeled endothelial cells. For endothelial cells prelabeled with 5-DOXYL-stearic acid in protein-free culture medium, Fig. 3 shows the ESR-signal intensity as a parameter for stability of the nitroxide label with time at 37°C. In the absence of albumin, results indicate a slow signal decay consistent with a half life of the label of $\sim 30$ min (Fig. 3A). Replacing the supernating medium with albumin solution further stabilized the signal intensity rendering a decay hardly determinable in the time course of the experiment and resulted in a step increase of the order parameter indicating a decreased mobility of the spin probe. Subsequent flushing with ascorbic acid reduced the signal intensity to nearly noise level between the two consecutive measurements (<3 min). In contrast, ascorbic acid did not rapidly extinguish the nitroxide label incorporated into cell membranes incubated in albumin-free medium (Fig. 3B) or label bound to albumin in the absence of cells (Fig. 3C).

ST-ESR measurements of spin-labeled albumin in presence and absence of cells. Temperature dependencies of rotational correlation time for albumin labeled with either 5- or 12-DOXYL-stearic acid are summarized in Fig. 4. As expected, rotational correlation time correlated inversely with temperature in all cases with a correlation coefficient ($r$) of $-0.764$ for 5-DOXYL-stearic acid and of $-0.887$ for 12-DOXYL-stearic acid-labeled albumin, but on different levels. From 37°C down to 3°C, rotational correlation time values for 5-DOXYL-stearic acid increased from 26 to 64 ns (Fig. 4A) and those for 12-DOXYL-stearic acid, from 48 to 98 ns (Fig. 4B). The rotational correlation time for 12-
DOXYL-labeled albumin was for all temperatures similar to values estimated from physical principles (Stokes-Einstein relation) based on medium viscosity and the molecular radius of albumin. This suggests a fairly rigid coupling of 12-DOXYL-stearic acid mobility to those of the albumin molecule.

Presence of endothelial cells led to an increase in rotational correlation times in case of the 5-DOXYL-stearic acid bound to albumin by ~15 ns throughout the temperature range investigated. Preincubation of cultured cells with blood plasma was followed by an even stronger increase (~9 ns) especially for higher temperatures (Fig. 4A). The result of multivariance analysis confirmed heterogeneity among the experimental groups for all temperatures ($F = 6.18, P < 0.0001$). The t-test showed statistical differences be-
tween plain albumin and cell preparations, whereas differences between the cells with and without albumin preincubation failed statistical significance. In contrast, rotational correlation times of 12-DOXYL-stearic acid bound to albumin did not increase significantly in the presence of cultured endothelial cells even after preincubation with whole plasma (Fig. 4B) as confirmed by multivariance test ($F = 0.77, P = 0.7143$) entailing all values at all temperatures. However, differences were observed below the physiological temperature range as shown by testing separately at each temperature ($F = 5.66, P = 0.0171$ at $20^\circ$C). At $20^\circ$C, plain albumin differed from the cell preparations ($t = 3.399, P = 0.0068$).

**DISCUSSION**

In the present study, ESR spectroscopy using nitroxide spin probes was used to characterize molecular interactions of albumin with the surface of endothelial cells. Two distinct spectroscopical effects caused by the anisotropy of the nitroxyl moiety are usually employed to measure molecular mobility: distortion of spectral lines and phase shift due to spin saturation. The range of rotational motions accessible with standard implementation of these two techniques was enlarged by introducing a two-dimensional interpretation of ST-ESR spectra. To our knowledge, this is the first study using ST-ESR spectroscopy to investigate molecular rotational motion in the range between $10^{-8}$ and $10^{-7}$ s typical for plasma proteins like albumin in aqueous solution at body temperature.

Interpretation of the rotational correlation time of spin probes necessitates knowledge about the location of the label within the system. Two initial locations for spin probes were used here: binding sites for fatty acids at the albumin molecule and endothelial cell plasma membranes. The result obtained with labeled endothelial cells (Fig. 3) was typical for biomembranes (41). Slow reduction of signal intensity with time can be explained by losses of the nitroxide spin probe due to inward translocation (28), distribution throughout the cell (25), and subsequent metabolic reduction (39). This behavior changed significantly on introduction of albumin into the system. Albumin has a significant affinity to bind fatty acids such as the spin probes used in this study (44, 45), and it removes spin labels from a variety of lipid membranes (19, 21). Accordingly, mobility of the spin label is slowed down after the addition of albumin as would be expected from uptake of the label from endothelial cells. The fast change in order parameter indicates that the label had been predominantly located in the outer cell membrane accessible to albumin. Furthermore, the label was apparently more stable with time when bound to albumin compared with cell membranes probably due to the absence of cellular degrading mechanisms.

For plain albumin incubated with ascorbic acid, a slow decay of the spin label with time (and a slight increase of the order parameter that might be linked to a slow protein precipitation process) is observed. In sharp contrast, the label was destroyed immediately on the addition of ascorbate in the presence of endothelial cells (Fig. 3). This could indicate that the spin probe is not permanently bound to albumin but in a fast mutual exchange with the cellular membrane (Fig. 5). Because albumin affinity is much higher than that of the plasma membrane, most of the label will be attached to the protein at any time. During fast transition between the two locations, the nitroxide would become vulnerable to destruction by the hydrophilic ascorbate.

The data shown in Fig. 3 also provide evidence that albumin added to the culture medium penetrates the ESL to take up the spin probe from the plasma membrane within the sample preparation time ($\sim 2–3$ min) as indicated by the rapid change of order parameter after the addition of albumin. This finding may be compared with observations by Vink and Duling (42) in intravitral microscopic studies observing diffusion of anionic macromolecules into the ESL. Albumin and fibrinogen equilibrated between blood and the ESL with a half time of $\sim 40$ min. The much shorter interval needed in the present approach for removal of fatty acids from the endothelial membranes may relate to the fact that no complete equilibration is needed for this process. Alternatively, it could indicate that the ESL present in vivo is more complex than that on the...
cultured endothelial cells (even in the presence of blood plasma) despite the fact that the existence of a glycocalyx on ECV304 cells has been reported in the literature (18, 33).

Interactions of albumin with the ESL were further investigated by comparing results obtained with two different spin labels. The nitroxyl moieties of 5- and 12-DOXYL-stearic acid are differentially attached to the protein resulting in different rotational correlation times (24, 26) over a large temperature range (Fig. 4). Differences in rotational freedom relate to the binding characteristics for fatty acids in hydrophobic pouches inside the albumin molecule (3, 10, 45). Nitroxide of the 12-DOXYL probe appears to be sufficiently immobilized to report rotational motion of the entire albumin molecule, whereas the label of the 5-DOXYL probe located at the surface of the albumin molecule is more susceptible to intermolecular contacts. As a consequence, the presence of an ESL matrix may influence the rotation of the peripheral 5-DOXYL label, whereas the 12-DOXYL label in a deeper position with restricted local rotation would mainly be affected by comparatively stable bonds of the carrier molecule albumin. The data for 12-DOXYL may hint at a slight change in the rotational dynamics on contact with the ESL expressed in an altered temperature dependency rather than an increase of rotational correlation time. Taken together, the experimental results support the concept of albumin being able to diffuse through the ESL while superficially interacting with the gel matrix macromolecules.

These results could help to understand the physiological double role of albumin at the endothelial surface: interaction with gel-like surface structures and shuttling poorly water-soluble substances (22) that would be excluded by persistent incorporation into local macromolecular networks. However, despite the lack of static links to a macromolecular network, albumin may contribute to the establishment of the spatial dimensions of the ESL (30), e.g., by transiently filling hydrophilic pores of the ESL. Weak and short-lived links sufficient to explain such effects do not necessarily impair rotation of the protein significantly. Without permanent binding to stationary molecules, albumin could frequently exchange with the luminal fluid phase and thus assume its transport function for hydrophobic substrates through the ESL.

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REFERENCES


2. Bureczynski FJ, Cai ZS, Moran JB, and Forker EL. Palmitate uptake by cultured hepatocytes: albumin binding and stig


13. Morrill GA, Doi K, and Kostellow AB. Progesterone induces transient changes in plasma membrane fluidity of amphi-


