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The influence of α-tocopherol and pyritinol on oxidative DNA damage and lipid peroxidation in human lymphocytes

J. Topinka[1], B. Binkova[1], R.J. Sram[1] and A.N. Erin[2]

[1] *Psychiatric Research Institute, Prague (Czechoslovakia) and* [2] *All-Union Research Center of Mental Health, Academy of Medical Sciences, Moscow (U.S.S.R.)*

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Summary

Unscheduled DNA synthesis (UDS) and lipid peroxidation (LPO) were measured in human peripheral lymphocytes from healthy volunteers. These processes were induced by the catalytic system Fe^{2+}-sodium ascorbate. The degree of induced LPO was measured spectrophotometrically by the thiobarbituric acid assay. UDS was detected by scintillometric measurement of the incorporation of ^{3}H-thymidine into DNA. The protective action by fat-soluble vitamin E (D,L-α-tocopherol) and the artificial antioxidant pyritinol on UDS and LPO was also investigated.

The system Fe^{2+} (2 μmole/l)–sodium ascorbate (30 μmole/l) increased the LPO level in healthy volunteers approximately 2.5 times and the incorporation of ^{3}H-thymidine by 60–70%. α-Tocopherol (0.2 mmole/l) very efficiently suppressed LPO processes ($p < 0.01$) and the oxidative damage of DNA measured as UDS was also significantly diminished ($p < 0.05$). Pyritinol had no effect on LPO and UDS under our experimental conditions.

In biological systems oxidative damage can occur due to the presence of high levels of superoxide-, hydroxyl-free radicals and hydrogen peroxide which can oxidize susceptible target molecules in living cells. These radicals initiate the process of membrane lipid peroxidation and react also with DNA, causing modifications of the molecular structure of the genetic material. Lately much attention has been paid to the investigation of the interactions between free oxygen radicals and living systems (Gutteridge, 1984; Dunford, 1987; Vuillaume, 1987; Halliwell and Gutteridge, 1984). The influence of active oxygen species on DNA triggers many yet unanswered questions (Ames, 1983; Friedberg, 1985; Elliot and Elcombe, 1987).

Living cells possess 3 species of protective enzymes which play a major role in the detoxification of free oxygen radicals — superoxide dismutases,

Correspondence: Jan Topinka, Psychiatric Research Institute, Ustavni 91, 181 03 Prague 8 (Czechoslovakia).

Abbreviations: UDS, unscheduled DNA synthesis; LPO, lipid peroxidation; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid; TBA, thiobarbituric acid; MDA, malondialdehyde; TP, . D,L-α-tocopherol; PYR, pyritinol; T, treated samples; C, controls.

catalases and peroxidases. The hydroxyl radical, against which a protective enzyme does not exist, is perhaps the most powerful oxidant to be found in biological systems. There are 3 main types of hydroxyl radical reactions in living cells: (1) hydrogen atom abstraction, (2) addition (e.g., it can be added onto aromatic ring structures such as purine and pyrimidine bases of DNA), (3) electron transfer reactions (Wilson, 1978). It seems that addition is the most important reaction associated with genetic damage, but the indirect effect of various compounds generated by the 2 other reaction types must also be considered.

Much effort has been given to the study of various types of free radical scavengers and protective agents (Logani and Davies, 1980; Erin et al., 1986; Liebler et al., 1986). The problem of the effective protection of cells against free oxygen radicals, however, is further complicated by the general toxicity and various side effects of some very efficient free radical scavengers. These effects cannot be tested in non-living systems, that is the great disadvantage of such studies.

The present paper deals with the protective influence of α-tocopherol and pyritinol on the oxidative damage in human peripheral lymphocytes from healthy volunteers.

Materials and methods

Chemicals

Ficoll 400 (Pharmacia, Sweden), 60% Verografin (Spofa, Czechoslovakia), MNNG, hydroxyurea, 5-fluoro-2′-deoxyuridine, sodium ascorbate, D,L-α-tocopherol (all Serva, F.R.G.), thiobarbituric acid, malondialdehyde-bis-diacetal (all Merck, F.R.G.), medium RPMI 1640 (USOL, Czechoslovakia), SLD 31 scintillation cocktail (Spolana, Czechoslovakia), and all other chemicals had the minimal degree of purity necessary for analysis.

Volunteers

The experiments were carried out on healthy volunteers of both sexes, one group, aged 43.1 years (SD 8.0), treated with α-tocopherol (N = 20) and another group, 43.5 years (SD 8.2), treated with pyritinol (N = 13). The effect of both compounds on lipid peroxidation (LPO) and unscheduled DNA synthesis (UDS) was tested in peripheral blood lymphocytes.

Sample collection

The lymphocytes were isolated on Ficoll 400-Verografin gradients (Harris, 1970). The content of the proteins in the suspension of isolated cells was determined by the Bradford assay using Coomassie blue G reagent (Bradford, 1976). The samples from each donor were diluted in medium RPMI 1640 to a cell density of 0.3 mg of proteins per ml and divided into several aliquots, in which molecular oxygen was activated by the addition of Fe^{2+} (2 μmole/l) and sodium ascorbate (30 μmole/l). On the basis of preliminary experiments, the influence of pretreatment with α-tocopherol and pyritinol in a final concentration of 200 μmole/l was investigated. Both compounds were added before the oxygen activating systems. The last aliquots of samples were used as controls without any induction of molecular oxygen. All treated samples and controls were incubated for 30 min at 37°C. After this incubation, the content of all tubes was separated into 2 parts for the determination of UDS and lipid peroxidation respectively.

Measuring of UDS

The level of the oxidative damage of DNA was estimated by measuring in vitro UDS. The method has been described in more detail by Martin et al. (1978). The induction of UDS by the alkylating agent 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) was used as positive control. After the activation of the molecular oxygen, samples were incubated for 30 min at 37°C with 10 mmole/l hydroxyurea and 2 μmole/l 5-fluoro-2′-deoxyuridine to inhibit semiconservative DNA synthesis. This incubation was followed by the addition of 400 kBq/ml of [methyl-^{3}H]thymidine (specific activity 2 TBq/mmole) to all tubes and 100 μmole/l MNNG dissolved in DMSO into the positive control samples. The cultures were incubated for 3 h at

37°C. DNA from cells was isolated by the alkaline hydrolysis of RNA in 0.3 mole/l KOH; after subsequent neutralization by HCl, washing and the addition of 5% TCA, DNA was thermally solubilized (90°C, 30 min) and separated from proteins by centrifugation. The incorporated radioactivity was measured in the supernatants on a Beckman LS 5801 liquid scintillation counter in SLD 31 liquid scintillation cocktail. In the second halves of the supernatants, the concentration of DNA in all the samples was estimated by the diphenylamine method. The specific activity of the samples was expressed as cpm/μg DNA and calculated for all treated (T) samples (LPO, LPO + TP, LPO + PYR, MNNG) and control (C) samples. Information about the increased incorporation of the radiolabelled nucleoside [methyl-^{3}H]thymidine as the consequence of DNA damage (excision repair) is given by the T/C ratio.

Measuring of LPO

The LPO level in lymphocytes was measured by a modified thiobarbituric acid (TBA) assay (Ohkawa et al., 1979). The TBA assay involves acid hydrolysis of lipoperoxides to malondialdehyde (MDA), which subsequently reacts with TBA producing MDA-TBA adducts suitable for sensitive spectrophotometric measurement. The maximum formation of the reaction products is attained in a 20% acetic acid solution at pH 3.5 upon raising the temperature to 95°C for 60 min. TBA-active products of lipid peroxidation were measured spectrophotometrically after extraction into butanol as the difference in the absorbances at 532 nm and 580 nm on Specord M 40 (G.D.R.). The obtained values were expressed as nmole of MDA per mg of the proteins using malondialdehyde-bis-diacetal as a standard.

Results and discussion

In the preliminary experiments, the influence of the sodium ascorbate concentrations on the levels of LPO and UDS was tested for a given Fe^{2+} concentration. An example of these effects for both LPO and UDS is given in Fig. 1. Both curves con-

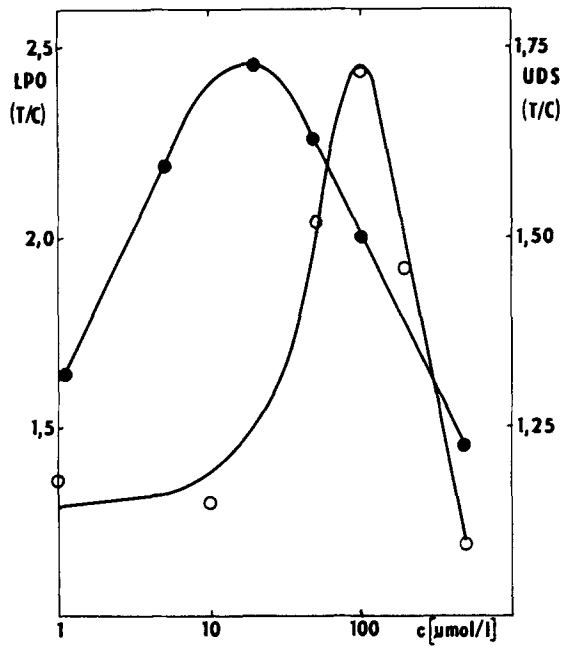

Fig. 1. Effect of sodium ascorbate concentration on LPO (●) and UDS (○) levels in peripheral lymphocytes of a healthy volunteer (40 years, woman). Both parameters are expressed relatively as the ratio T/C (T values for samples treated with ascorbate and 2 μmole/l Fe^{2+}, C controls with Fe^{2+} only). Samples were incubated for 30 min at 37°C.

firm the assumption that sodium ascorbate at lower concentrations moves the redox equilibrium $Fe^{2+/3+}$ in the direction of Fe^{2+} ions, which participate in the formation of free oxygen radicals (Dunford, 1987; Minotti and Aust, 1987). With increasing concentrations of sodium ascorbate LPO and UDS levels increase until a saturation level is reached, and then the antioxidant properties of sodium ascorbate in an ever increasing concentration start to decrease LPO and UDS levels. The maximum LPO level was reached at a lower ascorbate concentration than the maximum UDS, but there are large individual differences between the LPO and UDS levels for a given ascorbate concentration (see Table 1).

Table 1 summarizes our results with the lymphocyte short-term cultures incubated with the Fe^{2+}-sodium ascorbate system and with TP in conditions described in a previous section. The

TABLE 1

INFLUENCE OF α-TOCOPHEROL (200 μmole/l) ON LPO AND UDS INDUCED IN PERIPHERAL LYMPHOCYTES OF HEALTHY VOLUNTEERS

C, control level of LPO and ^{3}H-thymidine incorporation without any activation.
LPO, samples with activated oxygen by Fe^{2+} (2 μmole/l) and sodium ascorbate (30 μmole/l).
LPO + TP, the same as LPO but in the presence of TP.
MNNG, control + 200 μmole/l MNNG.

Sample No.	Lipid peroxidation			Unscheduled DNA synthesis			
	C (nmole MDA[a])	LPO T/C	LPO + TP	C (cpm/μg[b])	LPO	LPO + TP T/C	MNNG
1	0.76	2.22	1.63	52.4	1.24	1.10	5.42
2	1.29	3.43	1.22	66.8	1.21	1.02	4.24
3	1.25	3.03	1.40	81.5	1.88	1.33	3.11
4	1.25	4.71	1.17	81.5	1.90	1.33	3.11
5	1.21	2.55	1.11	50.7	1.30	1.21	5.23
6	1.30	2.31	1.70	49.4	1.29	1.01	4.06
7	1.03	1.86	1.07	40.5	1.78	1.45	4.13
8	1.03	1.78	1.21	40.5	1.21	1.06	4.13
9	1.34	2.70	1.34	81.8	1.31	1.00	1.83
10	1.18	3.44	2.44	85.0	1.71	1.22	3.62
11	1.18	3.90	2.60	85.0	1.81	1.00	3.62
12	0.99	2.69	1.61	52.3	1.62	1.33	2.63
13	0.99	2.40	1.61	52.3	1.28	0.90	2.63
14	0.98	1.89	1.00	52.1	1.16	1.03	4.10
15	0.98	1.59	1.11	52.1	1.19	1.02	4.10
16	0.75	1.89	1.17	70.7	2.89	2.35	3.50
17	0.75	1.96	1.45	70.7	2.06	1.93	3.50
18	0.88	1.91	0.97	75.8	1.17	1.01	3.62
19	1.01	2.13	0.87	45.6	2.78	1.82	3.66
20	0.92	1.65	1.25	44.1	1.39	1.03	2.32
Mean	1.05	2.50	1.40*	61.5	1.61	1.26**	3.63
SD	0.19	0.83	0.45	15.9	0.51	0.38	0.88

[a] nmole MDA per mg of proteins in the cell suspension.
[b] cpm/μg DNA.
*$p<0.01$.
**$p<0.05$.

results are expressed in relation to control cultures from the same donor and without any activation of the molecular oxygen. From these results it may be seen that the level of LPO in lymphocytes, expressed as nmole MDA/mg of proteins, is 2.5 times higher after the activation of molecular oxygen (LPO samples), and that this high level of LPO is very efficiently ($p<0.01$) suppressed by adding TP (LPO + TP samples).

The treatment with the oxygen-activating agents is also connected with an increased incorporation of the radiolabelled nucleoside ^{3}H-thymidine into DNA molecules. The cpm values were increased by 60% in comparison with the control samples (see Table 1). Higher levels of thymidine incorporation are connected with excision repair which is probably induced by a direct interaction of some active oxygen species with DNA. It has, for example, been described that in vitro hydroxylation of guanine residues in DNA occurs in the C-8 position in the presence of the oxygen and its activating agents (Vuillaume, 1987). This reaction may pro-

ceed directly with the hydroxyl radical, for which a protective enzymatic system does not exist. Hydroperoxides of fatty acids, which are formed during LPO processes, may also be decomposed into non-radical compounds (alkanes, aldehydes, ketones and other oxidative products). Many of these compounds are sufficiently reactive to cause indirect DNA damage through the formation of adducts with DNA. From the results obtained with TP (LPO + TP samples), we may deduce the protective effect of this compound on DNA, which may be connected with the structurally stabilizing effect of vitamin E on the lymphocyte membranes and with its radical-scavenging properties (Erin et al., 1986).

Results in Table 2 were obtained with lymphocytes treated in the same manner as the samples in Table 1, except that pyritinol was used as a possible protective compound, since pyritinol is known to be an effective hydroxyl radical scavenger from experiments in non-living systems (Pavlik and Pilar, 1988). As may be seen from Table 2, no effect of pyritinol on LPO processes and UDS was detected.

The protective action of TP in our experiments may be explained on the basis of the structural differences between TP and pyritinol. It seems that efficient protective action against the effect of free oxygen radicals is connected not only with the scavenging of these radicals, but also with the stabilization of the membrane lipid bilayers in the cells. The structural formulae of TP and pyritinol are illustrated in Fig. 2. It is obvious that the hydrophobic isoprenoic chain with the hydrophilic chromanol nucleus of the TP is structurally similar to the phospholipids which form the main part of

TABLE 2

INFLUENCE OF PYRITINOL (200 μmole/l) ON LPO AND UDS INDUCED IN PERIPHERAL LYMPHOCYTES OF HEALTHY VOLUNTEERS

C, control level of LPO and ^{3}H-thymidine incorporation without any activation.
LPO, samples with activated oxygen by Fe^{2+} (2 μmole/l) and sodium ascorbate (30 μmole/l).
LPO + PYR, the same as LPO but in the presence of PYR.
MNNG, control + 200 μmole/l MNNG.

Sample No.	Lipid peroxidation			Unscheduled DNA synthesis			
	C (nmole MDA[a])	LPO T/C	LPO + PYR	C (cpm/μg[b])	LPO	LPO + PYR T/C	MNNG
1	0.99	2.31	1.79	47.2	1.28	1.16	2.98
2	0.75	1.89	2.48	70.7	2.89	2.91	3.50
3	0.75	1.96	2.55	70.7	2.06	2.21	3.50
4	1.30	3.15	1.59	57.0	1.14	1.13	4.18
5	1.03	2.37	1.92	40.5	1.21	1.29	4.86
6	1.03	1.10	1.12	40.5	1.78	1.68	4.86
7	1.21	2.48	1.99	80.1	1.69	1.74	3.62
8	1.21	2.12	1.95	80.1	2.82	2.16	3.62
9	1.08	4.71	3.98	146.8	1.54	1.94	3.92
10	0.99	2.69	3.61	52.3	1.62	1.39	2.63
11	0.99	2.40	3.16	52.3	1.28	1.36	2.63
12	0.88	1.75	1.53	46.8	2.37	2.12	5.59
13	0.92	1.55	1.55	44.1	1.39	2.38	2.32
Mean	1.01	2.35	2.25	63.9	1.77	1.81	3.71
SD	0.17	0.88	0.87	28.6	0.59	0.54	0.97

[a] nmole MDA per mg of proteins in the cell suspension.
[b] cpm/μg DNA.

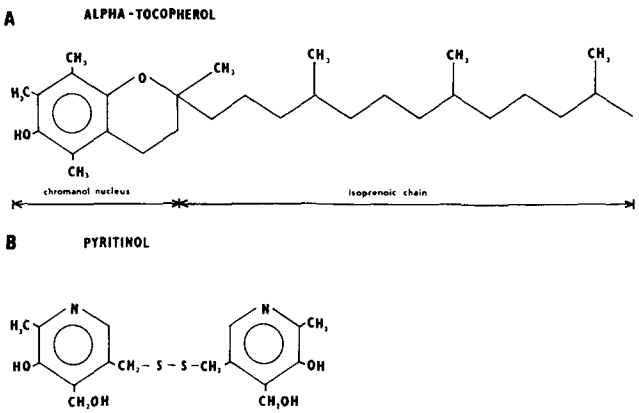

Fig. 2. Structural formulae of α-tocopherol (A) and pyritinol (B).

the membrane lipid bilayer. Thus the molecules of TP may be directly incorporated into this bilayer, and may affect the structural stability of the membrane. The localization of TP directly in the membranes is very important because of its bioavailability. The antioxidant activity of TP is explained by the presence of an OH-group in the chromanol nucleus (see Fig. 2), which may eliminate the hydrogen ion. The mechanism of the inhibition of LPO processes by TP was formulated for the first time by Tappel (1973) and is based on the interactions between free lipid radicals and TP. In comparison with pyritinol the advantages of TP as a free oxygen radical scavenger as well as a universal stabilizer of biological membranes are obvious. These theoretical approaches were confirmed in our experiments with lymphocyte short-term cultures with activated molecular oxygen, in which only the TP had a protective effect in LPO processes and in the oxidative damage of DNA measured as UDS.

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