Potential of hyperthermia-induced haemolysis of human erythrocytes by photodynamic treatment

Evidence for the involvement of the anion transporter in this synergistic interaction

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Heat treatment of human erythrocytes led to increased passive cation permeability, followed by haemolysis. K^+ leakage was linear up to a loss of about 80 % in the temperature range 46–54 °C. Kinetic analysis of the results revealed an activation energy of 246 kJ/mol, implicating a transition in the membrane as critical step. Pretreatment of erythrocytes with 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate, chymotrypsin or chlorpromazine caused a potentiation of subsequent heat-induced K^+ leakage. Photodynamic treatment of erythrocytes with Photofrin II, eosin isothiocyanate or a porphyrin- Cu^{2+} complex as sensitizer also induced an increase in passive cation permeability, ultimately resulting in colloid osmotic haemolysis. The combination of photodynamic treatment immediately followed by hyperthermia had a synergistic effect on K^+ leakage. Analysis of the results by the Arrhenius equation revealed that both the activation energy and the frequency factor of heat-induced K^+ leakage were decreased significantly by preceding photodynamic treatment, suggesting that hyperthermia and photodynamic treatment have a common target for the induction of K^+ leakage. Several lines of reasoning indicate that this common target is band 3. A model is thus proposed for the observed potentiation of hyperthermically induced K^+ leakage by photodynamic treatment, in which photo-oxidation of band 3 results in increased sensitivity to subsequent thermal denaturation. These phenomena may be of more general significance, as photodynamic treatment and hyperthermia interacted synergistically with respect to K^+ leakage with L929 fibroblasts also.

INTRODUCTION

Erythrocytes have been extensively used as a model to study membrane damage by various cancer therapies, such as hyperthermia and photodynamic therapy (PDT). Hyperthermia is used in the treatment of various malignant tumours, as a single mode of treatment or in combination with other treatments [1,2]. Although the cause of heat-induced cell death remains unknown, the high activation energy of hyperthermic cell killing suggests that the denaturation of a protein is the critical step [3,4]. This could be a membrane protein, as suggested by evidence implicating membranes as sites of hyperthermic damage [5]. Heat treatment of erythrocytes induces K^+ loss and haemolysis [6–8]. Recently, Lepock *et al.* [8] have presented evidence indicating that the denaturation of the anion transporter protein, band 3, is probably the critical target for hyperthermia-induced haemolysis.

PDT is a cancer therapy, in which a sensitizer-loaded tumour is irradiated with visible light [9]. At present, Photofrin II is being used in phase III clinical trials for PDT of various tumours. In model studies with erythrocytes, photohaemolysis appeared to be based on increased passive cation permeability, which can be induced by several sensitizers [10–14]. Despite numerous studies, the molecular mechanism of photodynamically induced K^+ leakage has not yet been unambiguously established. Several studies indicate that photohaemolysis is related to photo-oxidation of proteins [12,13]. Deuticke *et al.* [14] demonstrated that oxidative alterations of membrane proteins may cause increased lipid mobility, leading to the formation of an aqueous pore. Finally, some studies, utilizing sensitizers that seem to be more or less specific for band 3, focus on this protein as the most probable critical site for photodynamically induced K^+ loss and haemolysis [15,16].

The combination of PDT with hyperthermia has received much interest, as synergistic interactions have been described in an experimental mouse tumour system and in cultured cells [17,18]. The background of this synergism is unknown. One of the possible mechanisms could be the concerted action of both treatments on the same cellular component. We have studied in detail the synergistic interaction between photodynamic treatment and hyperthermia on some proteins, and the experimental results were consistent with a model in which photodynamic treatment affects the conformational characteristics of the protein molecule, thus changing its susceptibility to subsequent hyperthermia [19,20].

In the present investigations, a synergistic interaction between photodynamic treatment and hyperthermia on K^+ leakage in erythrocytes was found. The results can be reconciled with the following model: slight photodynamic damage to band 3 causes a conformational change, and this conformational change sensitizes band 3 to thermal denaturation, leading to potentiation of heat-induced K^+ leakage.

MATERIALS AND METHODS

Photofrin II was obtained from Photofrin Medical Inc., Raritan, NJ, U.S.A.; haematoporphyrin derivative (HPD) was from Paisley Biochemicals, Paisley, Scotland, U.K.; eosin isothiocyanate (EYNCS) and chlorpromazine were from Sigma; chymotrypsin was from Boehringer and 4,4'-di-isothiocyano-2,2'-stilbene disulphonate (DIDS) was from Pierce Chemical Co. The Cu^{2+} complex with HPD was prepared as described previously [16]. NCTC mouse fibroblasts (clone L929, ATCC number CCL 1) were obtained from Flow Laboratories.

Abbreviations used: DIDS, 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate; DPBS, Dulbecco's phosphate-buffered saline; EYNCS, eosin isothiocyanate; HPD, haematoporphyrin derivative; PBS, phosphate-buffered iso-osmotic NaCl solution (140 mM-NaCl, 10 mM- Na_2HPO_4/KH_2PO_4 , pH 7.4); PDT, photodynamic therapy.

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Fig. 1. Effects of hyperthermia on erythrocytes

(a) Effect of hyperthermia on K^+ leakage from erythrocytes. Temperatures used: $\circ$, 20 °C (control); $\bullet$, 46 °C; $\square$, 49 °C; $\blacksquare$, 52 °C; $\triangle$, 54 °C; $\blacklozenge$, 52 °C. Treatment was for 60 min, followed by further incubation at 20 °C. (b) Heat treatment of erythrocytes at 52 °C in the presence ($\blacksquare$, $\bullet$) or in the absence ($\square$, $\circ$) of dextran 4. Intracellular K^+ ($\square$, $\blacksquare$) and haemoglobin ($\circ$, $\bullet$) concentrations are expressed as percentages of the initial value ($[K^+]$, 145 mequiv/l; [haemoglobin], 5.1 mM).

Fig. 2. Effects of various pretreatments of erythrocytes on heat-induced K^+ leakage at 52 °C

$\circ$, Control; $\blacktriangle$, chymotrypsin; $\square$, DIDS; $\blacksquare$, chlorpromazine.

Tissue culture products and newborn bovine serum were purchased from Gibco. All other chemicals were of analytical grade and were used without further purification.

Freshly drawn heparinized human blood was centrifuged and the erythrocytes were washed three times and resuspended in phosphate-buffered saline (PBS). K^+ efflux from erythrocytes was determined with a flame photometer. The initial intracellular K^+ concentration was measured by lysis of the cells in distilled water. Haemolysis was measured as described by Lepock *et al.* [8]. Photodynamic treatment of erythrocytes was performed by illumination of the erythrocyte suspension, in the presence of a sensitizer, with a standard Rollei slide projector equipped with a 150 W quartz halogen light bulb, at $300 \text{ W} \cdot \text{m}^{-2}$. In control experiments it was established that illumination of cells in the absence of sensitizer, or incubation with sensitizer in the dark, did not cause K^+ leakage, and did not affect subsequent heat-

Fig. 3. Arrhenius plot of heat-induced K^+ leakage from erythrocytes

For symbols, see the legend to Fig. 2. v is K^+ leakage ($\% \cdot \text{min}^{-1}$).

induced K^+ leakage. Heat treatment of the erythrocyte suspension at a 10% haematocrit was done in a temperature-controlled water-bath (accuracy better than ± 0.1 °C). The samples reached the desired temperature within 3 min.

Incubation of the erythrocytes with DIDS was done as described by Jennings & Passow [21], at a 10% haematocrit, with $5 \mu\text{M}$ -DIDS in PBS for 30 min at 5 °C. Treatment of intact erythrocytes with chymotrypsin was carried out as described by Steck *et al.* [22], at a 10% haematocrit, with 0.2 mg of enzyme/ml for 20 min at 37 °C. Chlorpromazine (final concentration 0.1 mM) was added to a 2% erythrocyte suspension for 30 min at room temperature before heat treatment. The drug was left in the suspension during heat treatment.

Mean cellular volume measurements were done with a Coulter Counter ZM (Coulter Counter Electronics, Mijdrecht, The Netherlands) and by determination of forward light scattering on a Becton-Dickinson FACStar flow cytometer.

L929 cells were cultured, incubated with Photofrin II (5 µg/ml) and illuminated as described previously [23]. Hyperthermic treatment of the cells was performed by placing a culture dish, containing a confluent cell layer covered with 2 ml of Dulbecco's phosphate-buffered saline (DPBS), in a temperature-controlled water-bath. The K⁺ content of the cells was measured as described before [23]. Protein was determined according to Lowry *et al.* [24].

RESULTS

Hyperthermic treatment of erythrocytes induced K⁺ leakage that was linear with time of exposure up to a loss of 80% in the temperature range 46–54 °C. Elevation of the temperature increased the velocity of K⁺ leakage: no K⁺ was lost on incubation

at room temperature for 2 h, whereas at 52 °C nearly 51% was lost in the same period of time. The leakage of K⁺ ceased after cooling the heated erythrocyte suspension to room temperature (Fig. 1a). The increased passive cation permeability led ultimately to haemolysis, as shown in Fig. 1b). Haemolysis was preceded by a swelling of the cells, as determined by both Coulter Counter and flow cytometry measurements. After 30 min at 52 °C an increase of 30% in mean cellular volume was measured (results not shown), although virtually no haemolysis had taken place (Fig. 1b). The addition of 40 mM-dextran 4 (*M_r* 4000–6000) was protective against haemolysis, but had no influence on cation leakage (Fig. 1b).

The relationship between the rate of K⁺ loss and the temperature (*T*) was analysed according to the Arrhenius equation:

$$\ln k = \ln A - E/RT, \quad (1)$$

in which *k* is the reaction constant, *A* is the frequency factor, *E* is the activation energy and *R* is the gas constant. For heat-induced K⁺ leakage, the activation energy was 246 kJ/mol and the frequency factor was e⁸¹. The high activation energy is in the range commonly found for the denaturation of proteins.

In further experiments the possible involvement of band 3 in heat-induced K⁺ leakage was studied. Treatment of erythrocytes with DIDS, chymotrypsin or chlorpromazine did not cause appreciable K⁺ leakage by itself, but it potentiated subsequent heat-induced K⁺ loss (Fig. 2). Analysis of the synergistic effects obtained with these agents indicated that the activation energy remained constant, whereas the frequency factor increased (Fig. 3).

Photodynamically induced K⁺ loss, with protoporphyrin, Photofrin II, EYNCS and the HPD-Cu²⁺ complex as sensitizers, is shown in Fig. 4. Photodynamic treatment with each sensitizer ultimately resulted in colloid osmotic haemolysis (results not shown).

The effect of combined photodynamic and heat treatments was dependent on the sequence in which they were applied. Hyperthermia followed by photodynamic treatment had an additive effect (Fig. 5a), whereas the effect of photodynamic treatment immediately followed by hyperthermia was synergistic with all sensitizers used. Fig. 5(b) shows the combination of

Fig. 4. Photodynamically induced K⁺ leakage from erythrocytes

Sensitizers used were: ○, Photofrin II (12.5 µg/ml at 10% haematocrit); ●, protoporphyrin (12 µg/ml at 10% haematocrit); □, EYNCS (1 µM at 2% haematocrit); ■, Cu²⁺-HPD complex (20 µg/ml at 2% haematocrit).

Fig. 5. Effect of photodynamic treatment with Photofrin II (12.5 µg/ml at 10% haematocrit), hyperthermia and a combination of these treatments on K⁺ leakage

(a) ○, Photodynamic treatment; ●, erythrocytes exposed for 15 min to 52 °C, followed by photodynamic treatment. (b) Photodynamic treatment for: ○, 0 min; ●, 2 min; □, 5 min; ■, 14 min, followed by exposure to 46 °C. The residual K⁺ contents after the first treatment are normalized to 100% in the Figure.

Fig. 6. Influence of photodynamic pretreatment on the Arrhenius parameters of thermally induced K⁺ leakage

The ordinate shows photodynamically induced K⁺ leakage up to 40% (provoked by various illumination periods), prior to heat treatment. (a) Activation energy, (b) frequency factor. Sensitizers: ○, Photofrin II (12.5 µg/ml at 10% haematocrit); ●, protoporphyrin (12 µg/ml at 10% haematocrit); □, EYNCS (1 µM at 2% haematocrit).

Fig. 7. Effects of heat and photodynamic treatments on K⁺ leakage in L929 cells

(a) Effect of hyperthermia and the combination of photodynamic and heat treatments on the K⁺ leakage in L929 cells. Heat treatment was at: ○, 43 °C; ■, 46 °C. Photodynamic treatment was for 10 min (●) or 15 min (□), followed by incubation at 43 °C. The residual K⁺ contents after the first treatment are normalized to 100%. (b) Influence of photodynamic treatment on the activation energy (●) and the frequency factor (○) of subsequent heat-induced K⁺ leakage. The initial K⁺ concentration in untreated cells was 0.97 µequiv./mg of protein.

photodynamic treatment with Photofrin II and heat treatment at 46 °C. The effect was clearly dependent upon the amount of photodynamic damage: illumination for 2 min, which resulted in 4% K⁺ loss, increased the velocity of K⁺ leakage during subsequent hyperthermia at 46 °C by a factor of 13, whereas illumination for 14 min, causing K⁺ loss of 32%, induced an increase of about 75-fold. Analysis of the results obtained at different temperatures revealed that preceding photodynamic treatment resulted in a significant decrease in the activation energy and in the frequency factor, in an illumination-time-dependent way (Fig. 6). After an illumination for 14 min, the activation energy decreased to 28 kJ/mol and the frequency factor to e^{13} . The effects on the Arrhenius parameters obtained with protoporphyrin and EYNCS as sensitizer are shown in Fig. 6.

Similar results were obtained with L929 fibroblasts. Photodynamic treatment (results not shown) and hyperthermia both induced K⁺ leakage in L929 cells (Fig. 7a). The activation energy for heat-induced K⁺ leakage in the temperature range 43–46 °C was 587 kJ/mol, and the frequency factor was e^{221} . Photodynamic treatment followed by hyperthermia also had a synergistic effect (Fig. 7a). The decreases in the activation energy and in the frequency factor for heat-induced K⁺ leakage are shown in Fig. 7(b).

DISCUSSION

As shown, heat-induced haemolysis was preceded by K⁺ leakage and pre-lytic swelling of the cells. The presence of dextran 4 did not affect heat-induced K⁺ loss, but delayed the

onset of haemolysis. These observations indicate a colloid osmotic mechanism of haemolysis, based on increased passive cation permeability of the erythrocyte membrane [25]. Several experimental observations indicate that a membrane constituent is the most likely target of hyperthermia [7,8]. In differential scanning calorimetry and thermal gel analysis studies, band 3 (the anion transport protein) could be identified as the most probable critical target of thermally induced haemolysis [8,26].

Photodynamic treatment of erythrocytes also induces increased passive cation permeability, resulting in colloid osmotic haemolysis [12,13]. Experiments using EYNCS and the HPD-Cu²⁺ complex as sensitizers indicated that band 3 is probably involved in photohaemolysis [15,16]. Such observations and a kinetic analysis of the process led Pooler [27] to the conclusion that band 3 dimers in the membrane are the most likely target of photohaemolysis.

Thus band 3 is presumably the critical target both in thermally induced and in photodynamically induced colloid osmotic haemolysis. In previous model studies the combined effects of photodynamic and heat treatments on a common protein target were investigated [20]. It was shown that with hyperthermia followed by photodynamic treatment an additive effect can be expected. With the reverse sequence, however, synergistic interactions are feasible, based on photodynamically induced changes in protein conformation. Such changes affect the structural stability of the protein molecule [19] and can easily affect its susceptibility to subsequent thermally induced conformational deterioration. Characteristically, this mechanism is, in the Arrhenius equation, reflected by photodynamically induced changes in the activation energy and/or the frequency factor of heat-induced damage [20]. The results described in the present paper are in agreement with such a mechanism. Both photodynamic and heat treatments of erythrocytes induced increased passive cation permeability, presumably via damage of band 3 as a common target. When hyperthermia preceded photodynamic treatment the effects were additive, whereas with the reverse sequence a synergistic interaction was found. In terms of the Arrhenius equation, significant decreases in both the activation energy and the frequency factor were observed. The decreased frequency factor would tend to inhibit thermal damage. The effect of the decreased activation energy, however, is much more pronounced, resulting in a strong potentiation of heat-induced colloid osmotic lysis.

As well as photodynamically induced changes, other agents that affect protein conformation may change the susceptibility of a protein to subsequent heat treatment [20]. In this context, the effects of chymotrypsin, DIDS and chlorpromazine on subsequent heat-induced K⁺ leakage are interesting. These agents evoked no or only very limited K⁺ leakage at room temperature, but clearly potentiated subsequent heat-induced K⁺ loss. Analysis of the results using the Arrhenius equation revealed an increase in the frequency factor, with no or only a slight effect on the activation energy. As shown in many studies, treatment of erythrocytes with chymotrypsin leads to cleavage of the extracellular fragment of band 3 [21,22,28–30]. It is quite conceivable that this will affect the susceptibility of band 3 to subsequent heat treatment via the described mechanism. Under the present experimental conditions, DIDS is a highly specific reagent for the band 3 protein [21,30–33]. Therefore the pronounced potentiation by DIDS of subsequent heat-induced K⁺ leakage with the concomitant increase in the frequency factor implicates band 3 as the critical target in this process. Further, Snow *et al.* [34] have demonstrated that a localized phospholipid region strongly interacts with band 3 and that several drugs with local anaesthetic properties, such as chlorpromazine, affect band 3 structure and function by intercalating in this phospholipid

region. The potentiation of heat-induced K⁺ leakage by chlorpromazine can thus be explained along these lines. Taken together, the potentiation of heat-induced K⁺ leakage by chymotrypsin, DIDS and chlorpromazine supports the notion that band 3 is critically involved in this process, as indicated by several other studies [8,26].

The results with L929 fibroblasts indicate that the described phenomena may be of more general interest. A synergistic interaction was also observed with these cells between photodynamic and heat treatments, with concomitant changes in the activation energy and the frequency factor. It could be speculated that in this case also an abundant transport protein is the target of this synergistic interaction.

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REFERENCES

- Hahn, G. M. (1982) *Hyperthermia and Cancer*, Plenum Press, New York
- Konings, A. W. T. (1987) *Radiat. Phys. Chem.* **30**, 339–349
- Westra, A. & Dewey, W. C. (1971) *Int. J. Radiat. Biol.* **19**, 467–477
- Dewey, W. C. (1989) *Radiat. Res.* **120**, 191–204
- Leyko, W. & Bartosz, G. (1986) *Int. J. Radiat. Biol.* **49**, 743–770
- Ham, T. H., Shen, S. C., Fleming, E. M. & Castle, W. B. (1948) *Blood* **3**, 373–403
- Grzelinska, E., Bartosz, G. & Leyko, W. (1982) *Int. J. Radiat. Biol.* **42**, 45–55
- Lepock, J. R., Frey, H. E., Bayne, H. & Markus, J. (1989) *Biochim. Biophys. Acta* **980**, 191–201
- Gomer, C. J., Rucker, N., Ferrario, A. & Wong, S. (1989) *Radiat. Res.* **120**, 1–18
- Lamola, A. A., Yamane, T. & Trozzolo, A. M. (1973) *Science* **179**, 1131–1133
- Girotti, A. W. (1990) *Photochem. Photobiol.* **51**, 497–509
- Schothorst, A. A., Van Steveninck, J., Went, L. N. & Suurmond, D. (1972) *Clin. Chim. Acta* **39**, 161–170
- Dubbelman, T. M. A. R., Haasnoot, C. & Van Steveninck, J. (1980) *Biochim. Biophys. Acta* **601**, 220–227
- Deuticke, B., Henseleit, U., Haest, C. W. M., Heller, K. B. & Dubbelman, T. M. A. R. (1989) *Biochim. Biophys. Acta* **982**, 53–61
- Pooler, J. P. & Girotti, A. W. (1986) *Photochem. Photobiol.* **44**, 495–499
- Van Steveninck, J., Boegheim, J. P. J. & Dubbelman, T. M. A. R. (1985) *Biochim. Biophys. Acta* **821**, 1–7
- Henderson, B. W., Waldow, S. M., Potter, W. R. & Dougherty, T. J. (1985) *Cancer Res.* **45**, 6071–6077
- Christensen, T., Wahl, A. & Smedshammer, L. (1984) *Br. J. Cancer* **50**, 85–89
- Prinsze, C., Dubbelman, T. M. A. R. & Van Steveninck, J. (1990) *Biochim. Biophys. Acta* **1038**, 152–157
- Prinsze, C., Dubbelman, T. M. A. R. & Van Steveninck, J. (1991) *Biochem. J.* **276**, 357–362
- Jennings, M. L. & Passow, H. (1979) *Biochim. Biophys. Acta* **554**, 498–519
- Steck, T. L., Ramos, B. & Strapazon, E. (1976) *Biochemistry* **15**, 1154–1161
- Dubbelman, T. M. A. R. & Van Steveninck, J. (1984) *Biochim. Biophys. Acta* **771**, 201–207
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 264–270
- Schothorst, A. A., Van Steveninck, J., Went, L. N. & Suurmond, D. (1970) *Clin. Chim. Acta* **28**, 41–49
- Lysko, K. A., Carlson, R., Taverna, R., Snow, J. & Brandts, J. F. (1981) *Biochemistry* **20**, 5570–5576
- Pooler, J. P. (1986) *Photochem. Photobiol.* **43**, 263–266
- Cabantchik, Z. I. & Rothstein, A. (1974) *J. Membr. Biol.* **15**, 227–248
- Passow, H., Fasold, H., Lepke, S., Pring, M. & Schuhmann, B. (1977) *Adv. Exp. Med. Biol.*, 353–379

30. Dubbelman, T. M. A. R., De Goeij, A. F. P. M., Christianse, K. & Van Steveninck, J. (1981) *Biochim. Biophys. Acta* **649**, 310–316
31. Cabantchik, Z. I. & Rothstein, A. (1974) *J. Membr. Biol.* **15**, 207–226
32. Ramjeesingh, M., Gaarn, A. & Rothstein, A. (1980) *Biochim. Biophys. Acta* **599**, 127–139
33. Lepke, S., Fasold, H., Pring, M. & Passow, H. (1976) *J. Membr. Biol.* **29**, 147–177
34. Snow, J. W., Brandts, J. F. & Low, P. S. (1978) *Biochim. Biophys. Acta* **512**, 579–591

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