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Active Oxygen Species Formation in Synaptosomes Exposed to an Aluminum Chelator

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BONDY, S. C., H. TSENG AND C. ORVIG. *Active oxygen species formation in synaptosomes exposed to an aluminum chelator.* NEUROTOXICOL TERATOL **20**(3) 317–320, 1998.—This study evaluates the potential of two chelators, 1,2-dimethyl-3-hydroxypyridine-4-one (Hdpp) and 1-*n*-butyl-2-methyl-3-hydroxypyridin-4-one (Hnbp), to modulate cerebral rates of free radical production. The fluorometric assay for 2′,7′-dichlorofluorescein, which is formed by oxidation of a nonfluorescent precursor (2′,7′-dichlorofluorescin diacetate), was used to assay reactive oxygen species (ROS) production. The chelator Hdpp alone and the aluminum complexes of each chelator, Al $(dpp)_3$ and Al $(nbp)_3$, all inhibited basal rates of generation of ROS within a rat cerebral synaptosomal fraction. In the presence of an iron salt (1 μM $FeSO_4$), a major enhancement of synaptosomal ROS formation was apparent. However, with the addition of an equimolar concentration of Hdpp, $Al(dpp)_3$, or $Al(nbp)_3$, this stimulation was completely abolished. The *N*-substituted-3-hydroxy-4-pyridinones have been proposed to be of clinical utility for the removal of iron or aluminum from tissues. The clinical potential of this class of chelator may be enhanced by their ability to inhibit iron-related oxidative events. © 1998 Elsevier Science Inc.

Aluminum Metal chelation Free radicals Reactive oxygen species Hyroxypyridinones

ALUMINUM salts are known to exhibit toxicity to a variety of organs including the nervous system. Although aluminum certainly forms the basis of dialysis dementia, the role of this nonessential element in the etiology of Alzheimer's disease is more controversial (4). It has been claimed that chelation of aluminum with deferoxamine may ameliorate this disease (24). The mechanistic basis of any aluminum neurotoxicity is also not obvious; however, several reports suggest that a key feature may be an interaction with iron leading to excessive formation of reactive oxygen species (ROS) (6,14,28). This concept finds further support from evidence that amyloid deposits and neurofibrillary tangles found in cortical tissues in Alzheimer's disease have been reported to contain aluminum (12,34), although not all studies confirm this. Amyloid deposits appear able to promote ROS formation (16,32). There is other evidence that this disease involves deleterious free radical-induced events (8,29).

These issues, together with problems associated with excess levels of aluminum following renal impairment, have driven a search for appropriate chelators to facilitate aluminum excretion. Deferoxamine, the most widely used metal chelator, is rather expensive, toxic, nonselective, and cannot be orally administered. A new class of chelators, the 3-hydroxy-4-pyridinones, have been developed initially as potential iron chelators in view of their high affinity for ferric iron (10,17,18). These agents can also form aluminum complexes of high stability (9), and may be appropriate for clinical testing as possible drugs for reducing the body burden of this element (2,36).

In view of both the potentiation of iron-effected ROS gen-

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eration by aluminum salts and the potential for certain metal chelators to enhance the pro-oxidant properties of metals (1,35), we have examined the ROS-promoting capacity of two hydroxypyridinones in an isolated cerebral particulate fraction. This potential promotion of oxidant events must be considered because the hydroxypyridinones have a bidentate metal-sequestering site implying incomplete ligation of iron coordinates (9,33). The two compounds initially selected for study were 1,2-dimethyl-3-hydroxypyridin-4-one (Hdpp) and 1-*n*-butyl-2-methyl-3-hydroxy-4-pyridinone (Hnbp). These were chosen for comparison because a previous study had shown that whereas both were formed chelates with aluminum that may remain intact in vivo, the former was significantly less toxic than the latter (2). Such agents have previously been found able to increase excretion of aluminum in experimental animals (11,37). Aluminum mobilization in renal dialysis patients by Hdpp has also been described (20), suggesting clinical utility of these chelators. The compounds tested (Fig. 1), one in its free form and both as aluminum complexes, inhibited basal ROS formation or quenched its expression, at micromolar concentrations.

METHOD

Tissue Preparation

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 150–175 g, were decapitated, the brains were excised quickly on ice, and the cerebrocortex was dissected out. Tissue was weighed and homogenized in 10 vol. of 0.32 M sucrose and centrifuged at 1800 × *g* for 10 min. The resulting supernatant fraction was then centrifuged at 31,500 × *g* for 10 min to yield the crude cerebral synaptosomal pellet (P2). The P2 pellet was taken up in HEPES buffer to a concentration of 0.1 g-eq/ml. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10; pH 7.4.

Assay for Oxygen Reactive Species Formation

Reactive oxygen species were assayed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which is de-esterified within cells to the ionized free acid, dichlorofluorescin, DCFH. This is trapped within cells and thus accumulated (5). DCFH is capable of being oxidized to the fluorescent 2′,7′-dichlorofluorscein by reactive oxygen. The utility of this probe in isolated subcellular cerebral systems has been described (23). P2 suspensions were diluted in 9 vol. of HEPES buffer. The diluted fractions were then incubated with 5 μM DCFH-DA (added from a stock solution of 0.5 mM in 10% ethanol) at 37°C for 15 min. After this loading with DCFH-DA, the fractions were incubated for a further 60 min in the presence of the various compounds. The aluminum compounds were dissolved in methanol initially, and subsequently diluted in the incubation buffer. The final concentration of methanol (0.01%) had no ROS quenching properties. At the beginning and at the end of incubation, fluorescence was monitored on a Farrand spectrofluorometer, with excitation wavelength at 488 nm (bandwidth 5 nm), and emission wavelength 525 nm (bandwidth 20 nm). The rate of generation was found to be linear over the incubation period (23). Autofluorescence of fractions was corrected for by the inclusion in each experiment of parallel blanks with no DCFH-DA. The correction for autofluorescence was always less than 11% of the total. Oxygen reactive species formation was quantitated from a 2′,7′-dichlorofluorescein (DCF) standard curve (0.05–1.0 mM) and results were expressed as nmol DCF formed/15 min/mg protein.

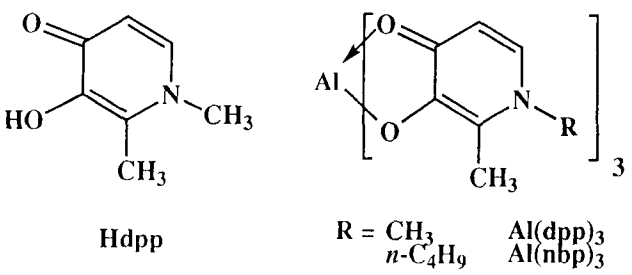

FIG. 1. Structure of compounds under study. Hdpp has also been referred to as L1 or CP20 and Hnbp has been referred to as CP24.

Materials

Hdpp, Al $(nbp)_3$, and $Al(dpp)_3$, were synthesized by previously published methods (9,18,26). 2′,7′-Dichlorofluorescin diacetate was purchased from Molecular Probes, Inc. (Eugene, OR), while DCF required for calibration was obtained from Polysciences, Inc. (Warrington, PA). Other materials were from Sigma Co. (St. Louis, MO).

Protein Determination

Protein concentration was assayed using the method of Bradford (7).

Statistical Analyses

Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference Test. The acceptance level of significance was $p < 0.05$ using a two-tailed distribution.

RESULTS

At a concentration of 1 μM, $Al(dpp)_3$, $Al(nbp)_3$, or Hdpp (not complexed with aluminum) all significantly depressed the rate of synaptosomal ROS generation below control levels (Fig. 2). A dose–relation study was performed with $Al(dpp)_3$ (Fig. 3). This compound inhibited ROS formation over a broad range of concentrations. When 1 μM $FeSO_4$ was present, ROS generation was markedly elevated over basal levels; this enhancement was completely abolished in the presence of an equimolar amount any of the three agents tested (Fig. 2). Increasing levels of chelators up to 50 μM in the presence of 1 μM Fe only reduced ROS production marginally.

DISCUSSION

In those studies where the $Al(dpp)_3$ and $Al(nbp)_3$ were compared, there was no major difference in their properties. Thus, the neurotoxicity of the more lipophilic $Al(nbp)_3$ complex (2) is not related to any free radical-producing potential. In the absence of exogenous metals, both compounds showed antioxidant rather than pro-oxidant qualities.

Hydroxypyridinones have been reported to depress lipid peroxidation in vivo and this effect is likely to be mediated by their ability to sequester iron (13,22,30). The ability of these chelators to protect against iron appears to be due to retardation of iron-dependent formation of the intensely pro-oxidant and very short-lived hydroxyl radical (21). In the absence of

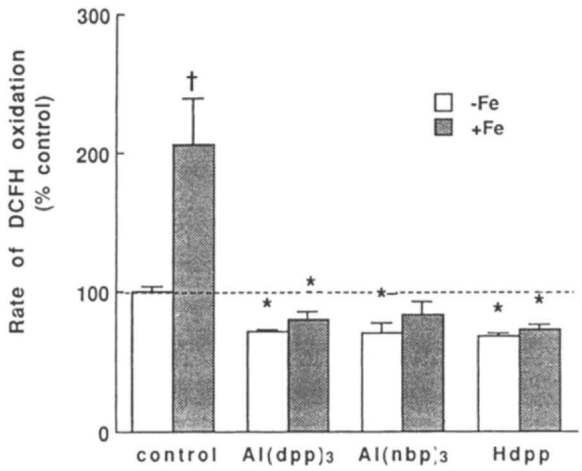

FIG. 2. Effect of 1 μM $Al(dpp)_3$, $Al(nbp)_3$, or Hdpp upon reactive oxygen species formation by cerebrocortical synaptosomes in the presence or absence of 1 μM $FeSO_4$. Each value represents the mean of three to five individual determinations ± SE. †Differs from corresponding value in the absence of $FeSO_4$ ($p < 0.05$). *Differs from basal control value. Basal level of activity was 3.46 ± 0.27 nmol DCF oxidized/mg protein/h.

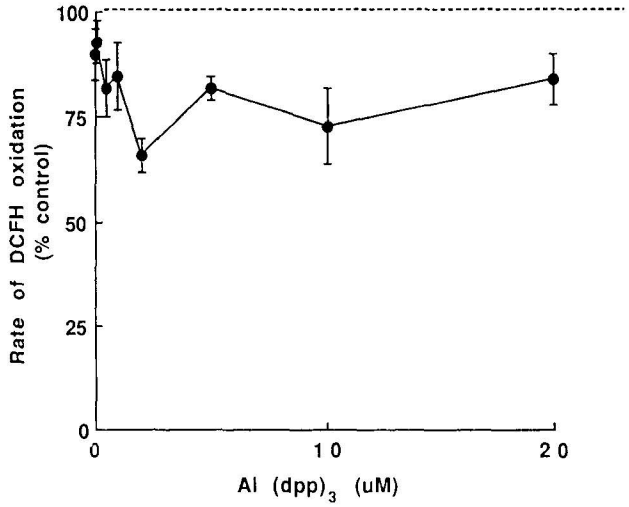

FIG. 3. Reactive oxygen species generation by the cortical synaptosomes together varying concentrations of $Al(dpp)_3$. Each value represents the mean of three to five individual determinations ± SE.

exogenous iron, Hdpp does not appear to possess intrinsic free radical-promoting characteristics because it did not promote lipid peroxidation in isolated hepatocytes (25). The lack of concentration-dependent response suggested that even a low concentration of the chelator may complex the small amount of soluble iron present, by exchanging Al for Fe, thereby reduced ROS formation. The pro-oxidant properties of Fe were abolished by both $Al(dpp)_3$ and $Al(nbp)_3$. This may have involved the formation of redox-inert iron complexes because Fe^{3+} has a greater affinity for both the chelators tested than Al^{3+} and is likely to have wholly or partially displaced aluminum (9,18).

The ability of Hdpp to quench Fe-promoted free radical formation is in contrast to many other chelators, such as ascorbate, which may powerfully promote iron-related oxidative events (3), but this result parallels the free radical-quenching properties of other chelators such as deferoxamine. At higher concentrations, in addition to chelating iron, deferoxamine is also able to directly act as a free radical quencher (15). The chelators studied here are unlikely to possess this property because there was no continuing dose response ROS-reducing relationship using increasing levels of chelator (Fig. 3).

In view of the potential of deferoxamine to promote ROS formation under some circumstances (1) and to precipitate dialysis dementia by mobilizing sequestered aluminum from tissues (31), the development of new metal chelators is of considerable importance. The possibility of promotion of metal-induced pro-oxidant events by novel chelators with putative clinical utility, therefore, needs to be taken into account. Hdpp is an effective chelator of both iron and aluminum and may be useful for the sequestration of either metal (19). The results reported here have failed to detect any tendency for the induction of major oxidative stress by the 3-hydroxy-4-pyridinone class of chelating agents, further enhancing the clinical potential of these compounds.

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