PART X

NEPHROLOGY 2

Chairmen: H Dutz
            F Tanman
A NEW ASPECT OF URAEMIC HAEMOLYSIS: INCREASED SUSCEPTIBILITY OF ERYTHROCYTES TO PEROXIDATION

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Apart from inadequate haemoglobin synthesis the increased haemolysis rate is acknowledged to be a main cause of anaemia in uraemic patients.

Several factors have been held to cause haemolysis in uraemia (acidosis, uraemic toxins, altered glucose metabolism). Although it is well known from other diseases that oxidative stress can cause haemolysis [1] up to now no information exists about the susceptibility of uraemic erythrocytes to activated oxygen. Investigations were therefore undertaken to find out whether the enzyme system for the detoxification of oxygen radicals in erythrocytes is altered under uraemic conditions.

During the autoxidation of oxyhaemoglobin to methaemoglobin toxic oxygen radicals are generated. One of the initial events during the autoxidation process is the generation of the very active superoxide radical [2,3] which is converted to hydrogen peroxide [4] by the enzyme superoxide dismutase. Hydrogen peroxide and organic hydroperoxides are in turn catabolised in erythrocytes mainly by the glutathione peroxidase system, and to a lesser degree by catalase [5]. The glutathione peroxidase system consists of the enzymes glutathione peroxidase (GSH-Px) and glutathione reductase (GR) and of NADPH which is generated through the pentose phosphate cycle [6]. If this oxygen detoxifying system is disturbed or if excess oxygen radicals are present cell destruction occurs either following haemoglobin denaturation, or from membrane damage due to an increased peroxidation of unsaturated fatty acids or through other mechanisms not elucidated so far [7]. Ferrihaemoglobin is reduced to haemoglobin under the influence of the enzyme ferrihaemoglobin reductase requiring NADH as cofactor which is supplied through the glycolytic pathway [7].

To elucidate the influence of uraemia on the oxygen detoxifying system the following parameters were investigated in vitro with erythrocytes from patients on haemodialysis and from healthy subjects: superoxide dismutase (SD), acetylphenylhydrazine (APHH) dependent haemolysis, hydrogen peroxide dependent haemolysis, lipid peroxidation, glutathione peroxidase (GSH-Px), and ferrihaemoglobin reductase.
Methods
Immediately after withdrawal the blood was kept between 0–4°C, plasma and the buffy coat were removed and the erythrocytes were twice washed with 0.9% saline. All the investigations were carried out within 6 hrs after blood withdrawal, no alterations were noticed during this time interval. The following estimation procedures were used: SD [8], APHH dependent haemolysis [9], hydrogen peroxide dependent haemolysis [10], lipid peroxidation [11], GSH-Px [12], ferrihaemoglobin reductase [13]. Details of the methods are published elsewhere [14].

Results and Discussion
Table I depicts the SD activity and the APHH dependent haemolysis.

<table>
<thead>
<tr>
<th></th>
<th>Superoxide dismutase</th>
<th>Per cent haemolysis/24 hr incubation</th>
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<th>Increase</th>
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<tr>
<td></td>
<td>Units/10 µg protein</td>
<td>Buffer</td>
<td>Buffer + APHH</td>
<td>Increase</td>
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<td>Controls</td>
<td>0.38 ± 0.15</td>
<td>2.5 ± 0.8a</td>
<td>4.5 ± 1.2b</td>
<td>80%</td>
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<td>Predialysis</td>
<td>0.43 ± 0.14</td>
<td>4.7 ± 1.6b</td>
<td>8.0 ± 0.9c</td>
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<tr>
<td>Postdialysis</td>
<td>0.44 ± 0.15</td>
<td>4.1 ± 1.2b</td>
<td>7.5 ± 1.3c</td>
<td>80%</td>
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Acetylphenylhydrazine represents, as model substance, a group of compounds which cause haemolysis by increasing the generation rate of oxygen radicals, especially that of superoxide radicals. After incubation in an appro-

<table>
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<tr>
<th></th>
<th>Buffer</th>
<th>Buffer + H₂O₂</th>
<th>Buffer</th>
<th>Buffer + H₂O₂</th>
<th>Glutathione peroxidase</th>
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<td>&lt; 1%</td>
<td>3.6 ± 1.1a</td>
<td>1.4 ± 0.4a</td>
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<td>&lt; 1%</td>
<td>10.3 ± 2.1b</td>
<td>1.3 ± 0.5a</td>
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<tr>
<td>postdialysis</td>
<td>&lt; 1%</td>
<td>4.2 ± 1.3a</td>
<td>1.4 ± 0.3a</td>
<td>2.7 ± 1.0b</td>
<td>1.7 ± 0.6</td>
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priate buffer system the haemolysis rate was significantly higher with uraemic erythrocytes than with control cells. Addition of APHH caused a significant augmentation of haemolysis both in uraemic and in normal erythrocytes. The APHH dependent increase of haemolysis was similar in all groups. From this finding it is concluded that uraemic erythrocytes are not more susceptible to superoxide radicals than those from healthy subjects.

In accordance with this observation the activity of SD was the same in all groups. Haemodialysis had no influence on the APHH dependent haemolysis rate or on the SD activity.

Erythrocytes withdrawn from uraemic patients predialysis had higher hydrogen peroxide dependent haemolysis rates than red cells from healthy subjects. After haemodialysis no differences could be detected between erythrocytes from uraemic patients and those from healthy subjects. This finding rules out the possibility that the higher peroxide induced haemolysis rate is due to differences in cell age. More likely, the differences are caused by some uraemic retention product removed during haemodialysis. In this connection it is of interest that the peroxide induced haemolysis rate can be augmented in normal erythrocytes by adding uraemic middle molecules to the peroxide containing incubation mixture [15]. The mechanism underlying this observation is not yet clear.

Lipid peroxidation, estimated by measuring malondialdehyde production, was significantly increased when hydrogen peroxide or organic peroxides were added to the incubation system. However, no differences could be detected between normal and uraemic erythrocytes. Therefore the observed difference in the susceptibility to peroxides cannot be attributed to an altered lipid peroxidation when uraemic and normal cells are compared. The activity of the enzyme GSH-PX was the same in all groups of patients. This result, however, does not necessarily represent the in vivo conditions, because in our in vitro investigations the essential co-factor, NADPH, was added in optimal concentrations. The possibility cannot be ruled out that the GSH-PX system is disturbed in vivo due to insufficient NADPH production in the pentose phosphate cycle. On the other hand it has been demonstrated that the activities of the enzymes glutathione reductase and ferrihaemoglobin reductase as well as the concentration of reduced glutathione are increased in uraemic erythrocytes compared with red cells from healthy subjects [14].

Conclusions

1. No differences exist between normal and uraemic cells with regard to the detoxification of, and the sensitivity to superoxide radicals, as measured by monitoring the SD activity and the APHH induced haemolysis.

2. A higher predialytic susceptibility of uraemic erythrocytes towards hydrogen peroxide could be demonstrated despite a normal GSH-PX activity. Lipid peroxidation increased in the presence of peroxides but was not different between uraemic and normal erythrocytes. Therefore, the reason for the increased peroxide induced haemolysis in uraemic cells remains open.
3. Hydrogen peroxide creating compounds should be avoided in uraemic patients.

References


Open Discussion

RITZ (Heidelberg)  Your results demonstrate increased predialysis susceptibility of erythrocytes to damage by peroxidation and such abnormality of erythrocytes is reversed by haemodialysis. This reversal of the erythrocyte abnormality by haemodialysis is somewhat reminiscent of the findings of Welt who demonstrated inhibition by uraemia and reversal by haemodialysis, of active Na extrusion. In your study the increased susceptibility to peroxide damage was not associated with increased generation of peroxidated membrane lipids. Is it not conceivable that peroxides constitute a non-specific damage to the erythrocyte membrane, with increased entry into the cell of external ions? The predialysis erythrocyte might then be unable to withstand such stress because of its impaired ability to maintain osmotic stability secondary to the reversible inhibition of the sodium pump.

LEBER  Certainly the osmotic vulnerability of the erythrocytes will decrease at the second event after oxidative stress. The question is whether the membrane damage (I have no doubt that this is primarily membrane damage because we do not find Heinz bodies in these cells) is caused by the increased or altered lipid peroxidation, which could be excluded in our experiments, or whether it is caused by other mechanical mechanisms. To mention the alteration of the protein moiety of the membrane, for instance the inactivation of sulphhydryl groups which change the fragility of the membrane and this in the second way leads to increased susceptibility to osmotic changes. I personally do not believe there is only a question of changing osmolality during dialysis. These cells are more or less vulnerable because if they undergo osmotic change, for example with glucose or with sodium you do not find this difference. It must be something which has to do with oxidation.
KOPP (Munich) Did you look at the phosphate content of your erythrocytes, because we have some evidence that phosphate-depleted erythrocytes might be more vulnerable than phosphate-repleted, with dependence on the acid base status of the patient, which is of course corrected after dialysis.

LEBER We have not measured phosphate in this experiment but we know from other investigations that the organic phosphates are increased in these cells. But these changes are not a consequence of difference in pH I think. Therefore I must refer again to the middle molecules. In one experiment, normal erythrocytes were incubated in a hydrogen peroxide containing buffer. We have added middle molecule fractions which were prepared and separated before dialysis. There is a significant increase of the haemolysis weight which is not due to pH or osmolality shifts as can be seen from this control experiment where the elution buffer of the same blood and the same column was used.