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Effects of oximes on mitochondrial oxidase activity

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ABSTRACT

Oximes, including 2-pyridinealdoxime methiodide (2-PAM), are reactivators of acetylcholinesterase (AChE) inhibited by organophosphate poisoning. Unfortunately, their clinical use has been limited by their toxicity. To investigate the mechanism of this toxicity, the effects of oximes on the enzymes choline oxidase (ChOD) and cytochrome *c* oxidase (CyCOD) of the respiratory chain in mitochondria were examined. The oximes 2-PAM, obidoxime, and diacetylmonoxime significantly ($P < 0.01$) inhibited ChOD activity, and the extent of inhibition correlated with the ability to reactivate inhibited AChE. When ChOD activity in mitochondrial extracts was tested, 2-PAM inhibited the activity by 75%, obidoxime and diacetylmonoxime did not significantly inhibit it, and 4-[(hydroxy-imino)methyl]-1-decylpyridinium bromide (4-PAD), which has greater toxicity, increased the amount of product generated in the assay to approximately 200% of normal levels. Similarly, 2-PAM inhibited the activity of CyCOD in mitochondrial extracts whereas obidoxime and diacetylmonoxime did not. One explanation for these findings is that, in addition to their inhibition of mitochondrial oxidases, the oximes may produce excessive reactive oxygen species such as H_2O_2 in the mitochondrial fraction, which may account for some of their toxicity.

This is a preliminary report related to the toxicities of oximes that may participate in the inactivation of mitochondrial oxidase enzymes. This hypothesis should be further investigated by in vivo study, including kinetic determination and free radical work.

1. Introduction

In the treatment of poisoning by nerve agents, the primary drugs used are anticholinergics that antagonize the effects of accumulated acetylcholine at the cholinergic synapses and cholinesterase reactivators to reactivate the inhibited AChE. In addition, central nervous system antidepressants such as benzodiazepines are used to treat convulsions. Among the anticholinergics, atropine is most frequently used; and 2-pyridinealdoxime methiodide (2-PAM), obidoxime, and HI-6 have been considered sufficiently effective as the reactivators. However, it is generally known that 2-PAM and obidoxime are ineffective, although HI-6 appears to be effective, against soman (Rousseaux and Dua, 1989; Koplovitz and Stewart, 1994). Furthermore, 2-PAM and HI-6 are ineffective, although obidoxime is effective, against tabun (Kassa and Vachek, 2002). Thus, it is thought that existing treatments do not effectively reactivate AChE inhibited by every type of nerve agent (Kassa, 2002; Kuca and Kassa, 2003). The differences in the efficacy of oximes against various nerve agents result mainly from the various aging rates at which inhibited AChE is converted to a form that can no longer be reactivated by oximes (Fleisher and Harris, 1965; Coult et al., 1966; Berends, 2001).

Another very important attribute of oximes is their ability to penetrate the blood–brain barrier (BBB) (Firemark et al., 1964; Hobbiger and Vojvodic, 1966; Sakurada et al., 2003; Petroianu et al., 2007; Lorke et al., 2008). We previously confirmed that the mean BBB penetration ratio of 2-PAM was approximately 10% in rats (Sakurada et al., 2003). However, in fact, it has been suggested that the actual ratio of 2-PAM concentration in the extracellular fluid of the brain vs. that in the blood would be lower after organophosphate poisoning because 2-PAM is used for reactivation of inhibited AChE in the blood before penetration of the BBB. Administration of higher 2-PAM concentrations might promote better reactivation of inhibited AChE in the brain. However, the blood concentration must be strictly maintained at several μg/mL by intravenous drip because of the toxicities of the oximes themselves (Askew, 1956; Marrs, 1991; Dawson, 1994; Eyer, 2003).

Recently, we have attempted to develop new oximes that have relatively high reactivation activity for AChE and can easily penetrate the BBB (Ohta et al., 2006; Okuno et al., 2008). However, almost all the synthesized compounds show strong toxicity. In general, respiratory paralysis is considered to be the major toxic effect caused

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by pyridine aldoximes, chemicals related to the common oximes (Faff and Bak, 1978). However, the mechanism of toxicity has not yet been examined and still remains unclear.

Because understanding the mechanism of oxime toxicity may assist in developing new antidote therapies, we have examined the effects of oximes on the activities of the mitochondrial oxidases choline oxidase (ChOD) and cytochrome *c* oxidase (CyCOD), which are key enzymes of the respiratory chain in mammalian mitochondria and play an essential role in generating ATP.

2. Materials and methods

2.1. Chemicals

2-PAM was purchased from Sigma (St. Louis, MO, USA). Obidoxime was purchased from Toronto research chemicals (North York, Canada). Diacetylmonoxime and 4-[(hydroxy-imino)methyl]-1-decylpyridinium bromide (4-PAD), the chemical structures of which are shown in Fig. 1, were synthesized in our laboratory. Peroxidase was purchased from Toyobo (Osaka, Japan). ChOD, hydrogen peroxide (H_2O_2), 4-aminoantipyrine, choline chloride, ascorbic acid, sucrose, and the tetrasodium salt of ethylenediamine tetraacetic acid (EDTA) were purchased from Wako (Osaka, Japan). Cytochrome *c* and *n*-dodecyl β-D-maltoside were purchased from Sigma (St. Louis, MO, USA).

2.2. Mitochondrial preparations

The hearts (1 g) were dissected from Wistar rats (250–300 g), suspended in 9 mL of 0.25 M sucrose, 10 mM Tris–HCl (pH 7.6), 0.2 mM EDTA, and homogenized slowly by moving a Potter-type homogenizer (AS ONE, Tokyo, Japan) up and down about 10 times. After centrifugation (600 × *g*) for 10 min, the supernatant was collected and centrifuged at 8000 × *g* for 10 min. The pellets were suspended in the same buffer and centrifuged again at 8000 × *g* for 10 min. The resulting pellets was resuspended in the same buffer and used for the experiment. The concentrations of proteins were determined by the copper reduction/bicinchoninic acid (BCA) reaction using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the Animal Experiments Ethics Committee, National Research Institute of Police Science.

2.3. Measurement of ChOD activity

The principle of the coupled assay procedure is as follows (Takatori et al., 1981). ChOD oxidizes free choline to produce H_2O_2 and betaine. The produced H_2O_2, in the presence of peroxidase as a catalyst, oxidizes and condenses phenol and 4-aminoantipyrine to generate a red quinone stoichiometrically. The concentration of red quinone is then determined by measuring the absorbance at 500 nm using a UV spectrophotometer (UV-2100, Shimadzu, Kyoto, Japan). The standard incubation mixture consisted of 4 mL of 0.1 M Tris–HCl buffer (pH 7.4) containing 5 units of ChOD, 50 units of peroxidase, 400 μmol of phenol, and 40 μmol of 4-aminoantipyrine. After addition of 10 μL of a 0.5 M choline chloride solution, the mixture was incubated at 37 °C for 20 min. The red quinone product was then measured at 500 nm using the UV spectrophotometer.

To examine the effect of 2-PAM on ChOD activity, 1 μmol of 2-PAM and 10 μL of choline chloride solution were added to the standard incubation mixture, and the mixture was incubated at 37 °C for 20 min.

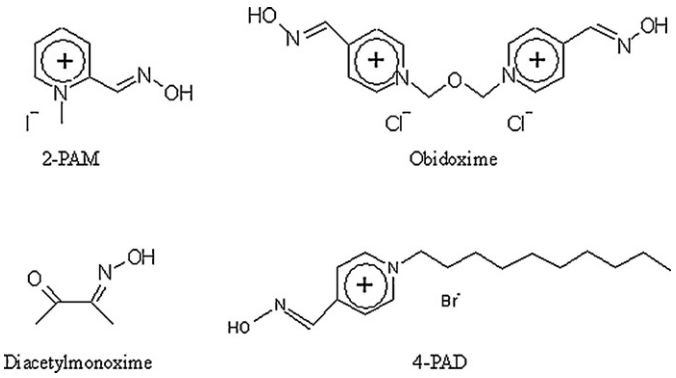

Fig. 1. Chemical structures of oximes.

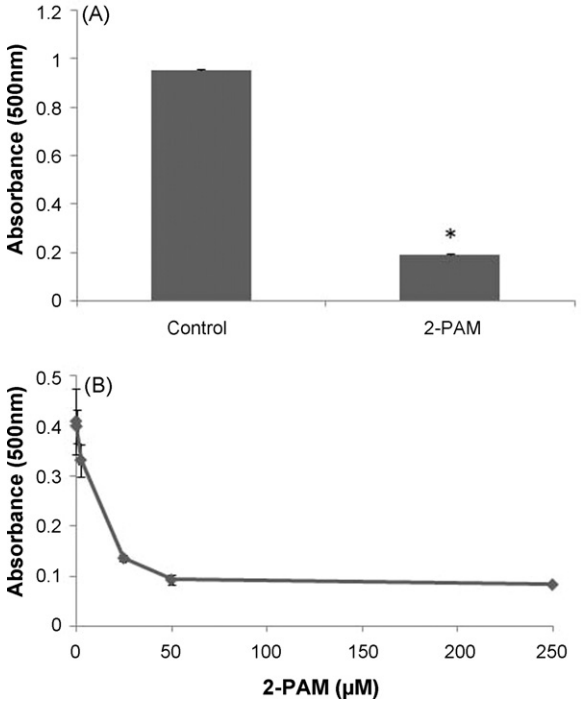

Fig. 2. Inhibitory effect of 2-PAM on ChOD activity. (A) To examine an effect of 2-PAM on ChOD activity, 1 μmol of 2-PAM and 10 μL of choline chloride solution were added to the standard incubation mixture, and the mixture was incubated at 37 °C for 20 min. The production of red quinine was significantly ($P < 0.005$) decreased by 2-PAM ($n = 4$). (B) Then, the inhibitory effects of 0–250 μM 2-PAM concentrations on ChOD activity were examined ($n = 4$). 2-PAM inhibited the production of red quinone in a concentration-dependent manner. Bar heights are means, and parentheses indicate standard deviation. $^*P < 0.005$.

2.4. HPLC analysis of the mixture of 2-PAM and choline chloride

The mixture of 300 μL of 0.5 mM 2-PAM and 300 μL of 1 mM choline chloride was incubated at 37 °C for 8 min. After the mixture was filtrated through the cellulose-acetate filter, a portion of (20 μL) of the filtrate was injected into the HPLC. 2-PAM was separated on a reverse-phase C18 column (puresil 5 μm C18, 250 nm × 4.6 mm, Waters) and detected by a UV detector at 290 nm (EICOM, EP-300 liquid chromatography system). The mobile phase, 0.2 M phosphoric acid containing 2 mM sodium 1-octanesulfonate and 0.1 M diethylamine, was delivered at a flow rate of 1.0 mL/min.

2.5. Measurement of CyCOD activity

Cytochrome *c* (63 μM) in buffer (10 mM Tris, 0.2 mM EDTA, and 0.05% *n*-dodecyl β-D-maltoside, pH 7.6) was incubated with 12.5 μM L-(+)-ascorbic acid for 30 min at room temperature (18 °C) to convert the ferric cytochrome *c* to ferrous cytochrome *c*. Twenty microliters of the 1 mg/mL mitochondrial protein solution was then added to 2 mL of the reaction mixture at 37 °C. The reaction was monitored spectrophotometrically (UV-2100, Shimadzu, Kyoto, Japan) at 550 nm for 2 min at 1 min intervals. Any change in the absorbance between 0 and 1 min was recorded, indicating CyCOD activity. This assay was carried as described in our previous report (Ikegaya et al., 2001).

2.6. Statistical analysis

The statistical analyses in Figs. 2–5 were performed using the Student's *t*-test. The comparison of effects of different oximes on ChOD activity in Fig. 3 was performed using a one-factor ANOVA, and then a post hoc test with the Tukey–Kramer method.

3. Results

3.1. Inhibitory effect of 2-PAM on ChOD activity

To examine an effect of 2-PAM on ChOD activity, 1 μmol of 2-PAM and 10 μL of choline chloride solution were added to the standard incubation mixture, and the mixture was incubated at 37 °C for 20 min. The result showed that the production of red