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INTERACTION OF HEXACHLOROPHENE AND OTHER COMPOUNDS WITH SPIN-LABELED BRAIN MEMBRANES

Gopa Rakhit and Joseph P. Hanig

Division of Drug Biology, Food and Drug Administration,
Washington, D.C. 20204

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Experiments reported here demonstrate that hexachlorophene influences oxidation-reduction events inside the brain membrane, possibly via a free radical mechanism. This was shown by nitroxide spin label quenching inside the rat cerebellum membrane bilayer due to the interaction between hexachlorophene and peroxidase-hydrogen peroxide system. Prior addition of antioxidants, e.g., vitamin E or butylated hydroxytoluene, prevented such membrane-bound fatty acid spin label reduction, presumably due to their free radical scavenging abilities. The 5-doxyl stearic acid spin probe attached to the brain membranes did not exhibit any detectable changes in their ESR spectra nor, consequently, in the microviscosity of the membranes when exposed to up to 40 mM hexachlorophene.

Chronic or acute exposure to hexachlorophene (HCP), a chlorinated methylenebisphenol germicidal agent, produces brain edema and neurotoxicity in humans (1) and in experimental animals (2-5), inhibits certain liver microsomal enzyme activities (6) and uncouples mitochrondrial oxidative phosphorylation (7). Several workers (8,9,10) have hypothesized that free radical formation and lipid peroxidation are fundamental processes in the development of edema in the CNS. Earlier work in our laboratory (11) had indicated that the food additive butylated hydroxytoluene (BHT), a potent antioxidant, protects against lethal doses of HCP in rats, and supported the hypothesis of free radical involvement as a possible cause of HCP toxicity. In this study, we have used electron spin resonance spectrometry (ESR), which determines free radicals in biological systems (12), to explore the mechanism of action of HCP at the molecular level. Spin labels are stable free radicals (13) that can be used as probes or reporter groups for biological macromolecules such as proteins (14), nucleic acids (15) and membranes (16) and can provide topographic information about the surrounding

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molecular environment (17). We have used fatty acid spin labels to examine the microviscosity (12) changes in the brain membranes caused by HCP. Stable nitroxide spin labels can undergo one electron reduction in chemical or biological systems and hence become invisible to electron spin resonance (18). This quenching or loss of ESR signal can be used advantageously to monitor the redox properties of xenobiotic compounds in biological systems. Using such spin labelled membranes isolated from rat cerebellum and rat frontal cortex, we have examined the effect of HCP on the oxidation-reduction events induced by horseradish peroxidase (HRP) and hydrogen peroxide to determine whether the transient HCP species such as free radicals can quench the ESR signal from the fatty acid spin label bound to the brain membrane. We also report the effect of prior addition of antioxidants (vitamin E and BHT) on membrane-bound spin label reduction due to HCP and possible mechanisms involved.

Because our preliminary work with triethyltin (TET), which also causes brain edema and uncoupling of oxidative phosphorylation in experimental animals (19), indicated a quenching effect similar to that obtained with HCP, we have explored the possibility that free radical mediation might be instrumental in the etiology of brain edema.

MATERIALS AND METHODS

Cerebellum and frontal cortex regions were identified and dissected from rat brain by the method of Glowinski and Iversen (20). The brain sections were then frozen in liquid nitrogen and weighed. A crude membrane fraction was prepared from brain regions (21) by homogenizing the tissue in 19 volumes of 0.32 M sucrose and centrifuging at 50,000 x g for 10 min. The pellet was rehomogenized in 19 volumes of ice-cold distilled water, pH 7.4, and recentrifuged as before. The final pellet was then suspended in 40 mM Tris-HCl buffer, pH 7.4, representing 50 mg of original tissue per ml of buffer. The total amount of protein in the membrane fraction was determined by the method of Lowry et al. (22).

The membranes were spin-labelled by vortexing the buffered suspension in a small glass tube on which 5-nitroxide stearate (1-oxyl-2,2-dimethyloxazolidine derivative of 5-ketostearic acid, 5 NS obtained from Syva Co., Palo Alto, Calif.) was deposited by evaporating the solvent ethanol. Free probe in the supernatant was removed by double washing and centrifuging in a Beckman microfuge at 4°C.

Horseradish peroxidase (type HPOFF) was used as purchased from Worthington Biochemical Corp., Freehold, N.J. The RZ value of horseradish peroxidase, defined as the absorptivity ratio at 403 and 275 nm, was above 3.0. All other chemicals were of the purest grade commercially available.

RESULTS AND DISCUSSION

Brain membranes were spin labelled with fatty acid spin probe 5-doxyl stearic acid. As shown in Fig. 1A, the strong asymmetric ESR signal is characteristic of the nitroxide spin label bound to the membrane bilayer (16). When such spin-labelled membranes obtained from either cerebellum or frontal cortex were reacted with HRP, HCP and H_2O_2 (added last), the nitroxide ESR signal was rapidly and completely quenched (Fig. 1C). All three components (HRP, HCP and H_2O_2) were necessary to quench the ESR signal from the nitroxide radical; any single component had no effect on the spin label signal. The combination HRP and H_2O_2 produced only a slight (if any) reduction in the bound spin label amplitude (Fig. 1B). Any other combination of only two components did not affect the spin label signal.

If, instead of adding the reactants directly to the spin-labelled membrane suspensions, the reaction between HCP, HRP and H_2O_2 was allowed to proceed for 5 min in isolation and the reaction mixture was subsequently added to the labelled membranes, there was only a slight (if

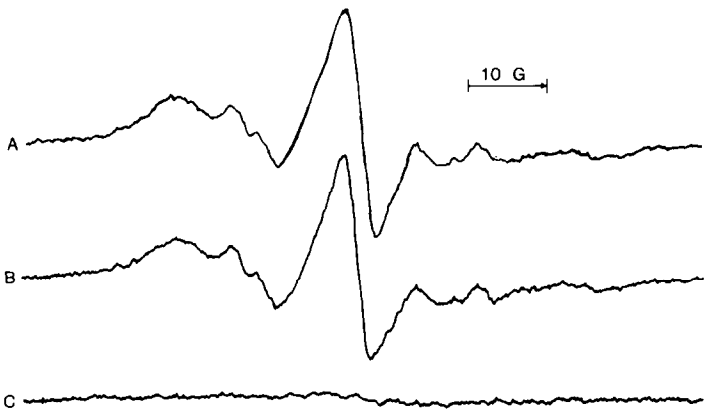

Figure 1. Quenching by hexachlorophene (HCP) in combination with horseradish peroxidase (HRP) and H_2O_2 of the ESR spectrum signal from nitroxide free radical spin label bound to brain membranes. A. ESR spectrum of 5-doxyl stearate spin label bound to rat cerebellum membrane (ca. 3 mg protein/ml) in 40 mM, pH 7.4 Tris-HCl buffer. B. ESR spectrum of spin-labelled membranes in presence of HRP (0.1 mg/ml final concentration) and H_2O_2 (0.03% final amount). C. ESR spectrum of spin-labelled membranes after reaction of HCP (0.05 mM final concentration) with HRP and H_2O_2. All ESR spectra were recorded at ambient temperature. Scan time 8 min/100 gauss, gain 6.3 x 10^3, 20 mwatt, 2 gauss modulation amplitude at 100 KHz, response time 1 sec. Arrow indicates direction of low to high magnetic field and length indicates 10 gauss.

any) reduction of the spin label signal. This finding demonstrates that a transient species formed during the reaction of HCP, HRP and H_2O_2 is responsible for rapid quenching of the spin-label signal. A complete loss of ESR signal from the spin label was also observed when the 5-doxyl stearic acid spin label was dissolved in 30% methanol and reacted with HCP and HRP/H_2O_2 system.

Rapid and complete spin label quenching was also observed when TET (3.0 mg/ml) was substituted for HCP in the reaction with HRP/H_2O_2 inside labelled brain membranes. Both HCP and TET produce brain edema and affect the membrane spin label in a very similar fashion. The formation and identity of free radical species from many electron donor compounds, e.g., chlorpromazine (23), ascorbate (24) and the carcinogen N-hydroxy-2-acetyl-aminofluorene (25), by HRP/H_2O_2 systems have been well documented by early investigators (26). Based upon such a well-known reaction mechanism as the HRP/H_2O_2 system, it is most likely that the transient intermediate responsible for spin label reduction in the HCP-HRP/H_2O_2 system is a free radical.

Recently, Floyd (18) has observed similar spin label quenching by erythrosine B (the food dye FD&C Red No. 3) in HRP/H_2O_2 system and suggested the possibility of free radical mediation in the dye-induced neurotoxicity.

Figure 2 shows the temporal effect of the HCP-HRP/H_2O_2 reaction system on the spin label signal reduction. In the presence of HCP, the loss of spin label ESR signal was complete within 10 min after H_2O_2 was added and no regeneration of signal was detected within 4 hr. In contrast, in the absence of HCP, the combination HRP and H_2O_2 produced only a slight reduction of ESR signal initially and thereafter the bound spin label signal remained stable for at least 1 hr. Figure 2 also shows that the prior addition of antioxidants, BHT, vitamin E, or ascorbic acid (not shown), prevented the HCP-induced spin label reduction. After initial loss of some spin label signal, the amplitude of ESR signal was stabilized and

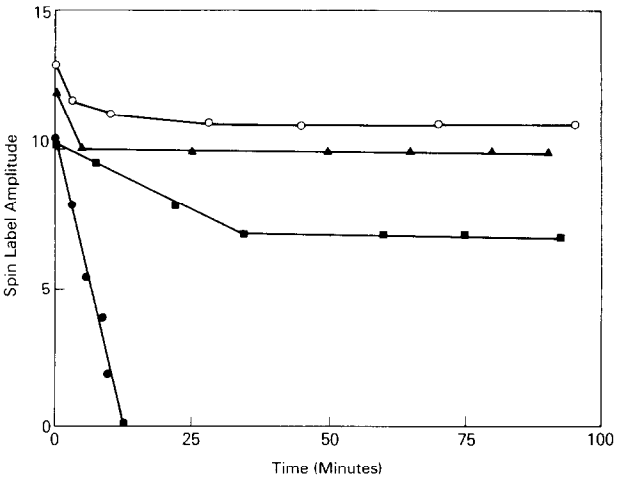

Figure 2. Membrane-bound spin label amplitude reduction as function of time during interaction of HCP with HRP, H_2O_2 and some antioxidants. Rat cerebellum membranes (3 mg protein/ml) were spin labelled with 5-doxyl stearate nitroxide free radical, and final concentrations of HRP and H_2O_2 were 0.1 mg/ml and 0.03%, respectively, in each sample. In all experiments, H_2O_2 was added last and ESR spectra were recorded within 2 min after H_2O_2 was added to the spin-labelled membranes. Membrane-bound spin label amplitudes (mid-field peak height of ESR spectrum) are in arbitrary units and plotted as function of time. ESR instrument settings were similar to those described in Fig. 1.

○ Reaction of HRP and H_2O_2 with spin-labelled membranes
● Interaction of HCP (0.05 mM final concentration) with HRP and H_2O_2
▲ Interaction of HCP with HRP and H_2O_2 in the presence of vitamin E (4 mM final concentration)
■ Interaction of HCP with HRP and H_2O_2 in the presence of BHT (4 mM final concentration)

at least 50% of the initial signal could be detected 1 hr after addition of H_2O_2 in the presence of vitamin E or BHT (Fig. 2). If, in contrast to the original experimental protocol, the antioxidants were added 10 min after the reaction of HCP with HRP/H_2O_2 had started, the spin label quenching could not be prevented.

Previous experiments with a rat animal model (11) had indicated that BHT could prevent or antagonize symptoms of HCP and the formation of brain edema, possibly by acting as a free radical scavenger.

The in vitro effect of HCP on brain membrane microviscosity was studied by recording the ESR spectra of the above fatty acid spin labelled brain membranes at ambient temperature after they were incubated with or without HCP in a shaking bath at 37°C for 30 min. No change in the shape or in the $2T_m$ values of the ESR spectra of the control membranes was detected

when compared to those of membranes receiving 40 mM HCP before incubation and under identical experimental conditions otherwise. These findings indicate that the interaction of HCP with the membrane bilayer must be minimal in altering the membrane fluidity, and hence the mechanism of action of HCP would be different from significant motional perturbation of brain membranes.

Preliminary efforts at direct detection of any free radicals produced by HCP in the above HRP/H_2O_2 reaction system inside brain membranes have not been successful, possibly because of the short lifetime of the free radicals and also because of the limitation of the present-day spin trapping technique (27).

The results of our experiments demonstrate for the first time that HCP influences the oxidation-reduction events occurring inside brain membranes. We have been able to monitor such redox changes by using a nitroxyl fatty acid spin label probe attached to the membrane bilayer. In addition, these experiments indicate that the mechanism of action of HCP and also TET in the development of brain edema may be associated with free radical metabolite formation in the brain. Further studies are needed to understand the exact nature and the extent of free radical damage *in vivo*.

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