Pathogen inactivation in cellular blood products by photodynamic treatment

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CHAPTER II

RELATION BETWEEN K^+ LEAKAGE AND DAMAGE TO BAND 3 IN PHOTODYNAMICALLY TREATED RED CELLS

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ABSTRACT

Potassium (K^+) leakage is one of the first events that appear after photosensitization of RBCs. This event may subsequently lead to colloid osmotic hemolysis. The aim of our study was to determine which photodynamically induced damage is responsible for increased membrane cation permeability. This was done by studying the effect of DMMB-mediated PDT on different membrane transport systems. Inhibition of band 3 activity (anion transport) showed a comparable light dose dependency as PDT-induced K^+ leakage, whereas glycerol transport activity was inhibited only at higher light doses. Dipyridamole (DIP), an inhibitor of anion transport, protects band 3 against DMMB-induced damage, and prevents the increase in cation permeability of the membrane. Damage to glycerol transport was partially reduced when PDT was performed in the presence of DIP. Because DIP has no affinity for the glycerol transporter, this protection might result from the reduced photodamage to band 3. These results support the hypothesis that band 3 might be involved in glycerol transport. Glucose transport was not affected by DMMB-mediated PDT. The present results are the first to show a causal relationship between DMMB-mediated photodamage to band 3 and increased cation permeability of RBCs.

INTRODUCTION

In spite of continuing improvement in laboratory tests to decrease the window phase and so to reduce the possibility of viral transmission, viral and bacterial contamination remains a major problem of the blood transfusion services.[1] The increasing costs cannot be met by many countries outside the western world. It is therefore necessary to investigate new strategies to improve the safety of blood component transfusion. Inactivation of the infectious pathogens may present an alternative method of ensuring pathogen-free blood. A heating or solvent–detergent treatment is already included in the processing of plasma products. These approaches have been shown to reduce the risk of virus transmission[2,3]; however, because of the fragility of the cells, they are not applicable to cellular blood products. The use of PDT as a method for pathogen inactivation in cellular blood products such as RBCC is being explored because various lipid-enveloped and non-enveloped viruses, gram-positive and gram-negative bacteria can be inactivated.[4-9] PDT is based on the use of light-activated agents, photosensitizers. The photosensitizers studied for pathogen inactivation in RBCC usually belong to the group of phthalocyanines[10] or to the group of phenothiazine dyes, such as MB[11] and DMMB.[12] On light activation, these molecules generate reactive oxygen species such as singlet oxygen ($^1O_2$), which are able to react rapidly with substrates present in their environment. Pathogens can be inactivated completely by PDT, but all photosensitizers tested so far also induce damage to RBCs.[13-17] A better understanding of the molecular mechanisms behind photodynamically induced damage is needed to improve the selectivity of the photodynamic decontamination technique and to reduce, or if possible to avoid, damage to RBCs. Increased permeability of the membrane to cations, as measured by K^+ leakage, is one of the first events that appear after photosensitization of RBCs. It is believed that this increased permeability will lead ultimately to colloid osmotic.[9,14-17] It is as yet unclear which photodynamically induced damage is responsible for the increased membrane permeability. Dubbelman *et al.*[18] have shown that inhibition of several carrier-mediated transport systems for glucose, glycerol and sulfate precedes K^+ leakage after protoporphyrin-mediated PDT. Based on the dose-squared relationship between fluence and photohemolysis, it

has been suggested that hemolysis results from damage to the anion transport protein of the RBC, band 3.[19] Recently, VanSteveninck *et al.* [20] have shown that the band 3 ligand dipyridamole (DIP) protects RBCs against DMMB-mediated PDT. This protection was selective for RBCs; DIP had no effect on the virucidal efficacy of DMMB. The aim of the present study was to gain a better insight into the molecular mechanisms behind DMMB-mediated photodynamically induced K^+ leakage in RBCs. As it is likely that damage to a membrane-spanning protein could be involved in K^+ leakage, the kinetics of photodynamically induced K^+ leakage and inhibition of carrier-mediated transport of glucose, glycerol and sulfate were compared. Because DIP is known to protect against DMMB-mediated K^+ leakage, the effect of DIP on the photoinhibition of the various transport systems was also investigated. Based on both the kinetics of inhibition and the effect of DIP, an attempt was made to identify the critical target for DMMB induced K^+ leakage.

MATERIALS AND METHODS

MATERIALS

RBCC were provided by the Red Cross Blood Bank Leiden-Haaglanden (Leiden, The Netherlands) after consent of human volunteer donors. The erythrocytes were centrifuged shortly after collection, washed three times with phosphate-buffered saline (PBS) containing 30 m*M* sucrose and resuspended at 10% hematocrit (Hct) in the same buffer. Sucrose was added to prevent photohemolysis.[21] DMMB was obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands. Stock solutions of 1 m*M* were made in 50 m*M* sodium phosphate buffer (NaP), pH 7.4, and stored at 4°C. DIP (2,6-bis(diethanolamino)-4,8-dipiperidino-[5,4-*d*] pyrimidine) (Sigma-Aldrich) was stored as a 200 m*M* stock solution in dimethylsulfoxide (DMSO) at 4°C. Sodium [^{35}S] sulfate ($Na_2{}^{35}SO4$) and D-[U-^{14}C-glucose] were obtained from Amersham, 's-Hertogenbosch, the Netherlands. All other chemicals were also purchased from Sigma-Aldrich.

PHOTODYNAMIC TREATMENT

Illumination was performed with a 300 W halogen lamp (Philips, Brussel, Belgium). The light passed a water and red filter to transmit only light with λ above 600 nm and to avoid heating of the sample. RBC suspensions were illuminated in 3 cm diameter culture dishes (Greiner, Alphen a/d Rijn, the Netherlands) containing 1 mL of RBC suspension (10% Hct). Irradiance was 10 mW/cm^2, as measured with an IL400A photometer equipped with an SEL033/F/U detector (International Light, Newbury, MA).
RBC suspensions were incubated with 100 µ*M* DIP in the dark. After 5 min, 15 µ*M* DMMB was added to the suspension. After another 5 min of incubation in the dark, illumination was started. All treatments were performed at a constant temperature of 20°C under continuous shaking (150 cycles/min). In control experiments, the same volumes of DMSO and NaP were added, without DIP and DMMB, respectively. These samples were illuminated as described previously.

POTASSIUM LEAKAGE AND HEMOLYSIS

K^+ leakage from RBCs was determined immediately after illumination, by means of a flame photometer (Clinical Flame Photometer 410C; Corning, Halstead, UK). Hemolysis was determined by measuring the optical density of the supernatant at 540 nm using a Novaspec II spectrophotometer (Pharmacia Biotech, Weesp, the Netherlands). Both K^+ and Hb release were expressed as a percentage of the total efflux evoked by lysis of the cells in distilled water.

BAND 3 ACTIVITY

Band 3 activity was measured by the influx of $Na_2{}^{35}SO4$ as described previously.[22] Briefly, after PDT, cells were centrifuged and resuspended (10% Hct) in PBS plus 1 m*M* Na_2SO4. The suspensions were incubated with 0.5 µCi/mL $Na_2{}^{35}SO4$ at 37°C. After 0, 10, 20 and 30 min of incubation, samples were taken and washed twice with PBS to remove free $Na_2{}^{35}SO4$. After precipitating the Hb with 5% trichloroacetic acid, radioactivity in the cells was determined by means of liquid scintillation counting (Tri-Carb 4000, Packard Instrument Co., Downers Grove, IL).

Band 3 activity of PDT-treated cells is expressed as percentage of control (cells without DIP or DMMB). Because DIP inhibits band 3 activity strongly, it must be removed before anion uptake can be measured. Therefore, cells incubated with DIP were centrifuged and resuspended to a 2% Hct solution in PBS containing 30 m*M* sucrose. After overnight incubation at 4°C, the cells were washed twice and resuspended in PBS plus 1 m*M* Na_2SO4. Band 3 activity was determined as described previously.

GLUCOSE TRANSPORT

Efflux of glucose was measured as described by Harris[23] and Sen and Widdas[24], with slight modifications. After illumination, cells were resuspended in buffer containing 135 m*M* NaCl, 10 m*M* KCl, 10 m*M* *Tris* and 100 m*M* glucose (Trisbuffered saline [TBS]–glucose), pH 7.4, at 10 % Hct. Cells were loaded with 0.25 µCi/mL D-[U-^{14}C-glucose] for 5 min at room temperature. The labeled cells were washed twice with TBS–glucose to remove free ^{14}C-glucose. After resuspension of the ^{14}C-glucose–loaded cells in 180 m*M* NaCl plus 12.5 m*M* glucose at 10% Hct, the efflux of ^{14}C-glucose was determined. For this, samples were taken every 30 s and centrifuged immediately for 10 s at 13.000 rpm. The amount of radioactivity in the supernatant was determined by means of liquid scintillation counting. Efflux was calculated as percentage of the total amount of ^{14}C-glucose taken up. Glucose transport activity is expressed as percentage of the activity of control (cells without DIP and sensitizer). The specificity of the glucose transport activity after DMMB-PDT was evaluated by incubating cells with the glucose transport inhibitor Cytochalasine B (cb; 50 µ*M*) for 10 min prior to loading with 0.25 µCi/mL D-[U-^{14}C-glucose]. After washing, cells were resuspended in buffer plus 50 µ*M* cb to maintain the inhibition during the measurement.

GLYCEROL TRANSPORT

Glycerol transport was measured by the hemolysis technique as described by Naccache and Sha'afi.[25] Briefly, RBCs were centrifuged and resuspended in 300 m*M* glycerol (2% Hct). Hemolysis was followed by the decreased scattering of the suspension, as measured at 620 nm (DU-64 spectrophotometer; Beckman,

Fullerton, CA). Nonspecific glycerol transport was measured in the presence of 2 m*M* CuSO4, which completely inhibits the facilitated diffusion of glycerol.[26] Active glycerol transport of the treated cells is expressed as percentage of the activity in control (cells without DIP and sensitizer).

STATISTICS

All experiments were performed three to five times with blood from different donors. Paired *t*-tests were used to determine the significance of differences between two groups. A *p*-value < 0.05 was considered significant. Means and standard deviations are reported.

RESULTS

EFFECT OF DMMB-MEDIATED PDT ON MEMBRANE PERMEABILITY

Photo-oxidation of human RBCs with DMMB resulted in increased permeability of the membrane toward cations, as measured by the leakage of K^+. This effect was detected already at low light doses. At 3 J/cm^2, the leakage amounted to 20% (Fig. 1). None of the light doses used induced any hemolysis. The K^+ leakage was inhibited by 80% when DIP was present in the cell suspension during the PDT with DMMB.

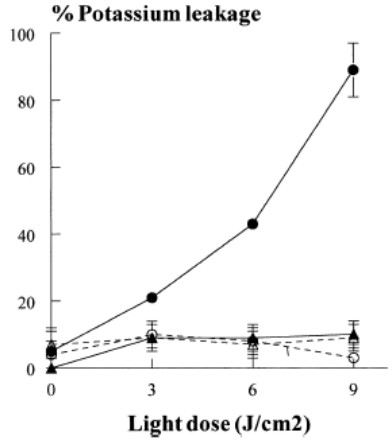

Figure 1. Effect of DIP on DMMB-mediated photodynamically induced K^+ leakage.
Ten percent erythrocyte suspensions in PBS and 30 m*M* sucrose were incubated in the dark with (△,▲) or without (○,●) 100 µ*M* DIP. After 5 min, DMMB (15 µ*M*) was added (●,▲) and the cells were incubated in the dark for another 5 min. In control cells (○), neither DIP nor DMMB was present. The RBC suspensions were exposed to light doses of 0 to 9 J/cm^2. K^+ leakage is expressed as percentage of the total efflux evoked by lysis of cells in distilled water.

EFFECT OF DMMB-MEDIATED PDT ON BAND 3 ACTIVITY

DIP is known to inhibit band 3 activity strongly. To study the effect of PDT on band 3 activity, DIP should be removed from the cells before the transport of anions can be measured. Therefore, cells containing DIP were subjected to extensive washing. This washing procedure resulted in complete restoration of anion transport activity in DIP-treated cells. The washing procedure had no effect on the band 3 activity of control cells (without DIP). Band 3 activity was inhibited strongly by DMMB-mediated PDT (Fig. 2). At 3 J/cm^2, a 30% inhibition of anion transport was observed, which increased to over 60% at 9 J/cm^2. When phototreatment was performed in the presence of DIP, even at a light dose of 9 J/cm^2, band 3 activity was not inhibited, and was maintained at the level of control cells (cells without DMMB or DIP).

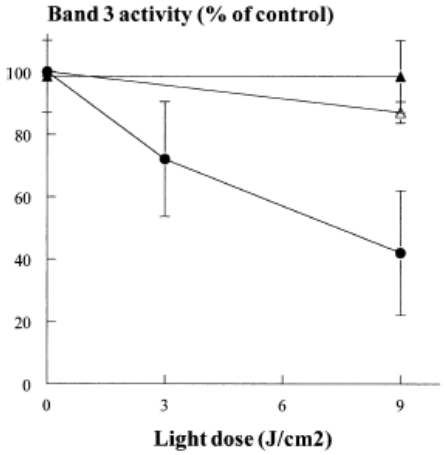

Figure 2. Protective effect of DIP against DMMB-induced inhibition of band 3 activity.
Ten percent erythrocyte suspensions in PBS and 30 m*M* sucrose were incubated in the dark with (△,▲) or without (●) 100 µ*M* DIP. After 5 min, DMMB (15 µ*M*) was added (●,▲), and the cells were incubated in the dark for another 5 min. The RBC suspensions were exposed to light doses of 0 to 9 J/cm^2. Samples treated with DIP were washed before band 3 activity was measured. Band 3 activity is given as percentage of control cells in which neither DIP nor DMMB were present.

EFFECT OF DMMB-MEDIATED PDT ON GLUCOSE TRANSPORT ACTIVITY

Incubation of the RBCs with DIP, which is known to bind with low affinity to the erythrocyte glucose transporter[27], has a small, but significant ($P< 0.05$), inhibitory effect on the glucose transport activity. In contrast to the results obtained with protoporphyrin, DMMB-mediated photosensitization of red cells did not affect the activity of the glucose transporter (Fig. 3). Even at light doses that induced 90%

leakage of K^+ (9 J/cm^2), glucose transport activity was not inhibited significantly. The efflux as measured at this high light dose was not because of nonspecific leakage of glucose. Cytochalasine B, an inhibitor of the Glut-1 glucose transporter[28], inhibited glucose exchange in treated cells to an extent comparable to that in control cells, suggesting that the protein conformation was not affected by DMMB-PDT.

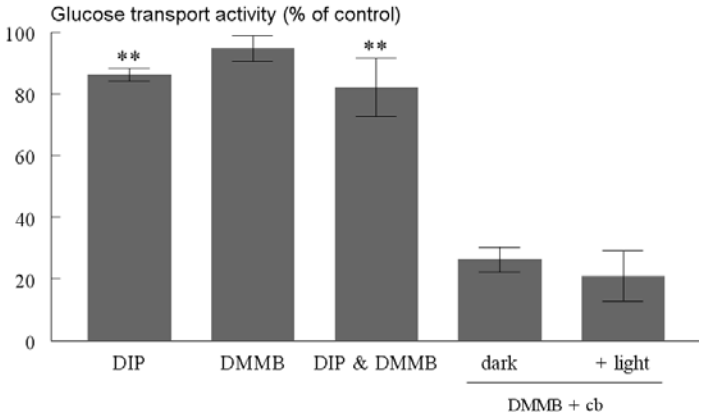

Figure 3. Effect of DMMB-PDT on the glucose transport of RBCs.
Ten percent erythrocyte suspensions in PBS and 30 m*M* sucrose were incubated in the dark for 5 min with DIP only (DIP), with DIP and DMMB (DIP & DMMB) or with DMMB only (DMMB). [DIP] = 100 µ*M,* [DMMB] = 15 µ*M.* Glucose transport of the treated cells was measured immediately after light exposure of 9 J/cm^2. Control for specificity of the activity measurement was performed with the use of 50 m*M* Cytochalasine B (cb) after incubation with DMMB in the dark or exposed to a light dose of 9 J/cm^2. Glucose transport activity is given as percentage of control in which neither DIP nor DMMB were present. ** $P < 0.05$.

EFFECT OF DMMB-MEDIATED PDT ON GLYCEROL TRANSPORT ACTIVITY

After an initial lag phase, glycerol transport was inhibited rapidly by DMMB-mediated photosensitization (Fig. 4). In contrast to what was observed with band 3 activity, DIP only partially protected against DMMB-mediated photodamage to the glycerol transport. DIP itself had no effect on glycerol transport activity. The

measured transport activity after DMMB-PDT was carrier mediated, as checked with the use of CuSO4, a specific blocker of the carrier. Passive transport was measured in this case and was negligible in comparison with the active transport. After 6 J/cm^2, the active transport was equal to the passive transport, suggesting a total inhibition of the carrier by DMMB-PDT (data not shown).

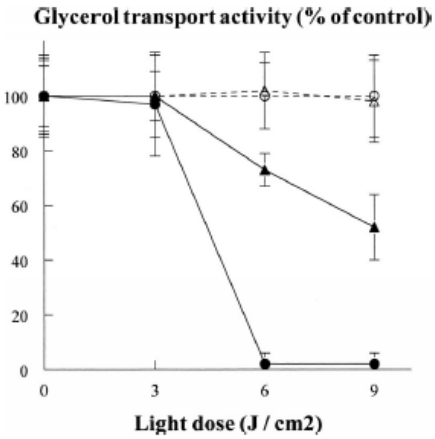

Figure 4. Partial protective effect of DIP against DMMB-induced inhibition of glycerol transport.
Ten percent erythrocyte suspensions in PBS and 30 m*M* sucrose were incubated in the dark with (△,▲) or without (○,●) 100 µ*M* DIP. After 5 min, DMMB (15 µ*M*) was added (●,▲) and the cells were incubated in the dark for another 5 min. In control cells (○), neither DIP nor DMMB were present. The RBC suspensions were exposed to light doses of 0 to 9 J/cm^2. Measurement of glycerol transport activity occurred immediately after light exposure from 0 to 9 J/cm^2. Glycerol transport activity is given as percentage of control.

DISCUSSION

A damaged band 3 has been suggested as being responsible for various forms of RBC lysis, including immunolysis[29], hyperthermic lysis[30], glycerol lysis[31] and osmotic lysis.[32,33] Based on the observation that photohemolysis obeys a second-order power dependence on light dose, and on the great potency of eosin isothiocyanate to sensitize photohemolysis, Pooler suggested a role in photohemolysis of band 3 protein, which exists as a dimer in the membrane.[19] Recently, VanSteveninck *et al.*[20] showed that the band 3 ligand DIP could protect RBCs against photodynamically induced K^+ leakage and delayed hemolysis. This again suggested an involvement of damage to band 3 in increased cation permeability and photohemolysis. Based on the comparable light dose dependency and response to DIP, we were able to show a direct correlation between DMMB-mediated damage to band 3 and K^+ leakage. Already, at a light dose of 3 J/cm^2, there was considerable K^+ leakage and inhibition of band 3

activity, as measured by decreased sulfate transport. Both K^+ leakage and inhibition of band 3 activity were completely prevented when illumination was performed in the presence of DIP. These results suggest strongly that DMMB-mediated photodamage to band 3 is indeed a causal factor in inducing K^+ leakage. The study of the transport systems for glucose and glycerol showed that they were not involved in the onset of DMMB-photodynamically induced K^+ leakage. Glucose transport was not affected by the PDT. Neither the rate of glucose efflux nor the ability of cytochalasin B to inhibit glucose transport was decreased. The facilitated diffusion of glycerol was inhibited by DMMB-mediated PDT. However, an involvement of this transport system in K^+ leakage is highly unlikely because of the difference in light dose dependency. At a light dose of 3 J/cm^2, K^+ leakage was already initiated, whereas glycerol transport was not affected. Notwithstanding the fact that DIP has no known preference for the glycerol transporter, photoinhibition of glycerol lysis was prevented partially by the presence of DIP in the illumination mixture. This was not caused by a general stabilization of the red cell membrane, because addition of DIP after the PDT had no effect on the inhibition of glycerol transport (data not shown). An alternative explanation for the protective effect of DIP could be the involvement of band 3 in glycerol transport as previously suggested.[31,34,35] Indeed, it has been observed that erythrocytes from patients suffering from systemic lupus erythomatosus have a decreased glycerol transport activity, which correlated with a decreased quantity of band 3 protein in the membrane.[34] Further evidence for the involvement of band 3 in glycerol transport is provided by the observation that the classical band 3 transport inhibitor 4.49-diisothiocyanostilbene-2,29-disulfonate also inhibits glycerol transport.[31,35] Not all band 3 inhibitors influence glycerol transport activity.

From our results it is clear that DIP has no effect on the glycerol transport activity (Fig. 3), which is in agreement with the finding that phenylglyoxal and 4.49-dinitrostilbene-2,29-disulfonate, which bind at the same location in band 3 as DIP[36], also had no effect on glycerol transport activity.[35] We hypothesize that the observed inhibition of glycerol transport activity by DMMB-mediated PDT is, at least partly, because of damage to band 3, and that DIP prevents such damage. In conclusion, our results are the first to show a direct correlation between DMMB-

mediated photodamage to band 3 and K^+ leakage. It remains to be elucidated whether this is specific for DMMB or is a general phenomenon for all photosensitizers, and whether band 3 is the only critical target. Also, it has to be established which photodamaged residues in band 3 are responsible for the leakage of potassium.

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