J. Biosci., Vol. 15, Number 3, September 1990, pp. 211–215.

Membrane lipid peroxidation by ultrasound: Mechanism and implications

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Abstract. Ultrasonic radiation produced a dose-dependent linear increase in lipid peroxidation in the liposomes membrane as reflected in the measurement of conjugated dienes, lipid hydroperoxides and malondialdehydes. Ultrasound induced malondialdehyde production could not be inhibited by any significant degree by superoxide dismutase or histidine or dimethyl furan but was very significantly inhibited by butylated hydroxytoluene, cholesterol, sodium benzoate, dimethyl sulphoxide, sodium formate and EDTA. The scavenger studies indicated the functional role of hydroxyl radicals in the initiation of ultrasound induced lipid peroxidation.

Keywords. Ultrasound; liposomes; lipid peroxidation; hydroxyl radicals; scavengers.

Introduction

Ultrasound is being increasingly used in biology and medicine for clinical diagnosis, physiotherapy and also in hyperthermic cancer therapy (Kremkau, 1979). The biophysical effects of ultrasound in aqueous solutions can be classified as thermal effects, cavitation and the direct effects (Hill, 1968). The thermal effects of ultrasound are utilized for hyperthermia. The effects of its non-thermal modes of action have not been investigated. Although degradation of DNA in aqueous solution and breaking of cells are induced by ultrasound mostly by shearing stress of cavitation (Coakley and Nyborg, 1978), the chemical effects of free radicals produced during collapse of ultrasound induced cavitation bubbles have mostly remained unexplored. In the context of the important and increasing use of ultrasound and in view of the advantages offered by liposomal systems (Chatterjee and Agarwal, 1988) in the study of free radical-mediated membrane damage, this study has used liposomes to show that ultrasound induces a free radical-mediated and dose-dependent lipid peroxidation. Preliminary results were published elsewhere (Jana *et al*., 1986).

Materials and methods

Multilamellar liposomes were prepared using egg lecithin (obtained from V. P. Chest Institute, Delhi) by the method described earlier (Chatterjee and Agarwal, 1988). Liposomes in the presence or in absence of scavengers/quenchers were exposed to 20 KHz ultrasound (Labsonic 1510, Braun, Melsungen AG, Germany) by the method of Jana *et al.* (1986). Ultrasound dose rate was measured by Fricke Dosimetry (Fricke and Hart, 1966). Ultrasound induced lipid peroxidation was estimated by the simultaneous measurement of malondialdehyde (MDA),

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Abbreviations used: MDA, Malondialdehyde; DMSO, dimethyl sulphoxide; SOD, superoxide dismutase.

conjugated dienes and hydroperoxide by the method of Chatterjee and Agarwal (1988). Electron microscopy of liposomes, sonicated or non-sonicated, was done by the negative staining technique (Banerjee and Chatterjee, 1983).

Results

Figure 1A shows the electron micrograph of the nonirradiated liposomes illustrating the presence of multi bilayers of lipid. After exposure to ultrasound of dose 913J/m^2 the liposomes got fragmented as evident in figure 1B. Many small spherules (presumably unilamellar liposomes) of diameter $\simeq$ 300 Å are seen in this figure as fragmentation products. Exposure of liposomal suspension to ultrasonic radiation (20 KHz) caused free radical-mediated lipid peroxidation as estimated by measurement of at least 3 reaction products, *viz.*, conjugated dienes, lipid hydroperoxides and MDA. A linear dose-effect relation was observed in all cases (figure 2). Production of MDA was confirmed by spectrophotometric and spectrofluorometric methods including the detection of the excitation (360 nm) and emission (435 nm) maxima characteristic of MDA-glycine adduct formed after addition of glycine in the system. Different radical scavengers, antioxidants or quenchers were introduced one by one and their effects on the ultrasound induced lipid peroxidation were noted. The use of different radical scavengers or antioxidants did not change the nature of the dose-response relation but reduced significantly the slope of the line thereby indicating significant inhibition of the lipid peroxidation reaction. Table 1 shows the values of maximum inhibition obtained by the different inhibitors used and the corresponding inhibitor concentrations. While cholesterol and butylated hydroxy toluene produced maximum inhibition (around 90%), dimethyl sulphoxide (DMSO) and-sodium benzoate produced about 80% inhibition of ultrasound induced lipid peroxidation. Sodium formate and EDTA produced comparatively less but very significant inhibition (64%) of lipid peroxidation. Histidine, dimethylfuran and superoxide dismutase (SOD) did not produce any significant inhibition of the ultrasound induced lipid peroxidation.

Discussion

This study has thus shown that ultrasonic radiation can cause lipid peroxidation in the liposomal membrane and that the yield was significantly reduced by the antioxidant, BHT, OH* radical scavenger, sodium formate, metal ion chelator, EDTA and cholesterol. Also, all the 3 reaction products of lipid peroxidation, conjugated dienes, lipid hydroperoxides and MDA, were detected in the ultrasound exposed liposomal membrane. The ultrasound induced lipid peroxidation thus appeared to be the result of free radicals and oxygen mediated chain reactions (Chatterjee and Agarwal, 1988) and which basically involves the following stages: (i) abstraction of hydrogen atom, (ii) formation of lipid free radical (R*), (iii) formation of lipid-peroxyl free radical (ROO*), (iv) formation of lipid hydroperoxide (ROOH) and (v) formation of endoperoxide and MDA.

Different reaction products, e.g., conjugated dienes, hydroperoxides, MDA, etc., are produced at different stages of free radical-mediated lipid peroxidation and can be estimated by the respective assay methods. However each method has its own merits and demerits (Chatterjee and Agarwal, 1988) and the results obtained by

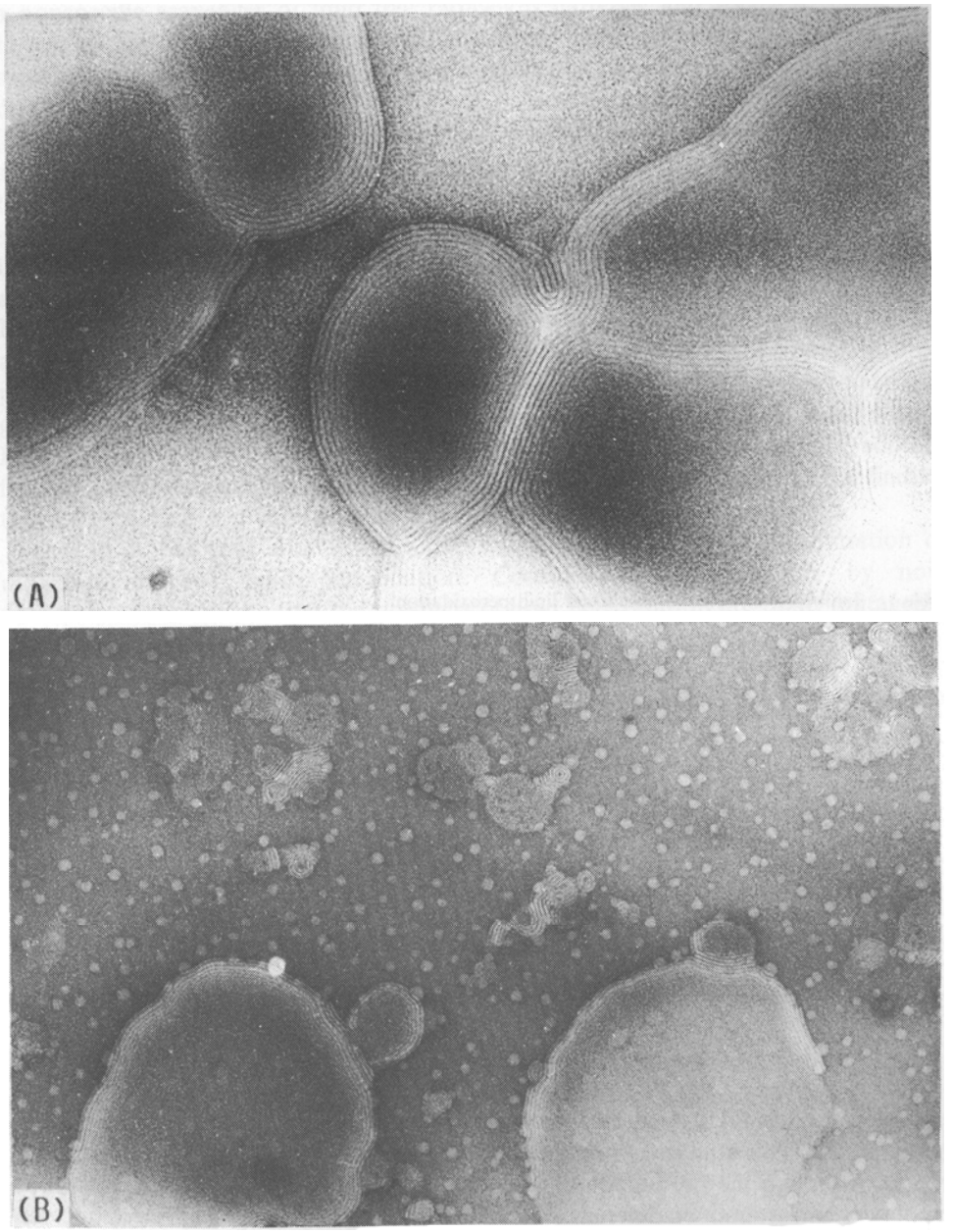

Figure 1. Egg lecithin liposomes negatively stained with PTA. (**A**), Nonirradiated (× 150,000) and (**B**), irradiated with ultrasound of dose 913 J/m^2 (× 65,600).

using any one of the assay methods may easily be misleading. With a view to obtaining greater confidence 3 reaction products, e.g., conjugated dienes, hydroperoxides and MDA, were assayed in parallel in this study. It is of interest to note that all the 3 methods of assay presented similar dose-dependent pattern of ultrasound induced lipid peroxidation

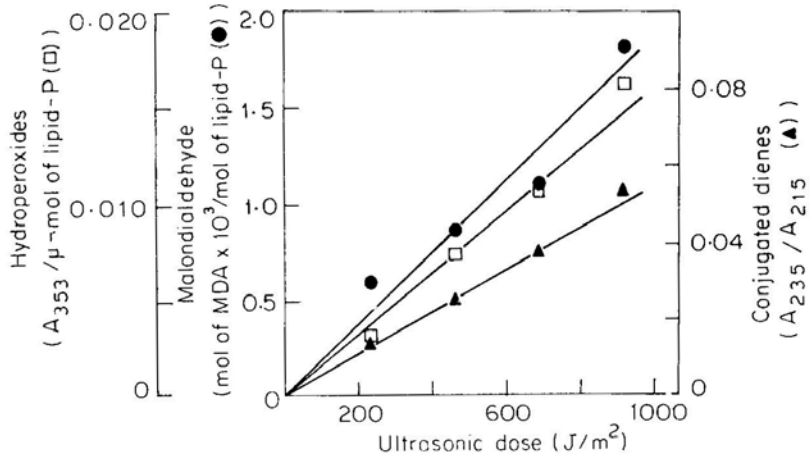

Figure 2. Increase in the production of conjugated dienes (A_{235}/A_{215},▲), hydroperoxides (A_{353}/μmol of lipid-P,□) and MDA (●) in the liposomal membrane with increasing dose of ultrasound (dose rate 7·61 J/m^2/s).

Table 1. Inhibition of ultrasound-induced lipid peroxidation.

Additions	Malonaldehyde content (mol of MDA × 10^3/mol lipid-P)	Inhibition (%)
Liposome + ultrasound of dose 913 J/m^2 (control)	1·94	—
Control + cholesterol (1 mol/mol lipid-P)	0·136	93
Control + BHT (0·41 mol/mol lipid-P)	0·233	88
Control + sodium benzoate (1·67 mol/mol lipid-P)	0·388	80
Control + DMSO (27·59 mol/mol lipid-P)	0·407	79
Control + sodium formate (14·22 mol/mol lipid-P)	0·698	64
Control + EDTA (1·35 mol/mol lipid-P)	0·698	64
Control + SOD (44·77 units/μmol lipid-P)	1·878	3
Control + DMF (4·57 mol/mol lipid-P)	1·9	~0
Control + L-histidine (1·44 mol/mol lipid-P)	1·949	~0

In a preliminary report (Jana *et al.*, 1986) we have shown evidence for the involvement of OH* radicals as the initiator of ultrasound-induced lipid peroxidation. This study has presented additional evidence in confirmation of that report. Although the production of OH* radicals and hydrogen atoms in aqueous media by ultrasound of different frequencies was demonstrated by electron spin resonance and spin trapping studies (Makino *et al.*, 1983; Edmonds and Sancier, 1983), these demonstrations do not necessarily prove the functional role of these radicals (Valenzeno, 1987). To determine a functional role, experiments aimed at demonstrating modulation of the reactants by use of radical scavengers seem appropriate (Valenzeno, 1987) and have been widely used (Chatterjee and Agarwal, 1988). In this study, no significant inhibition of lipid peroxidation resulted by use of SOD or histidine or DMF. The involvement of superoxide radicals O^- or singlet oxygen ($^1O_2$) in the ultrasound-induced lipid peroxidation thus appears unlikely. BHT and cholesterol produced nearly 90% inhibition of lipid peroxidation. BHT is

a nonspecific scavenger of lipid free radicals (Chatterjee and Agarwal, 1988) and hence no specific information could be derived there from about the initiation mechanism. The role of cholesterol in the inhibition of lipid peroxidation has been debated by many investigators and several possible mechanisms were suggested (Szebeni and Toth, 1986). However, none of the mechanisms suggested are so specific as to suggest the involvement of any particular radical in the initiation of lipid peroxidation. On the other hand, each one of the agents, sodium formate, sodium benzoate, DMSO and EDTA, produced significant inhibition of ultrasound induced lipid peroxidation. Sodium formate, sodium benzoate and DMSO are already known as scavengers of OH* radicals (Chatterjee and Agarwal, 1988). EDTA is known to inhibit metal catalyzed lipid peroxidation (Tien *et al.*, 1982), although under some circumstances EDTA was also reported to stimulate lipid peroxidation (Tien *et al.,* 1982). On the other hand, EDTA is also known to act as a good quencher of OH* radicals (Blazek and Peak, 1988). Although the exact role of EDTA in the present case may be debated, parallel use of these agents, sodium formate, sodium benzoate, DMSO and EDTA, lends greater support to the finding that OH* radical is involved in the ultrasound induced lipid peroxidation.

This study has thus shown that OH* radicals are involved in the initiation of ultrasound-induced lipid peroxidation. Considerable evidence has by now accumulated to indicate that the products of lipid peroxidation are toxic, mutagenic and carcinogenic (Pryor, 1976; Yagi, 1982; Chatterjee and Agarwal, 1988). In view of the wide use of ultrasound in human diagnosis and therapy, the present finding should have wider and important relevance and should sound a note of caution against indiscrete use of this radiation.

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