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Peroxidative membrane damage in human erythrocytes induced by a concerted action of iodoacetate, vanadate and ferricyanide

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Human erythrocytes incubated without substrate in the presence of iodoacetate (0.2 mM), vanadate (0.5 mM) and ferricyanide (5 mM) form aqueous membrane leaks of equivalent radii of 0.5–0.8 nm leading to complete colloid-osmotic lysis within 180 min. All three components are indispensable for the effect. Inosine but not glucose markedly enhances the rate of hemolysis. These effects are due to oxidative damage, as indicated by concomitant destruction of polyunsaturated fatty acids and suppression of both effects by radical scavengers. Hemoglobin is not oxidized under these conditions. GSH and membrane SH levels remain almost normal, and no crosslinking or irreversible aggregation of membrane proteins is observed. In the absence of O_2 no membrane damage can be observed. It is proposed that radical formation originates from reduction of O_2 by NADPH, analogous to processes described in microsomal membranes. NADH seems not to be involved, since leak formation occurs in spite of the blockage of NADH formation by iodoacetate. Vanadate and ferricyanide are probably required to amplify the peroxidative reaction sufficiently to overcome the cellular antioxidative capacity.

Introduction

The reactions initiating the formation of activated oxygen species (O_2^-, $OH^\cdot$, H_2O_2) and the modes of attack of these species on biological material are subjects of intensive research [1,2]. The deleterious effects of oxidative damage on proteins and lipids are of paramount interest in biological and biomedical research [3–6]. Oxidative stress induces numerous types of alteration in biological membranes. Among the structural manifestations, peroxidation of lipids [7,8] and oxidation, crosslinking, or scission of proteins [8–10] are most important. Functionally, changes of membrane fluidity [11], rigidification of the membrane skeleton [12], inhibition of membrane enzymes [13,14] and of carrier-mediated transport [15], as well as loss of the barrier function have been described [14,16,17].

Many of these perturbations are well established for mammalian erythrocytes treated with inorganic oxidants (O_2^- [18,19]; H_2O_2 [12,20]), with compounds inducing the formation of these oxidants [21,22], and with compounds that lead to

Abbreviations: EGTA, ethyleneglycol bis(2-aminoethyl ether)-*N*,*N*′-tetraacetic acid; r_{SE}, Stokes–Einstein radius; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; PCMBS, *p*-chloromercuriphenylsulfonate.

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a selective oxidation of SH-groups [23,24] or are precursors of alkoxy radicals [16,25,26].

Besides these established entrances into the pathways of oxidative membrane damage, there are other less direct ways of initiating oxidative chain reactions. One such way is described in the following.

Fuhrmann and Knauf [27] have shown that erythrocytes simultaneously exposed to ferricyanide and vanadate in the presence of adenosine and iodoacetic acid release K^+ and take up Na^+. The modulation of a cation pathway, somehow related to the operation of the red-cell transmembrane NADH-dependent oxidoreductase using ferricyanide as electron acceptor [28,29], has been proposed as an explanation for these cation movements. Vanadate is known as an oxidant of various intra-erythrocytic constituents, including GSH and NADH [30,31]. It has also been shown to induce or stimulate free-radical-mediated and peroxide-producing oxidation in enzyme-catalysed [32,33] and non-enzymatic reactions [34] and to promote lipid peroxidation in endomembranes [35,36].

In the present paper we demonstrate that incubation of erythrocytes with vanadate and ferricyanide in the presence of iodoacetic acid in fact induces peroxidative damage of the red cell membrane, resulting in the formation of aqueous leaks and cell lysis.

Materials and Methods

Materials

Human blood from healthy donors was from the local blood bank. [^{36}Cl]Chloride and [^{14}C]erythritol were from Amersham-Buchler. Metavanadate ($NaVO_3$) was from Merck, diethyldithiocarbamate, superoxide dismutase and butylated hydroxytoluene from Sigma, desferrioxamine (Desferal®) from Ciba, 4,4′-dinitrostilbene 2,2′-disulfonate (DNDS) from K & K Chemicals, 4,4′-diisothiocyanostilbene 2,2′-disulfonate (DIDS) from Calbiochem, 4-bromomethyl-7-methoxycoumarin from Aldrich. Solutions containing metavanadate were prepared by dissolving the salt by heating and adjusting the pH to 7.4.

Methods

Freshly taken human blood, anticoagulated with citrate, was stored at 4° C in a conventional storage medium containing glucose (20 mM) and adenine (25 μM) and used for experiments within 5 days. Erythrocytes were isolated by centrifugation (5 min, $6000 \times g$), plasma and buffy coat were removed and the cells were washed three times with isotonic saline at room temperature.

Oxidative treatment

Washed erythrocytes were incubated at 37° C, pH 7.4, in 10 vol. of the following medium (concentrations in mM): NaCl (130), NaH_2PO_4/Na_2HPO_4 (12.5), containing routinely iodoacetate (0.2), vanadate (0.5), and ferricyanide (5.0). This medium is termed IVF medium in the following. Further supplements or pretreatments are given in the Results. At the end of the incubation period butylated hydroxytoluene, dissolved in a small volume of ethanol, was added at a final concentration of 100 μM to stop lipid peroxidation and leak formation [16]. The addition of butylated hydroxytoluene was omitted when rates of hemolysis were measured.

Characterization of the modified cells

Leak permeability: hemolysis curves. For hemolysis curves samples of cells suspended in IVF medium at 37° C were taken at appropriate intervals and centrifuged. Hemoglobin released was quantified photometrically after conversion to cyanomethemoglobin and expressed relative to the content in the total cell suspension (= % lysis). Rates of hemolysis were defined as the reciprocal of the time required for 50% hemolysis (t_{50}^{-1}).

Leak permeability: leak size. The apparent size of the induced leaks was characterized by following the colloid-osmotic lysis of damaged cells during incubation, after addition of butylated hydroxytoluene (100 μM), in isotonic saline (100 mM KCl, 50 mM NaCl) and testing nonelectrolytes of varying molecular size for their ability to act as osmotic protectants [24]. Cells were suspended in isotonic saline containing 40 mosmol/l of either mannitol, sucrose, raffinose, Dextran 1 ($M_r = 970$), or Dextran 4 ($M_r = 4000$–6000) and incubated for 20 h at 4° C. The extent of lysis was

then determined. The size of the induced holes was set equal to the radius of the nonelectrolyte protecting 95% of the cells against lysis. Radii of protecting solutes were adopted from Ref. 16.

Leak permeability: tracer fluxes. Increases in membrane leak permeability were quantified by measuring the increase of the stilbene disulfonate-insensitive Cl^- permeability or the cytochalasin-B-insensitive erythritol permeability at 0 °C using tracer fluxes as described earlier [24]. In control cells this permeability is extremely low ($P < 10^{-9}$ cm · s^{-1}) at 0 °C.

Activity of transmembrane NADH oxidoreductase. The reduction of extracellular ferricyanide by intact erythrocytes, followed spectrophotometrically as A_{420}, was taken as a measure for the activity of the transmembrane NADH oxidoreductase. Erythrocytes (10% hematocrit) were incubated at 37 °C in NaCl/phosphate buffer (150 mM and 12.5 mM resp.) containing 0.5 mM ferricyanide and further additives as given in the legends. At intervals, samples were taken, deproteinized with 50 μl 3 M $HClO_4$ and centrifuged, and the A_{420} was measured in the supernatant.

Oxidative changes of hemoglobin. At appropriate intervals, 50 μl of the cell suspension incubated as described above were diluted into 3 ml of 75 μM phosphate buffer (pH 7.4). From the absorption at 560, 577, 630 and 700 nm the fractional amounts of oxidized hemoglobin were calculated using formulae given by Szebeni et al. [37].

Glutathione, membrane SH-groups and binding of heme compounds to the erythrocyte membrane. Washed cells were lysed in 40 vol. of 5 mM phopshate buffer (pH 8) at 4 °C. Washed membranes were solubilized by addition of 6 vol. of the buffer containing 1% (w/v) SDS. Membrane SH content and amounts of heme compounds bound to the membrane were determined as described in Ref. 38; cellular glutathione contents were quantified according to Ref. 39.

Membrane protein patterns. Ghost membranes prepared as described above were solubilized in buffer (0.3 M Tris (pH 7)/5% (w/v) SDS) [40] in the presence of 5 mM *N*-ethylmaleimide [41]. After addition of 20% (v/v) glycerol, samples containing between 0.2 and 0.8 mg protein per ml were incubated at 37 °C for 50 min. When desired, disulfide bonds were cleaved by incubating the ghosts with 40 mM dithioerythritol for 30 min at 37 °C. Subsequently, 50 mM *N*-ethylmaleimide were added to prevent aggregation of protein material [41]. 30 μg of membrane protein were applied per slot of a conventional linear gradient slab gel. The composition of the stacking and the running gel, the buffer used, the running condition and the staining procedure were precisely as described in Ref. 38.

Disappearance of phospholipids. Cells were lysed by 2 vol. of H_2O, and extracted [42]. Phospholipids were separated by two-dimensional TLC as described in Ref. 38. Phospholipid fractions were quantified by phosphorus determination.

Fatty acid patterns. The fatty acid pattern of membrane phospholipids was quantified by a slight modification (Jüngling, E., personal communication) of the procedure of Voelter et al. [43]. Briefly, fatty acyl groups of extracted phospholipids (see above) were converted to the respective esters of the fluorescent label 4-bromomethyl-7-methoxycoumarin and separated by HPLC on a Hibar LiChroCART column filled with Lichrosphere 60CH8 (Merck, Darmstadt) using an isocratic eluent of acetonitrile/water (83 : 17 (w/w)). The data were processed by a Merck-Hitachi Chromato-Integrator D 2000.

Fluorescence. Formation of fluorescent chromolipids in peroxidizing membranes was assessed by measuring the relative fluorescence in isopropanol/chloroform extracts [42] at an excitation wavelength of 358 nm and an emission wavelength of 434 nm.

Results

Leak formation in IVF medium

Incubation of human erythrocytes in a medium containing 0.2 mM iodoacetate, 0.5 mM sodium metavanadate and 5 mM potassium ferricyanide (IVF medium) leads to the formation of membrane leaks, permeable to small ions (Cl^-), and to nonelectrolytes (erythritol) as indicated by tracer flux measurements in the presence of inhibitors of the specific transport system for the test solutes (Fig. 1). None of the three components alone or in combination with one of the other two induces leaks. It takes an exposure period of about 60 min

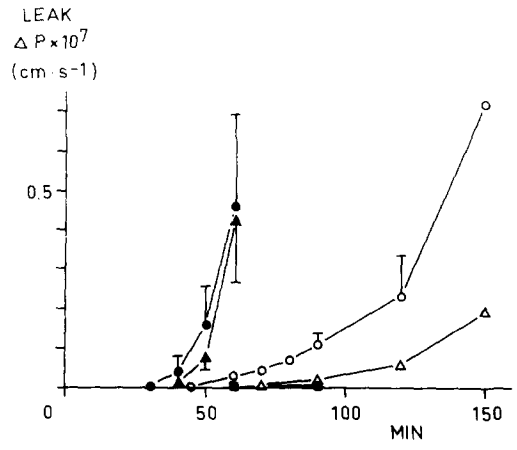

Fig. 1. Time-dependent development of peroxidative damage in human erythrocytes incubated in IVF medium (iodoacetate 0.2 mM, vanadate 0.5 mM, ferricyanide 5 mM). Cells were incubated at 37 °C, for the time periods given, in IVF medium (open symbols) or in IVF medium containing 2 mM inosine (closed symbols). The reaction was stopped by addition of 100 μM butylated hydroxytoluene and leak permeabilities for $^{36}Cl^-$ (circles) or [^{14}C]erythritol (triangles) were determined as described in Methods. ■, $^{36}Cl^-$ permeability in cells incubated in IVF medium containing 2 mM inosine and 100 μM butylated hydroxytoluene.

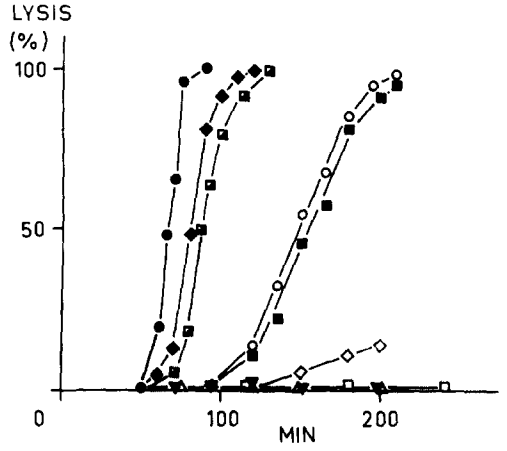

Fig. 2. Hemolysis of human erythrocytes incubated in IVF medium. Red cells were exposed at 37 °C to IVF medium under the following conditions: with no further additives (○); with 5 mM glucose (■); with 2 mM inosine (●); with 2 mM inosine and either 100 μM butylated hydroxytoluene (□), or 2 mM thiourea (△); with 2 mM inosine after conversion of hemoglobin to methemoglobin by pretreatment with nitrite (30 min, 150 mM $NaNO_2$) and thorough washing (◇); under the same condition in the presence of 2.5 mM NaN_3 (◆); with 2 mM inosine after conversion of hemoglobin to carbonmonoxyhemoglobin in a N_2 atmosphere (▼) or O_2 atmosphere (◪). Samples were taken at intervals and hemolysis was measured as described in Methods.

before the leaks appear. Within the next 75 min of incubation the defect becomes so pronounced that hemolysis occurs, which reaches completion after about 200 min (Fig. 2).

The processes leading to leak formation can be accelerated by the addition of inosine (Fig. 1 and 2), but are only slightly stimulated by glucose (Fig. 2). Inosine decreases the time required for 50% hemolysis from about 145–155 min to about 65–70 min (Fig. 2). The half-times decrease with increasing temperature according to a Q_{10} value of about 2. Addition of butylated hydroxytoluene, a powerful hydrophobic oxy-radical scavenger, completely suppresses the formation of leaks (Fig. 1) and prevents hemolysis (Fig. 2). The same is true for the antioxidant thiourea. When hemoglobin was converted to carboxyhemoglobin, lysis was only slightly delayed in an O_2-containing atmosphere but completely disappeared under N_2. These findings suggest the involvement of peroxidative reactions requiring molecular oxygen but not oxyhemoglobin. Attempts to demonstrate formation of malondialdehyde as an indicator of lipid peroxidation were unsuccessful because vanadate interferes with the color-forming thiobarbituric acid reaction. Analysis of the membrane phospholipid's fatty acid patterns, however, revealed a marked decrease in the content of polyunsaturated fatty acids (Fig. 3). After a 75 min incubation of erythrocytes in IVF medium in the presence of inosine (a time period just short of massive lysis) 22 : 6 is diminished to 20% of the level in native cells, 20 : 4 to about 40% (Fig. 3A). As a further indicator of peroxidative breakdown of unsaturated phospholipids, the relative fluorescence intensity of membrane lipid extracts increases more than 10-fold after treatment in IVF medium containing inosine (Fig. 3B). Both degradation of unsaturated fatty acids and formation of fluorescent chromolipids can be suppressed completely by butylated hydroxytoluene and are markedly diminished in cells containing carbonmonoxyhemoglobin in the absence of oxygen (Fig. 3A + B). Simultaneously, the levels of the aminophospholipids, phosphatidylethanolamine and phosphatidylserine, decrease by about 40% during incubation in IVF medium (Fig. 3C).

In line with the involvement of a peroxidative

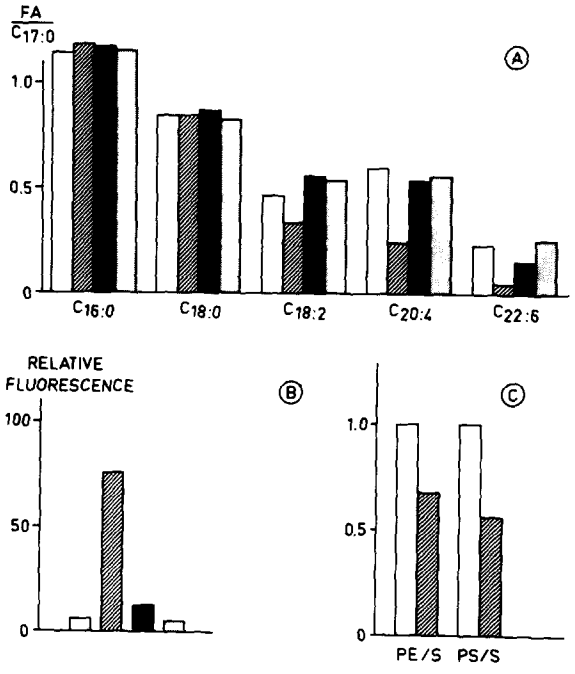

Fig. 3. Degradation of unsaturated fatty acids and phospholipids in human erythrocytes incubated in IVF medium. Erythrocytes were incubated in IVF medium containing 2 mM inosine at 37 °C for 75 min. The reaction was stopped by addition of 100 μM butylated hydroxytoluene. The content of fatty acids (A) normalized to the contents of heptadecanoic acid (17:0) added as an internal standard, of fluorescent chromolipids (B), and the ratio of phosphatidylethanolamine and phosphatidylserine over sphingomyelin (PE/S, PS/S, (C)) were determined as described in Methods. □, control cells; ▨, IVF-treated cells; ■, cells containing only CO-hemoglobin treated with IVF medium under nitrogen; ▩, cells treated with IVF in the presence of 100 μM butylated hydroxytoluene.

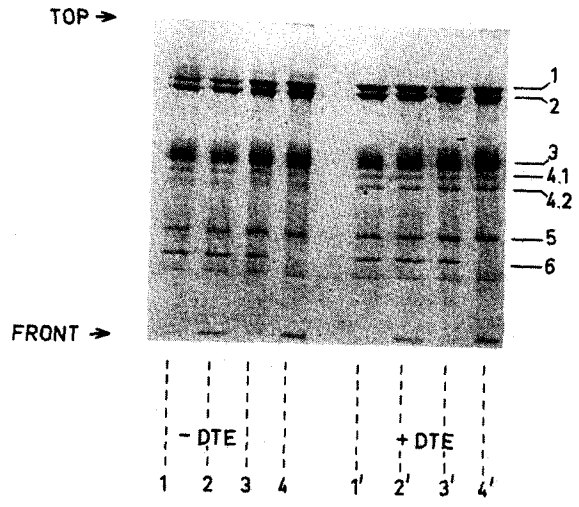

Fig. 4. Membrane polypeptide patterns of erythrocytes incubated in IVF medium in the presence of inosine. Human erythrocytes were exposed to IVF medium at 37 °C *not at all* (1,1'), or for 30 min (2,2'), 60 min (3,3') or 100 min (4,4'). After termination of the reaction by butylated hydroxytoluene, cells were washed and lysed, ghosts were isolated and membranes solubilized and treated with *N*-ethylmaleimide (NEM) as described in Methods. 30 μg of membrane protein were applied per slot. Slots 1'–4': the solubilized membrane proteins were reduced with 40 mM dithioerythritol (= DTE) followed by addition of 50 mM *N*-ethylmaleimide [42]. Nomenclature of polypeptide bands according to Ref. 64.

chain reaction, leak formation proceeds when cells are washed after a short period of treatment (40 min) with IVF medium containing 2 mM inosine and are then incubated further without any additives. Under these conditions formation of malondialdehyde can also be demonstrated (35 nmol/ml cells during 90 min incubation).

Less prominent manifestations of the damage exerted by the IVF medium concern the thiol status of the cell. The level of glutathione decreases slightly (−0.35 μmol/ml cells ($n = 3$)), thus reaching a value of about 85% of the control level, before leak formation becomes evident. This decrease, however, can be produced by the mere presence of iodoacetate. Membrane protein SH-groups diminish to a minor extent (−20%) before the onset of lysis.

Intracellular hemoglobin is not oxidized to methemoglobin to any detectable extent in cells incubated in IVF medium, and no significant precipitation of denatured hemoglobin at the membrane inner surface was observed. Moreover, the SDS-gel-electrophoretic membrane polypeptide patterns remained essentially normal (Fig. 4), in contrast to several forms of oxidative red cell damage [24,40,44,45]. In view of these observations, an unusual set of reactions can be suspected as the event initiating the peroxidative membrane damage.

Further experiments addressed the properties of the induced leak sites and the role of the components of the IVF medium for its effectivity.

Properties of the induced leak sites

Size. An apparent radius of the induced membrane defects was derived from the ability of

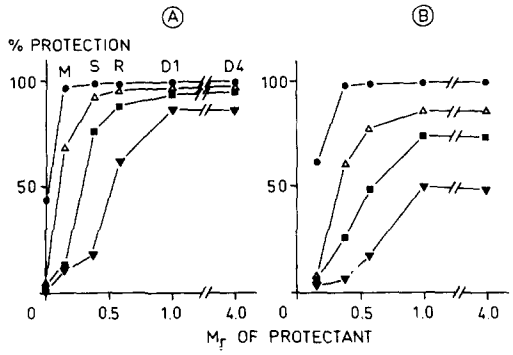

Fig. 5. Estimation of the size of the leaks induced by treatment in IVF medium. (A) Cells incubated in IVF medium for 60 min (●), 80 min (△), 100 min (■) or 120 min (▼) before the reaction was stopped by butylated hydroxytoluene. (B) Cells incubated in IVF medium in the presence of 2 mM inosine for 40 min (●), 50 min (△), 60 min (■) and 70 min (▼) before addition of butylated hydroxytoluene. Cells were then washed and incubated for 20 h at 4° C in KCl/NaCl (100/50 mM) in the presence of 40 mM of protectant: M = Mannitol; S = Sucrose; R = Raffinose; D1 = Dextran 1; D4 = Dextran 4. M_r in kDa.

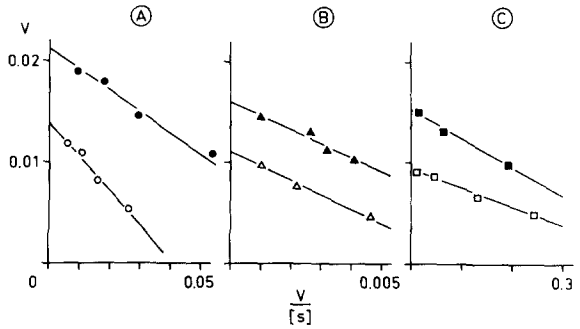

Fig. 6. Kinetic evaluation of rates of hemolysis of erythrocytes exposed to IVF medium as a function of the concentration (mM) of components of the system (Hofstee plot). (A) Erythrocytes incubated with varying concentrations of vanadate in the presence of 5 mM ferricyanide and 0.2 mM iodoacetate, without (○) or with (●) 2 mM inosine. (B) Erythrocytes treated with various concentrations of ferricyanide in a medium containing 0.5 mM vanadate and 0.2 mM iodoacetate without (△) or supplemented with (▲) 2 mM inosine. (C) Erythrocytes exposed to varying concentrations of iodoacetate in a medium containing 5 mM ferricyanide and 0.5 mM vanadate without (□) or with (■) 2 mM inosine. The reciprocal of the time (min) required for 50% hemolysis (at 37° C) was taken as V.

nonelectrolytes of different molecular size to protect treated cells against colloidosmotic lysis. The rationale behind this approach has been outlined in Ref. 16 and 24. According to Fig. 5, larger protectants are required with increasing time of exposure to IVF medium. In cells treated without inosine, sucrose (r_{SE} = 0.46 nm) still provides complete protection against lysis after 60 min of treatment, indicating an apparent radius of the induced leak site below 0.5 nm. After longer incubations part of the cell population cannot be protected, even by Dextran 4. In cells treated for 70 min in the presence of inosine, Dextran 4 (M_r = 4000, r_{SE} = 1.75 nm) protects only 50% of the cells and raffinose has almost no protective effect.

This pattern indicates that the size of the individual holes increases with increasing time of exposure to the injurious medium and the leak sizes are not homogeneously distributed in the cell population.

Enhancement of transbilayer reorientation rates of lipids in parallel to leak formation. Under a number of conditions that we have studied previously, formation of membrane leaks by oxidants was accompanied by an enhancement of the rates of flip-flop of phospholipids or their analogs [46,47]. In cells treated with IVF medium, the transmembrane movement of palmitoylcarnitine, a recently introduced probe for transbilayer mobility (Classen, J., M.D. thesis, unpublished) also proved to be enhanced (data not shown).

(C) Role of the components

In order to characterize our oxidative system in more detail we first determined the concentration dependency for each of the three agents at a saturating concentration of the two others, using the rates of hemolysis as an indicator. From the Hofstee plots in Fig. 6 the following concentrations for a half-maximal effect can be deduced: vanadate, 0.3 mM (without inosine) or 0.2 mM (with inosine); ferricyanide, 1.4 mM; iodoacetate, 0.02 mM; inosine, 0.2 mM. More specific information concerning the three components is compiled below.

(1) Vanadate. Vanadate enters the red cell, at least to a considerable extent, via the anion-exchange system (band 3) [48]. Its uptake and presence as VO_3^- is required to support the injurious effect of IVF, since inhibition of the anion transporter by DIDS or DNDS delays lysis (Table I) and intracellular vanadyl cannot replace vanadate (data not shown). Intracellular vanadate might

TABLE I

RATES OF HEMOLYSIS (t_{50}^{-1}) OF ERYTHROCYTES INCUBATED IN IVF MEDIUM CONTAINING 2 mM INOSINE, IN THE PRESENCE OF VARIOUS ADDITIVES

Values normalized to lysis in the absence of the additives.

Additive	Final concentration (mM)	Rate	Number of experiments
None (control)		1.0	
4,4′-Dinitrostilbene 2,2′-disulfonate	0.1	0.57	4
4,4′-Diisothiocyanostilbene 2,2′-disulfonate (pretreated 30 min 37 °C)	0.5 [a]	0.32	1
EDTA	5	0.24	4
EGTA	5	1.00	2
Butylated hydroxytoluene	0.1	< 0.01	4
Desferrioxamine	0.2	0.64	3
Diphenylamine	0.2	0.60	2
NaN_3	2.5	1.11	3
NaF	10	0.97	2
NaCN	2.5	1.37	2
Quinacrine	0.1	< 0.01	2
Quinine	2	1.26	2
Superoxide dismutase	100 [b]	1.00	2
Bathophenanthroline disulfonate	2	0.60	1
N,N′-Bis(2-chloroethyl)-*N*-nitrosourea (BCNU) (pretreatment 30 min, 37 °C)	0.3	1.00	1
Chlorodinitrobenzene (pretreatment 30 min, 37 °C)	0.3	0.72	2
PCMBS	0.1	0.96	2
Diethyldithiocarbamate (pretreatment 45 min, 37 °C)	4	0.87	2
Diethyldithiocarbamate (present during treatment)	1	< 0.01	2

[a] μmol/ml cells.
[b] U/ml cells.

catalyze a formation of superoxide [49], that in turn could induce membrane damage. To check for this possibility we tested whether inhibition of superoxide dismutase would interfere with leak formation. Cells were pretreated with 4 mM diethyldithiocarbamate, a condition known to inhibit superoxide dismutase by removing Cu^{2+} [50], washed free of the chelator and exposed to IVF medium. This procedure caused only a minor time delay in the development of membrane damage (Table I). In contrast, 1 mM diethyldithiocarbamate present during incubation in IVF medium provided full protection against lysis. These observations are in line with the well-established high antioxidant capacity [51] of diethyldithiocarbamate, they substantiate the peroxidative nature of leak formation but provide no evidence for a requirement of superoxide dismutase. Superoxide dismutase added to the extracellular medium was also ineffective (Table I).

Contribution of radicals arising from a reaction of vanadate with glutathione, in analogy to mechanisms described in Ref. 52, could also be excluded, since pretreatment of erythrocytes with either chlorodinitrobenzene, which blocks glutathione irreversibly [53], or *N,N′*-bis(2-chloroethyl)-*N*-nitrosourea which prevents GSSG reduction due to inhibition of GSH reductase [54], has essentially no influence on the development of membrane damage (Table I).

Ferricyanide. The erythrocyte membrane is impermeable to ferricyanide [55]. Therefore, its effect must originate from a transmembrane action. Ferricyanide cannot be replaced in our experiments by ferrocyanide, by Fe^{3+}/ADP, Fe^{3+}/ATP (5 mM), or by ferricytochrome *c* (0.8 mM). This excludes an unspecific contribution of ferricyanide as an extracellular redox catalyst. Ferrocyanide (and sodium nitroprusside ($Na_2[Fe(CN)_5NO]$)) in fact inhibit leak formation in IVF medium (data not shown).

A transmembrane NADH oxidoreductase in the erythrocyte membrane has been shown to reduce ferricyanide [28,29]. Oxidation of NADH by this enzyme, however, is not involved in the effect of IVF medium. In agreement with others [29,55], we could demonstrate reduction of ferricyanide by intact erythrocytes which was stimulated by inosine (Fig. 7). However, when either vanadate or iodoacetate or both were also present, for instance, in the complete IVF system, no reduction of ferricyanide could be measured. Quinacrine, known as an inhibitor of NADH oxidoreductase [56], suppressed leak formation considerably (Table I). The concentrations required, however, did not inhibit NADH oxidoreductase activity (Fig. 7). Quinacrine thus probably acts by scavenging

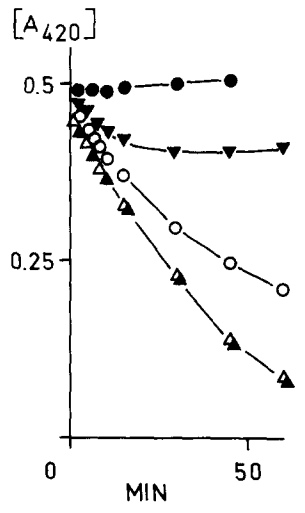

Fig. 7. Reduction of ferricyanide by human erythrocytes. The transmembrane transfer of electrons to ferricyanide, as measured by the change in A_{420}, was followed at 37° C in NaCl/phosphate buffer (pH 7.4) (150 mM and 12.5 mM, resp.) containing 0.5 mM ferricyanide with no further supplement (○), supplemented with 2 mM inosine in the absence (△) or the presence (▲) of 0.1 mM quinacrine or supplemented with 0.5 mM vanadate (●) or 0.2 mM iodoacetate (▼).

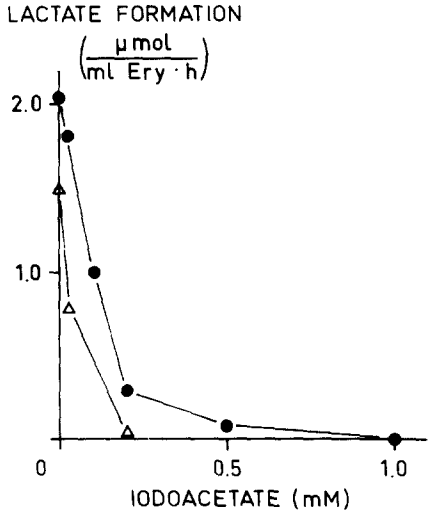

Fig. 8. Inhibition of lactate formation by iodoacetate. Human erythrocytes (10% hct) were incubated at the given concentration of iodoacetate in phosphate-buffered saline at pH 7.4 in the presence of 2 mM inosine (●) or 5 mM glucose (△). The production of lactate was followed over a period of 5 h and the rate was determined. Lactate was measured by routine methods [65].

radicals, in analogy to its suppressive effects on membrane damage by *t*-butylhydroperoxide (Heller, K.B. and Deuticke, B., unpublished results). PCMBS, reported to inhibit NADH oxidoreductase [28], also had no inhibitory effect on lysis is IVF media (Table I). In conclusion, a contribution of ferricyanide via mechanisms other than transmembrane NADH oxidoreductase must be envisaged.

Iodoacetate. At the low concentrations used routinely in our study, an almost complete inhibition of glyceraldehyde-3-phosphate dehydrogenase is the predominant effect of the inhibitor (Fig. 8). Therefore, glycolytic reactions and intermediates beyond this step of glycolysis are probably not relevant for the process investigated here. Other permeable SH-reactive agents (diamide, *N*-ethylmaleimide) could replace iodoacetate (data not shown).

Stimulation by inosine: the role of metabolism. Leak formation in IVF medium occurs, although slowly, in the virtual absence of metabolic substrate and is not stimulated by addition of glucose (Fig. 2). On the other hand, inosine and other purine nucleosides, but not pyrimidine nucleosides (data not shown) markedly stimulate leak formation. Nucleosides taken up by the erythrocyte are cleaved to ribose-1-*P* (and purine base) and further metabolized via the hexose monophosphate shunt and the Embden-Meyerhof pathway. Since the lower part of glycolysis, starting with the glyceraldehyde-3-phosphate dehydrogenase reaction and formation of NADH is essentially blocked under our experimental conditions (Fig. 8), one might assign the stimulating effect of inosine either to an increased formation of NADPH or the accumulation of one of the many intermediates of the hexose monophosphate shunt.

Modulation of the injurious effect of IVF medium. In many types of oxidative damage to erythrocytes, hemoglobin and its oxidation products are supposed to play an important role as initiators or scavengers of oxy-radicals [25]. As already shown in Fig. 2, the availability of oxyhemoglobin is not required for oxidative damage in IVF medium. On the other hand, conversion of hemoglobin into methemoglobin by pretreatment of the cells with nitrite strongly suppressed the effect of IVF medium without and with inosine. This may be due to the well-established [25] oxy-radical

scavenging properties of methemoglobin (Fig. 2). Azide, which markedly stimulates oxidative damage by H_2O_2 and organic peroxides [16,20], due to inhibition of catalase [20] or formation of azidomethemoglobin and consecutive relief of its radical scavenger properties [16], had only a slightly stimulating effect in cells containing oxyhemoglobin (Table I). This is in line with the virtual absence of methemoglobin in IVF-treated cells. In methemoglobin-containing cells azide restitutes the injurious effect of IVF medium (Fig. 2).

Desferrioxamine, a potent chelator of ferric ions which forms catalytically inactive Fe complexes [57] suppresses leak formation in IVF medium (Table I). Since, however, desferrioxamine also forms complexes with vanadate [58], it may just act by preventing the uptake of vanadate. This contention is also borne out by the finding that the impermeable chelator, EDTA, which forms complexes with both vanadate and divalent cations, also delays leak formation in IVF, while EGTA, which does not form a complex with vanadate [58], is ineffective. The lack of influence of EGTA also excludes a contribution of Ca^{2+} to the ion leak formation in IVF medium.

Discussion

We have shown here that exposure of erythrocytes to a mixture of iodoacetate, vanadate and ferricyanide induces a membrane alteration that is peroxidative in nature. This is indicated by (a) the parallelism between leak formation and the degradation of polyunsaturated fatty acids, (b) the suppression of this damage by antioxidants such as butylated hydroxytoluene, thiourea, diethyldithiocarbamate or diphenylamine, (c) the dependence on the availability of oxygen, and (d) the progress of peroxidative damage after removal of the noxious agents.

The sequence of events leading to the initiation of the peroxidative chain reaction presumably involves metabolic reactions. This is indicated by the requirement for iodoacetate and the stimulation by inosine. In the range of concentrations required here, iodoacetate is assumed to be a rather specific inhibitor of glyceraldehyde-3-phosphate dehydrogenase [59]. Reactions, or (accumulation of) metabolites, 'upstream' of the glyceraldehyde-3-phosphate dehydrogenase-reaction therefore seem to be required for the effect of IVF. The further elucidation of these reactions has to take into account, however, that damage develops in the absence of any added substrate and glucose accelerates only slightly (+15%), while purine nucleosides stimulate markedly. This constellation suggests that the hexose monophosphate shunt plays a role in the effect of IVF.

In the presence of suitable electron acceptors products of the phosphorylytic cleavage of inosine are channelled at a high rate through the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reactions, which produce NADPH [60]. Production of glucose 6-phosphate from inosine is not limited by hexokinase, in contrast to glucose 6-phosphate formation from glucose and ATP [61].

NADPH is a well-established link between metabolism and lipid peroxidation by oxy-radicals in microsomal and other endomembrane systems (for a review see Ref. 7). In plasma membranes, including that of the erythrocyte, however, radical formation sustained by reaction of O_2 with NADPH has never been detected to our knowledge. If such a reaction in fact occurs in erythrocytes, the additional presence of vanadate and ferricyanide seems to be required for a production of oxy-radicals in amounts sufficient to induce detectable membrane damage. Vanadate is known to amplify the oxy-radical-mediated oxidation of NADH and NADPH [32–36,49,57], particularly in the presence of membanes, including the erythrocyte [32,62]. Stimulation of an NADPH : oxidoreductase [63] or non-enzymatic amplification of O_2^--mediated oxidation of NADPH [33] have been proposed as the underlying mechanisms.

Previous in vitro determinations of the enzymatic oxidation of NADPH by erythrocyte membranes in the presence of vanadate indicate very low enzymatic activities which were strongly inhibited by cyanide [62] but not by thiourea [32], a pattern opposite to that found in our study for the leak formation in IVF medium. Moreover, the in vitro oxidation of NAD(P)H by erythrocyte membranes in the presence of vanadate is suppressed by superoxide dismutase [62] and should

therefore either not occur in native erythrocytes containing this enzyme in large amounts, or should at least be activated by inhibition of superoxide dismutase, e.g., after pretreatment with diethyldithiocarbamate (cf. Ref. 22). Such activation was not observed in our experiments. It is therefore unlikely that the hitherto described enzymatic pathways of generation of oxyradicals by oxidation of NAD(P)H [33,62] are involved under our conditions. Moreover, if O_2^- is part of the vanadate-induced amplification of oxidative membrane damage, it must somehow be protected from superoxide dismutase.

These considerations do not yet provide a role for ferricyanide. Under our experimental conditions this impermeable anion does not operate as an electron acceptor in a transmembrane electron flow. Iodoacetate and vanadate prevent enzyme-catalyzed electron transfer from the cell interior to ferricyanide (Fig. 7). A transmembrane effect has to be postulated, nevertheless. An unspecific pro-oxidative effect may be involved. In preliminary experiments we have demonstrated that the progress of oxidative damage in erythrocytes pretreated only with *t*-butylhydroperoxide [38] is enhanced by ferricyanide. This observation suggests actions of ferricyanide not involving transmembrane oxidoreductases.

In conclusion, the results suggest the following working hypothesis. Under metabolic conditions generating NADPH (through the hexose-monophosphate shunt and the upper part of the Embden-Meyerhof pathway) but not NADH (due to inhibition of glyceraldehyde phosphate dehydrogenase), the erythrocyte membrane is attacked by endogenously evolving radicals when vanadate and ferricyanide are present to enhance the peroxidative chain reaction. The initiating radicals may arise from a direct or enzyme-catalyzed reduction of O_2 by NADPH. Preliminary experiments to verify this hypothesis by altering the turnover of hexose monophosphate shunt provide support for this concept.

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