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EFFECT OF FREE RADICAL SCAVENGERS AND METAL ION CHELATORS ON HYDROGEN PEROXIDE AND PHENYLHYDRAZINE INDUCED RED BLOOD CELL LIPID PEROXIDATION

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Desferrioxamine a well-known iron chelator was found to decrease hydrogen peroxide and phenylhydrazine induced lipid peroxidation of red blood cell membranes assessed by hydrocarbon gas release and loss of polyunsaturated fatty acids. Hydroxyl radical scavengers like mannitol and thiourea and proteins like albumin were unable to reduce peroxidative reactions to our system. Addition of uric acid (in an unphysiological concentration of 5 mM) to the incubation medium resulted in a slight reduction in H_2O_2/phenylhydrazine mediated break-down of arachidonic (20:4) and docosahexaenoic acid (22:6) in the erythrocyte membrane and consequently in a decreased alkane release and haemolysis.

KEY WORDS: Radical scavenger, uric acid, red blood cells, lipid peroxidation, alkane release.

INTRODUCTION

Oxygen-derived radicals have been shown to be involved in degradation of DNA and hyaluronic acid, membrane lipid peroxidation, destruction of endothelial cells and induction of increased vascular permeability.[13,15] Toxicity of oxygen radicals has therefore been suggested as a major cause of cancer, heart disease, aging, rheumatoid arthritis and other autoimmune diseases, air pollution-induced damage, ethanol-induced liver damage, degenerative disease of the nervous system, haemolysis, antigen-antibody mediated damage and complement activation.[1,2,6,7,11,15,16,19,26,27] Free radical activity is also involved in the action and side effects of many drugs (i.e. antitumour agents, antimalarial drugs.[1,14,15,18,22,28] Therefore it is very important to develop free radical scavengers suitable for clinical use.

Our experiments were designed to analyse the effect of hydroxyl radical scavengers and chelating agents on oxygen-derived free radical-induced lipid peroxidation using alkane release and polyunsaturated fatty acid breakdown as parameters of lipid perioxidation.

METHODS AND MATERIALS

Ethanol, methanol, thiourea, hydrogen peroxide were from Merck, desferal (desferrioxamine) from Ciba-Geigy, uric acid, albumin from Sigma, pheylhydrazine from EGA-Chemie, FRG.

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Isolation of Red Blood Cells

Blood was drawn from healthy donors and erythrocytes were isolated with microcristalline cellulose and α-cellulose (1:1/w/w) as described by Beutler *et al.*[4]

Alkane Assay

Erythrocyte suspensions (haematocrit 2.5%) or haemolysates (haemoglobin concentration 0.8 g/dl) were incubated in phosphate buffered saline and perioxidation started by addition of 10 mmol/l hydrogen peroxide plus 0.25 mmol/l sodium azide or 2 mmol/l phenylhydrazine. After incubation of the suspension in 10 ml head space vials for 2 h at 37°C analysis of hydrocarbon gases was performed as previously described.[24]

Antioxidants

To evaluate the influence of various antioxidants, erythrocyte suspensions and haemolysates (containing "ghosts") were preincubated for 15 min. with the tested antioxidant prior to the initiation of peroxidation.

Haemolysis

2 hours after initiation of the peroxidative reactions, haemolysis was determined photometrically (at 540 nm) by measuring the haemoglobin concentration in the supernatant of red blood cell suspension after conversion to cyanomethaemoglobin.

Fatty Acid Analysis

Isolation of ghosts, lipid extraction and fatty acid analysis were performed as previously described.[25]

RESULTS

In the first experiment the effect of well-known hydroxyl radical scavengers on hydrogen-peroxide induced lipid peroxidaton of erythrocytes was tested. After preincubation of red blood cells with mannitol, ethanol or thiourea for 15 min. no significant reduction of hydrocarbon gas release could be found. Addition of albumin and desferrioxamine to the incubation medium did not result in a decrease in alkane production. Only the preincubation of red blood cells with uric acid was able significantly to reduce H_2O_2 induced pentane and ethane release (Table I). Similar results were obtained by determining the hydrogen peroxide induced haemolysis of red cells preincubated with the above-mentioned antioxidants: only the addition of uric acid to the cell suspension significantly decreased haemolysis (Table II). To exclude the possibility of the added scavenger not being able to permeate the red blood cell membranes, haemolysed erythrocytes with the same haemoglobin concentration as the erythrocytes in the previous experiments were prepared and supplied with the same concentration of antioxidants. The results obtained under these experimental conditions were exactly the same as the previous system apart from the experiment

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TABLE I
Hydrogen peroxide induced lipid peroxidation of intact red blood cells — effect of different antioxidants

Addition of	Number of experiments	Ethane	Pentane
		nmol/g Hb/2 h	
none	44	0.21 ± 0.04	0.84 ± 0.19
mannitol 100 mmol/l	4	0.24 ± 0.06	0.68 ± 0.22
ethanol 100 mmol/l	4	0.20 ± 0.005	0.74 ± 0.24
thiourea 2 mg/ml	3	0.28 ± 0.07	0.70 ± 0.25
albumin 1 mg/ml	2	0.23 ± 0.06	0.80 ± 0.20
uric acid 2 mg/ml	3	0.10 ± 0.06	0.44 ± 0.18
desferrioxamine 0.5 mg/ml	4	0.20 ± 0.05	0.78 ± 0.21

Experimental conditions: isolated washed red blood cells haematocrit: 2.5%, sodium azide 0.25 mmol/l, final H_2O_2 concentration: 10 mMol/l incubated in PBS pH 7.4 for 2 h.
Preincubation with antioxidant before addition of H_2O_2 for 15 min.
$\bar{X} \pm SD$.

TABLE II
Decrease in H_2O_2-induced haemolysis due to preincubation with antioxidants

Addition of	Number of experiments	Haemolysis
controls	44	36.1 ± 7.8%
+ mannitol 100 mmol/l	4	35.0 ± 9.9%
+ ethanol 100 mmol/l	4	38.1 ± 8.2%
+ thiourea 2 mg/ml	3	41.0 ± 7.6%
+ albumin 2 mg/ml	2	36.2 ± 8.6%
+ uric acid 2 mg/ml	3	17.2 ± 6.3%
+ desferrioxamine 0.5 mg/ml	4	38.9 ± 10.1%

Experimental conditions: see Table I.

TABLE III
Hydrogen peroxide induced lipid peroxidation of haemolysates — effect of different antioxidants

Addition of	Number of experiments	Ethane	Pentane
		mmol/g Hb/2 h	
controls	6	0.22 ± 0.05	0.88 ± 0.22
mannitol 100 mmol/l	3	0.19 ± 0.04	0.79 ± 0.23
ethanol 100 mmol/l	3	0.23 ± 0.05	0.89 ± 0.20
thiourea 2 mg/ml	3	0.27 ± 0.04	0.76 ± 0.21
albumin 1 mg/ml	2	0.24 ± 0.06	0.84 ± 0.21
uric acid 2 mg/ml	3	0.12 ± 0.02	0.20 ± 0.08
desferrioxamine 0.5 mg/ml	4	0.08 ± 0.02	0.10 ± 0.08

Experimental conditions: Haemolysates containing ghosts (haemoglobin concentration 0.8 g/dl) were incubated as described.

with a preincubation with desferrioxamine: gas release was found to be markedly decreased, significantly less even than that measured after preincubation with uric acid (Table III).

To exclude a hydrogen-peroxide specific phenomenon another oxidative substance, phenylhydrazine, was tested under the same experimental conditions. (Table IV and

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TABLE IV
Effect of antioxidants on phenylhydrazine induced lipid peroxidation of intact red blood cells

Addition of	Number of experiments	Ethane	Pentane
		in nmol/g Hb/2 h	
controls	8	5.73 ± 0.82	9.6 ± 1.72
mannitol 100 mmol/l	3	5.96 ± 1.11	9.5 ± 2.10
ethanol 100 mmol/l	3	5.28 ± 1.32	10.2 ± 1.91
thiourea 2 mg/l	3	4.77 ± 1.0	8.8 ± 1.61
albumin 1 mg/l	2	5.33 ± 0.88	8.3 ± 2.08
uric acid 2 mg/ml	3	3.78 ± 0.79	2.21 ± 0.48
desferrioxamine 0.5 mg/ml	3	5.92 ± 1.41	9.40 ± 2.42

Experimental conditions: Isolated red blood cells (haematocrit: 2.5%) were incubated with sodium azide 0.25 mmol/l, phenylhydrazine 2 mmol/l in PBS pH 7.4
Preincubation with the antioxidants for 15 min.

TABLE V
Effect of desferrioxamine on phenylhydrazine induced peroxidation of red blood cell membranes.

Haemolysate + ghosts + 2 mmol/l phenylhydrazine plus	Ethane	Pentane
	in nmol/g Hb/2 h	
none	5.2 ± 1.0	10.1 ± 3.1
mannitol 100 mmol/l	5.7 ± 2.1	9.6 ± 1.9
uric acid 2 mg/l	3.4 ± 1.3	2.7 ± 0.8
desferrioxamine 0.5 mg/ml	2.3 ± 1.1	1.4 ± 0.3

Red blood cells were haemolysed in salt free phosphate buffer and peroxidation was started adding phenylhydrazine 2 mmol/l.

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V) Again preincubation with desferrioxamine markedly decreased hydrocarbon gas release in the system with haemolysed erythrocytes, whereas to a lesser extent uric acid inhibited alkane production in both systems. Again neither the hydroxyl radical scavengers nor albumin showed any effect. As previously shown ethane and pentane are oxidative degradation products of membrane lipids. Therefore we analysed fatty acid composition of red blood cell membranes before and after exposure to hydrogen peroxide (Table VI). When added to intact red blood cells uric acid in the suspension medium slightly inhibited loss of polyunsaturated fatty acids. Preincubation with desferrioxamine nearly totally prevented break-down of arachidonic (C20:4) and docosahexaenoic acid (C22:6) in experiments using haemolysates with membranes as a target for lipid peroxidation (Table VII).

DISCUSSION

In our experiments hydroxyl radical scavengers were unable to inhibit H_2O_2 induced peroxidation of erythrocyte membranes. Neither alkane production nor polyunsaturated fatty acid break-down were decreased when red blood cells were preincubated with a hydroxyl radical scavenger. Previously we showed that ethane and pentane are degradation products of polyunsaturated fatty acids of the red blood cell

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TABLE VI
Effect of different antioxidants on polyunsaturated fatty acid break-down

		Fatty acids (%)					
		16:0	18:0	18:1	18:2	20:4	22:6
before incubation with H_2O_2	$n = 8$	24.2 ± 1.1	16.2 ± 2.0	16.1 ± 0.7	14.9 ± 2.1	20.3 ± 0.8	8.5 ± 0.9
after incubation with H_2O_2	$n = 10$	34.1 ± 1.9	18.5 ± 1.2	20.1 ± 0.8	13.1 ± 1.3	10.1 ± 1.4	2.1 ± 1.0
after incubation with H_2O_2							
plus preincubation with mannitol 100 mM	$n = 4$	33.1 ± 1.3	17.7 ± 0.8	19.5 ± 1.3	14.8 ± 0.8	11.5 ± 1.2	3.6 ± 1.3
uric acid 2 mg/ml	$n = 4$	32.0 ± 0.7	17.5 ± 0.9	19.1 ± 2.1	11.9 ± 1.0	15.9 ± 1.1	4.7 ± 1.1
desferrioxamine 0.5 mg/ml	$n = 4$	32.5 ± 1.2	18.5 ± 1.2	18.7 ± 1.5	15.1 ± 2.3	11.1 ± 1.8	2.5 ± 1.2

Isolated red blood cells were incubated with sodium azide 0.25 mM, H_2O_2 10 mM in PBS pH 7.4 at 37 C. Preincubation with antioxidants for 15 min. Fatty acid analysis of red blood cell membranes after 2 hours of incubation. $\bar{X}$ + SD.

TABLE VII
Effect of desferrioxamine on hydrogen peroxide induced polyunsaturated fatty acid breakdown

	Number of experiments	16:0	18:0	18:1	18:2	20:4	22:6
before incubation with H_2O_2	$n = 4$	23.8 ± 1.2	15.8 ± 1.1	17.2 ± 1.3	13.1 ± 1.2	21.8 ± 2.5	8.6 ± 1.8
after incubation with 10 mM H_2O_2	$n = 4$	35.1 ± 1.3	18.0 ± 1.2	19.1 ± 1.1	13.8 ± 1.4	11.1 ± 2.3	2.8 ± 1.0
after incubation with 10 mM H_2O_2							
plus preincubation with desferrioxamine 0.5 mg/ml	$n = 4$	24.7 ± 0.5	18.7 ± 1.1	17.1 ± 0.5	12.6 ± 1.0	19.3 ± 0.5	7.6 ± 0.7

Haemolysate with ghosts incubated with 10 mM H_2O_2 and 0.25 mM NaN_3 for 2 hours in PBS. Preincubation with desferrioxamine 0.5 mg/ml. X + SD.

membrane.[31,32] The inability of hydroxyl radical scavengers to inhibit ferrous ion-stimulated peroxidation of phospholipids was demonstrated before;[12] also no effect on iron-stimulated lysis of erythrocytes[21] and haemolglobin-catalysed H_2O_2 induced lipid peroxidation[22] could be found. Only linoleic peroxide-induced TBA-production in erythrocytes showed a marked decrease when thiourea was added to the incubation medium.[7] Because mannitol, ethanol and thiourea have been clearly shown to be hydroxyl radical scavengers[5] involvement of hydroxyl radicals in hydrogen peroxide or phenylhydrazine induced peroxidation seems to be unlikely. Uric acid had been shown before to decrease MDA-production in a copper catalysed peroxidation of

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polyunsaturated fatty acids[29] and in t-butylhydroperoxide-induced lipid peroxidation.[30] In our system, not only alkane release was significantly reduced, but also fatty acid break-down was found to be partially inhibited. Due to its antioxidant effect plasma uric acid has been suggested to contribute to the increased life-span which has occurred in human evolution.[29] By far the most efficient substance to prevent hydrogen peroxide or phenylhydrazine induced lipid peroxidation in our assay was desferrioxamine. Alkane release and polyunsaturated fatty acid break-down revealed nearly total inhibition of peroxidative reactions after addition of desferrioxamine.

Transition metal catalysts in non-protein bound, low-molcular weight form are required for the formation of highly reactive free radicals like the hydroxyl radical and for the promotion of scission of peroxides and hydroperoxides.[15] Iron bound to desferrioxamine does not react with the superoxide radical to form hydroxyl radicals[20] and therefore reduces the low molecular weight iron for the production of more reactive radical species. These catalytically active forms of iron can be reductively released from body iron store by leukocyte mediated superoxide radical formation in inflammatory reactions f.e. rheumatoid arthritis.

At higher concentrations desferrioxamine starts scavenging free radicals, mainly hydroxyl radicals but also to a lesser degree superoxide radicals.[19] Desferrioxamine is a specific iron chelator approved for clinical use, and is absorbed by tissues and excreted when complexed with iron.[23] According to our results, its site of action is limited to the extracellular space because of its inability to cross cellular membranes. Desferrioxamine has already been used for inhibition of hyaluronic acid degradation in rheumatoid arthritis,[6,15] treatment of states of increased low molecular weight iron[3,6,8,11,17] such as haemochromatosis with peroxidative damage to the myocardium and pancreatic islet cells. Another indication for its use is in the reduction in side effects of antitumour agents because bleomycin and anthracycline derivatives form complexes with iron and induce via redoxcycling the formation of oxygen radicals possibly responsible for pulmonary fibrosis and cardiotoxicity. The excellent protection of membrane fatty acids against free radical attack demonstrated in our experiments should encourage the wider use of this drug in free radical-mediated disease states.

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