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PROTECTION OF CELL PROTEINS AGAINST FREE-RADICAL ATTACK BY NOOTROPIC DRUGS: SCAVENGER EFFECT OF PYRITINOL CONFIRMED BY ELECTRON SPIN RESONANCE SPECTROSCOPY

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Summary—The potency of nootropic drugs to protect cell proteins and lipids against free radical attack was studied. In an *in vitro* system, generating hydroxyl radicals by a Fenton-type reaction, the soluble proteins (bovine serum albumin and cytosol protein from brain) were quickly insolubilized and precipitated. Pyritinol (and tamitinol) exhibited the best protection against insolubilization of protein while centrophenoxine (meclophenoxate) and its dimethylaminoethanol moiety were less effective, piracetam (and oxiracetam) being without effect.

The lipid peroxidation induced by free radicals from cyclic redox reactions of iron-ascorbate was not influenced by pyritinol, indicating the selectivity of its scavenger action.

The efficient scavenging of hydroxyl radicals by pyritinol was confirmed by electron spin resonance spectroscopy measurement of hydroxyl radicals entrapped by spin trap. Millimolar concentrations of pyritinol competitively decreased the formation of spin adducts.

The results suggest that the protective effect of pyritinol against free-radical induced derangement of cell proteins may be an important part of its antirheumatic, as well as nootropic, action.

Key words—pyritinol, centrophenoxine, piracetam, oxygen, free-radicals, protein precipitation, ESR spectroscopy.

There is growing evidence that free-radical interactions are implicated in the pathogenesis of many diseases including radiation injury, astherosclerosis, arthritis, cancer and ageing (Wolf, Garner and Dean, 1986; Halliwell and Gutteridge, 1986a). The most dangerous species of oxygen radicals is the hydroxyl radical that can attack proteins, lipids, nucleic acids and, actually, almost any molecule of a living cell. If the production of hydroxyl radical escapes the control mechanisms, then the local antioxidant defence may be overcome and substantial damage to cell functions may occur. Conceivably, the pharmacological protection of important cellular components against attack by free-radicals should be a fruitful strategy in the management of the consequences, provided there is a suitable free-radical scavenger drug available.

Within the group of nootropic drugs, free-radical scavenger properties of centrophenoxine (meclophenoxate) were claimed as the molecular mechanism of its nootropic action (Zs.-Nagy and Nagy, 1980; Zs.-Nagy and Floyd, 1984). It was of interest, therefore, if other nootropic drugs possessed free-radical scavenger properties as well. With the use of simple *in vitro* methods for the detection of free-radical activity, it was found that pyritinol exerted a pronounced scavenger action against hydroxyl radicals which was confirmed by the electron spin resonance spectroscopic technique in spin trapping experiments.

METHODS

Protein polymerization and insolubilization assay

The protection afforded by nootropic drugs against free-radical-induced insolubilization of proteins was tested in an *in vitro* system generating hydroxyl radicals during homolysis of hydrogen peroxide by redox coupling with the ferrous (Fe^{2+}) to ferric (Fe^{3+}) transition. The procedure and conditions used were identical with those described by Zs.-Nagy and Nagy (1980). Soluble proteins, such as bovine serum albumin or those from the brain, were grossly crosslinked and insolubilized with increasing concentration of ferrous ammonium sulphate. The remaining soluble proteins were determined by the method of Miller (1959).

In vitro lipid peroxidation assay

The method of Rehncrona, Smith, Akesson, Westerberg and Siesjo (1980) was used. Briefly, the brain from an adult rat was homogenized in 10 volumes of 50 mM phosphate buffer, pH 7.0 and 0.5 ml aliquots were incubated for 1 hr, with or without drug. The free-radical generating system consisted of 0.1 mM ascorbic acid and 0.01 mM ferrous sulphate. The product of lipid peroxidation was assayed by thiobarbituric acid method, as modified by Rehncrona *et al.* (1980), with the detection limit set at 0.01 μmol of malonyldialdehyde.

Electron spin resonance measurement of hydroxyl free-radicals entrapped by dimethyl pyrroline N-oxide (DMPO)

The protocol of the method of Floyd and Lewis (1983) as described by Zs.-Nagy and Floyd (1984) was followed. Briefly, electron spin resonance spectra were recorded with a JEOL PE-3X spectrometer. The measurements were performed at 2 mW microwave output with 100 kHz magnetic modulation. The temperature of the cavity was set to 25°C and stabilized with a JES-VT-3A temperature controller to ±0.5°C. The samples were measured in aqueous solution sample tubes JES-LC-01. The relative concentrations of spin adduct were determined by comparing the amplitudes of lines in the spectra of the samples. No changes of linewidths in electron spin resonance spectra of different samples and of the cavity quality factor when determined from the intensities of a manganese (Mn^{2+}) internal standard line were observed.

Chemicals and drugs

The chemicals used in all experiments were of analytical grade. Dimethylaminoethanol (DMAE) and DMPO were from Koch Light and Sigma, respectively. Pyritinol and tamitinol were from E. Merck, centrophenoxine from Bracco, piracetam from UCB and oxiracetam from CIBA-GEIGY.

RESULTS

Protection of soluble proteins against free-radical attack by pyritinol

The screening of nootropic drugs for the protective effect against insolubilization of protein induced by free-radicals showed that at 10 mM concentration of the drug, the protection afforded by pyritinol and its derivative tamitinol was superior to other nootropics tested (Table 1). Actually, piracetam and oxiracetam did not exert any protection at all. It was confirmed that centrophenoxine, as well as its dimethylaminoethanol (DMEA) moiety, afforded some protection but in substantially larger concentrations than pyritinol or tamitinol. Pyritinol was then investigated in greater detail. Figure 1 shows the results of three experiments with bovine serum albumin. The precipitation of bovine serum albumin increased rapidly with the amount of iron present in the assay. Above 4 mM Fe^{2+}, only the small portion of bovine serum albumin remained soluble. It may be seen that neither Fe^{2+} nor Fe^{3+} ions alone interacted with bovine serum albumin. Pyritinol, at 10 mmol/l, afforded complete protection and at 2 mmol/l it still protected more than 50% of bovine serum albumin against 8 mM Fe^{2+}. Included in this figure are the results with ascorbic acid (a well-known antioxidant), which neither increased precipitation of bovine serum albumin nor protected against it.

Table 1. Effect of nootropic drugs on precipitation of bovine serum albumin induced by hydroxyl radicals. The assay contained 0.8 mg/ml of bovine serum albumin, 10 mM ferrous ammonium sulphate, 0.2% H_2O_2 and drugs in the concentrations as indicated. The protection of bovine serum albumin against insolubilization is expressed as a percentage of the total concentration of protein (mean values from 3 experiments with SEM less than 5% of the means)

Drug	Protection (%) 10 mM	50 mM
Pyritinol	100	100
Tamitinol	100	100
Centrophenoxine	16	100
Dimethylaminoethanol	6	51
Piracetam	0	0
Oxiracetam	0	0

The soluble proteins from the brain were insolubilized by free radicals but they were more sensitive than bovine serum albumin to Fe^{2+} induced precipitation. While bovine serum albumin was still

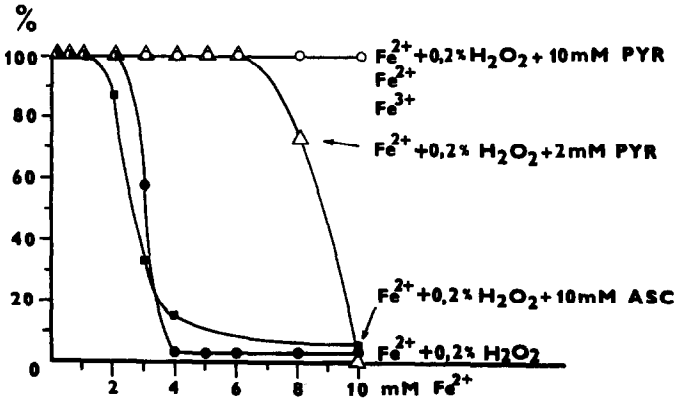

Fig. 1. Effect of pyritinol (PYR) and ascorbic acid (ASC) on insolubilization of bovine serum albumin induced by hydroxyl radicals. The assay mixture contained bovine serum albumin (0.8 mg/ml), ferrous ammonium sulphate (0.5–10 mM), 0.2% hydrogen peroxide, pyritinol or ascorbic acid as indicated. Reaction was completed during 10 min at 25°C, the samples were centrifuged and the supernatants were assayed for protein. The mean values from three experiments were plotted as a percentage of the total concentration of bovine serum albumin against increasing concentration of Fe^{2+}.

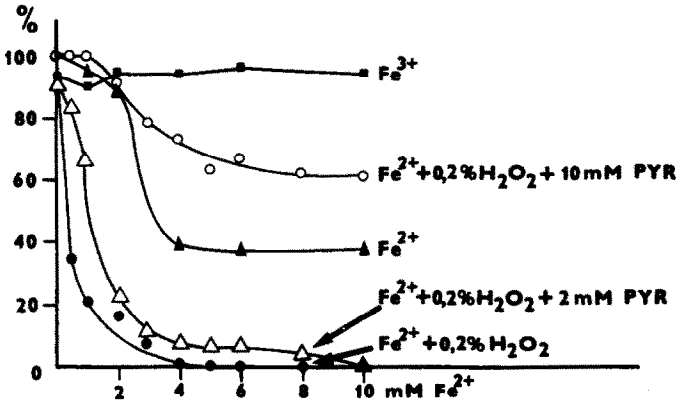

Fig. 2. Effect of pyritinol (PYR) on precipitation of soluble proteins in brain induced by hydroxyl radicals. The conditions were the same as described in Figure 1, but the soluble fraction of the homogenate of rat brain in H_2O was used instead of bovine serum albumin.

soluble at 3 mM Fe^{2+} (Fig. 1), the soluble proteins from the brain had been precipitated by 0.5 mM Fe^{2+} (Fig. 2) at the same concentration of peroxide (H_2O_2) (0.2%) in both cases. However, 10 mM pyritinol protected only half of the soluble proteins from brain against precipitation with 10 mM Fe^{2+}, while 2 mM pyritinol was efficient only at the smallest concentrations of Fe^{2+} used (less than 2 mM). Furthermore, Fe^{2+} ions alone, in concentrations from 4 to 10 mM, insolubilized half of the soluble proteins from brain in the assay (see Fig. 2). It is evident that the formation of free-radicals was supported by substances present in the soluble fraction of the homogenate of brain.

Effect of pyritinol on lipid peroxidation in vitro

It was of interest to know if pyritinol protected against lipid peroxidation. A simple *in vitro* assay was used in which lipid peroxidation of a homogenate of brain was brought about by ascorbic acid-ferrous sulphate system. From Table 2 it is clear that pyritinol, in concentrations between 0.01–1 mmol/l, did not affect substantially the formation of the lipid peroxidation product.

Scavenging of hydroxyl radicals by pyritinol

From previous experiments it seemed that pyritinol might be the selective scavenger of hydroxyl radicals. This problem was tackled by means of spin-trapping technique and electron spin resonance spectroscopy.

Table 2. Effect of pyritinol on lipid peroxidation in homogenates of brain. The mixture consisted of 1 ml of a homogenate of brain (1:10, w/v, in phosphate buffer, pH 7.2), 0.1 mM ascorbic acid, 0.01 mM ferrous sulfate and pyritinol as indicated. Incubation (37°C) was terminated after 1 hr, the peroxidation product assayed by thiobarbituric acid reagent and the absorbance read at 532 nm (mean values with SEM and number of estimations in parentheses)

Pyritinol (mM)	Thiobarbituric acid reaction (A)
0	0.898 ± 0.014 (6)
0.01	0.964 ± 0.026 (3)
0.1	0.972 ± 0.012 (3)
1	0.830 ± 0.021 (3)

For the generation of hydroxyl radicals the Fenton reaction mixture, consisting of Fe^{2+}-adenosine diphosphate (Fe^{2+}-ADP) complex and H_2O_2 was used (Zs.-Nagy and Floyd, 1984). These radicals can be trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), resulting in a typical electron spin resonance signal. Figure 3(A) is the control spectrum of the DMPO-OH spin adduct. Figures 3(B) and (C) shows the spectra in the presence of 3.25 and 6.5 mM pyritinol, respectively. A clear-cut decrease in the amplitude of lines of the electron spin resonance spectra, B and C, dependent on the concentration of pyritinol is seen. If the amplitudes of the spectral lines in Figure (3A) are considered as 100%, then pyritinol in the concentrations mentioned above, reduced their size by about 40 and 80%, respectively. The omission of Fe^{2+} ions or H_2O_2 extinguished the spectra completely, i.e. no hydroxyl radicals were trapped.

DISCUSSION

Attack by hydroxyl radicals usually sets off a radical chain reaction *in vivo* but its very high reactivity imposes theoretical and practical restraints on the detection *in vivo* (Wolf *et al.*, 1986). However, the outcome of the interaction with particular type of molecule may be measured in an *in vitro* system. Furthermore, the scavenger behaviour of various agents may be quantified, provided they do not react with the individual components of the assay system.

In the first series of experiments, precipitation of proteins induced by hydroxyl radicals was chosen as a clear-cut indicator of the presence and action of hydroxyl radicals. No precipitation of bovine serum albumin was seen in the assays with either ferrous or ferric ions, or H_2O_2 or with nootropic drugs alone. The pyridine nootropics, pyritinol and tamitinol, were superior to the rest. The DMAE moiety of centrophenoxine (meclophenoxate) was already shown to be effective in this essay (Zs.-Nagy and Nagy, 1980), but larger concentrations than those of pyritinol were used. Better protection of bovine

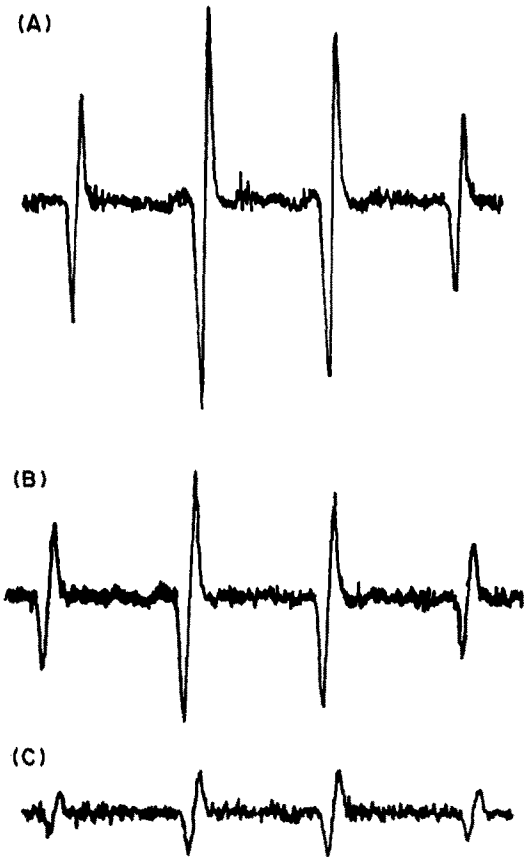

Fig. 3. Effect of pyritinol on electron spin resonance spectra of DMPO-OH spin adduct, obtained in ADP-Fe^{2+}-H_2O_2 mixture. (A) The control system containing 50 mM DMPO, 2 mM ADP, 0.1 mM ferrous chloride and 10 mM hydrogen peroxide in bicarbonate buffer (100 mM NaCl, 25 mM $NaHCO_3$, pH 7.1). Usually 1 min elasped between mixing the assay components and the beginning of the measurement of the spectra. (B) The same as (A) but 3.25 mM pyritinol was added before hydrogen peroxide. (C) The same as (A) but 6.5 mM pyritinol was added.

serum albumin by centrophenoxine (as compared to DMEA) in this study may be explained by the hydroxyl radical scavenging property of its *p*-chlorphenoxyacetic acid moiety (Zs.-Nagy and Floyd, 1984). The classical nootropic piracetam and the chemically similar oxiracetam were without effect. The lack of protection against precipitation of bovine serum albumin in assays where ascorbic acid was added indicated that this antioxidant could not be used indiscriminately as a scavenger in an *in vitro* system.

The protective effect of pyritinol on precipitation of protein induced by hydroxyl radicals has not been reported. Since the hydroxyl radical acts through direct addition (e.g. ring-hydroxylation), hydrogen atom abstraction and electron transfer, it is supposed that the pyritinol molecule is modified as a result of its interaction with the hydroxyl radical.

The control experiments with cytosol from brain, instead of bovine serum albumin, confirmed that Fe^{2+} ions alone could induce a substantial damage to proteins since cytosol from brain contained an effective source of auto-oxidisable reducing agents such as thiols, reduced nucleotides, ascorbate etc, which together with Fe^{2+} ions gave rise to free radicals.

The increased susceptibility of protein in brain to free radical-induced polymerization (as compared to that of bovine serum albumin) may be related to the elevation of protein cross-linking in the aged brain (Zs.-Nagy and Nagy, 1980). Furthermore, in some neurodegenerative diseases, e.g. Alzheimer's and Parkinson's disease there is a conspicuous neuronal accumulation of abnormal proteins (Wischik and Crowther, 1986) which are polymerized by a process not excluding non-enzymatic cross-linking by free radicals.

In the following series of experiments it was found that the addition of pyritinol was not effective protection against lipid peroxidation *in vitro*, at least in the concentrations used. It is supposed that the low lipid solubility of pyritinol (Nowak and Schorre, 1969) might be the main cause, since lipid-soluble free radical scavengers exerted a pronounced effect in this type of assay (Baba, Lee, Ohta, Tatsuno and Iwata, 1981).

The ADP-Fe^{2+}-H_2O_2 system generating hydroxyl-free radicals and their trapping by DMPO were used to confirm the competitive scavenging of hydroxyl radicals by pyritinol. The assay conditions suggested by Zs.-Nagy and Floyd (1984; see also Floyd, 1983; Floyd and Lewis, 1983) were rigorously adhered to. In the *in vitro* experiments, the generation of free radicals was dependent on levels of Fe^2. Estimations of the concentration of free-radicals reported by Zs.-Nagy and Floyd (1984) from similar experiments indicated the ratio 100:15 between Fe^{2+} and free radicals entrapped by DMPO, respectively. The competition between 50 mM DMPO and 1–12 mM pyritinol showed the concentration-dependent scavenging of hydroxyl radicals with an ED_{50} about 4 mmol/l. The scavenging of hydroxyl radicals by DMAE or centrophenoxine, in an identical system, was reported by Zs.-Nagy and Floyd (1984). If the concentrations of DMEA (100 mM) and centrophenoxine (50 mM), which markedly competed with DMPO, are compared with those of pyritinol (3.25 and 6.5 mM), then it was evident that pyritinol was by about an order of magnitude a more efficient scavenger of hydroxyl radicals than centrophenoxine or DMAE.

The present experiments have produced evidence that pyritinol possessed significant hydroxyl free-radical scavenger activity in *in vitro* systems. The fact must be considered that there are many problems in demonstrating the effectiveness of free-radical scavengers *in vivo*. The level of free radicals in the tissues is also dependent on the availability of metal ion catalysts. Since the levels of metal chelates *in vivo* are not greater than 5–10 μmolar, the significant production of free-radicals may not start before these levels are reached (Halliwell and Gutteridge, 1986a

and b). There are some cases where more than enough iron may be present or released during pathological conditions, e.g. in the cerebrospinal fluid or in the brain, respectively.

It is difficult to estimate the level of scavenging drugs which should be reached at the place of free-radical production, in order to prevent damage to cellular components. In the brain, however, the level of bound iron is high and that of antioxidants is low and, therefore, micromolar concentrations of scavenger drug may help to ameliorate free radicals generated during disruptive events. There are some clinical reports that may be viewed as supporting the opinion that pyritinol may also have a scavenger effect *in vivo*. Camus, Yaffe, Crouzet, Prier, Mercier and Dubois (1978) and Berry (1986) used pyritinol successfully, instead of the other well-known but more toxic scavenger penicillamine, for the treatment of some cases of rheumatoid arthritis. The protection of cartilage and synovial protein against free-radical-induced degradation (Wolf *et al.*, 1986) may be an important factor in the treatment of rheumatoid arthritis. The same line of reasoning may be applied to some cases of stroke or brain trauma (notwithstanding perinatal brain damage), where the generation of hydroxyl free-radicals by reactive iron/oxygen radical complexes is abundant (Halliwell and Gutteridge, 1986b) and where pyritinol was successfully used for treatment (Benesova, Pavlik and Petova, 1984).

Finally the potency of pyritinol to protect proteins in brain against radical induced polymerization, in conjunction with recent reports that pyritinol enhanced cholinergic transmission in brain (Martin and Vyas, 1987, Pavlik, Benesova and Dlohozkova, 1987), substantiates its use for the treatment of cognitive disorders.

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