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FREE RADICAL FORMATION INDUCED BY ULTRASOUND AND ITS EFFECTS ON STRAND BREAKS IN DNA OF CULTURED FM3A CELLS

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Recent sonochemical studies have revealed that active oxygen species are formed by pyrolysis of water molecules due to high temperature cavitation bubbles. When aqueous solutions of DNA were sonicated, single-strand breaks and double-strand breaks of DNA were observed. Formation of double-strand breaks due to mechanical effects of cavitation and formation of single-strand breaks mostly due to free radicals were indicated. The sonochemically generated radicals from DNA constituents due to H atom and OH radical reactions, and pyrolysis processes, were identified by spin trapping with 3,5-dibromo-2,6-dideuterio-4-nitrosobenzene sulfonate. When suspensions of mouse mammary carcinoma FM3A cells and aqueous solutions of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were exposed to 1 MHz ultrasound in the presence of Ar, a good correlation between DMPO-OH formation and the cell killing induced by ultrasound were observed. Although single-strand breaks of DNA in the cells were observed at the maximum intensity of DMPO-OH formation and cell killing, double-strand breaks were not. Free radical formation by ultrasound in aqueous solutions and its relation to DNA strand breaks *in vitro* and *in vivo* are discussed.

KEY WORDS: Hydroxyl radicals, DNA strand breaks, Acoustic cavitation, EPR-spin trapping.

INTRODUCTION

Ultrasound is widely used in bio-medical fields for diagnosis, surgery and hyperthermic cancer therapy and also for cell disruption and making liposomes and emulsions in the laboratory. Biophysical modes of ultrasonic action are classified into thermal and nonthermal mechanisms. The nonthermal includes cavitational and other mechanisms (radiation pressure, acoustic streaming, radiation torque etc.). Cavitation involves the formation, growth and collapse of small gas bubbles in liquids exposed to ultrasound, and can be divided into two types, stable and transient. The very high temperature (several thousand degree K) and pressures (several hundred atmospheres) induced by cavitation in collapsing gas bubbles in aqueous solutions exposed to ultrasound lead to the thermal dissociation of water molecules into H atoms and OH radicals[1]. Combustion processes occur in the gas phase in the presence of volatile solutes and also in the interfacial regions when even non-volatile solutes (eg. DNA) are present[2]. In this paper, the roles of H atoms and OH radicals on DNA strand breaks when aqueous DNA solutions are sonicated are described, and the possibility of DNA strand breaks in the cells after sonication is examined.

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SPIN TRAPPING STUDIES OF THE SONOCHEMISTRY OF AQUEOUS SOLUTIONS

The formation of H atoms and OH radicals induced by ultrasound in aqueous solutions was confirmed by spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)[3], and subsequently by α-(4-pyridyl-1-oxide) N-*tert*-butyl nitrone (POBN) for H atoms. 4-(N-methylpyridinium) *tert*-butyl nitrone (PYBN) was used for H atoms and OH radicals[4]. Recently, spin adducts of α-phenyl-N-*tert*-butyl nitrone (PBN) and α-(4-nitrophenyl) N-*tert*-butyl nitrone (NPBN) for H atoms were observed[5]. When the yield of OH radicals in aqueous solution in the presence of different rare gases was measured by spin trapping with DMPO after sonication with 50 kHz ultrasound, the effectiveness of the dissolved rare gases on the formation of OH radicals was in the order Xe > Kr > Ar > Ne > He[6]. The formation of OH radicals increases with decreasing thermal conductivity of the rare gases and with increasing final temperature of the collapsing cavitation bubbles. This result is consistent with the dependency of sonoluminescence thermal conductivity[7].

SPIN TRAPPING STUDIES OF DNA CONSTITUENTS IN AQUEOUS SOLUTION

Free radical formation by ultrasound in aqueous solutions of DNA constituents has been studied by using ESR-spin trapping. First of all, the spin trap 3,5-dibromo-4-nitorosobenzene sulfonate (DBNBS) was found to be particularly suitable for sonolysis study of aqueous solutions of amino acids[8]. The sonolysis of nucleic acid bases and nucleosides was investigated with DBNBS as the spin trap. Spin adducts were also generated by OH radicals produced by UV-photolysis of aqueous solutions containing hydrogen peroxide. For dilute solutions of nucleic acid bases and nucleosides, the ESR spectra of the spin adducts were identical for UV-photolysis and for sonolysis. Hydrogen-abstraction reactions and addition to the C5-C6 double bond of pyrimidines due to H atoms and OH radicals were shown[9]. The possibility of new radicals specifically generated by pyrolytic processes in highly concentrated solutions of nucleotides was examined by using 3,5-dibromo-2,6-dideuterio-4-nitrosobenzene sulfonate (DBNBS-d_2) as a spin trap. At low concentrations, the spin-trapped radicals produced by sonolysis are due to H atoms and OH radicals, typically addition to the 5,6 double bond of the base moiety. On the other hand, at high concentrations methyl radicals due to pyrolysis were found for thymidine 5′-monophosphate, uridine 5′-monophosphate, 2′-deoxy-uridine 5′-monophosphate, and cytidine 5′-monophosphate[10]. These results indicate that pyrolysis radicals can be detected when the nucleotides are accumulated at high concentrations in the interfacial regions of the cavitation bubbles.

In summary, the features of free radical formation induced by ultrasound in aqueous solution are: 1) free radicals form randomly depending on cavitation occurrence, 2) free radical formation depends on the final temperature at which cavitation bubbles collapse and 3) hydroxyl radicals and hydrogen atoms from water, secondary reactants and additional pyrolysis radicals from solutes can be formed.

DNA STRAND BREAKS INDUCED BY ULTRASOUND *IN VITRO*

When aqueous solutions of DNA isolated from calf thymus were sonicated with 1.2 MHz continuous waves, the number of double-strand breaks and single-strand breaks were determined by neutral and alkaline sucrose gradient sedimentation analyses[11]. Higher yields of DNA double-strand breaks compared with that of single-strand breaks were observed at the lower intensities less than 2.3 W/cm^2. At the higher intensities above 2.3 W/cm^2, the yield of single-strand breaks increased. To elucidate the mechanism of DNA strand breaks induced by ultrasound *in vitro*, effects of cysteamine on DNA strand breaks induced by ultrasound was examined. When aqueous DNA solution was sonicated in the presence of cysteamine, the number of double-strand breaks of DNA was not influenced, but the number of single-strand breaks was reduced to about one-fifth that of the sonicated control. These results suggest that the double-strand breaks were exclusively induced by the mechanical effect, and most of the single-strand breaks were produced by water radicals arising from cavitation.

EFFECT OF FREE RADICALS INDUCED BY ULTRASOUND ON DNA STRAND BREAKS IN THE CELLS

It is interesting to note the effects of free radicals induced by ultrasound on the DNA strand breaks in the cells. This study was performed to examine whether OH radicals and H atoms or other mechanical effects arising from ultrasonic cavitation induce strand breaks of DNA in the cultured cells or not. First, the relationships between free radical formation and the cell-killing of mouse mammary carcinoma FM3A cells were examined, and then the DNA double- and single-strand breaks in the cells after sonication were studied.

Mouse mammary carcinoma FM3A cells were obtained from the Japanese Cancer Research Resource Bank. The cells were maintained in Eagle's MEM supplemented with 10% calf serum, and were grown in incubators at 37°C in a 5% CO_2, water saturated atmosphere. Highly purified DMPO was purchased from Labotec Co. Ltd. and 3,3,5,5-tetramethyl-1-pyrroline-N-oxide (M_4PO) was obtained from Aldrich Co. Ltd. M_4PO was used to attempt to detect new active oxygen species. The sample solutions were irradiated by using 1 MHz ultrasound. The polystyrene tubes containing sample solutions saturated with O_2, Ar or N_2O were rotated at 30 rpm during sonication. These methods have been previously described in detail[12]. After sonication in the presence of Ar, O_2 or N_2O, sample solutions of DMPO or M_4PO were transferred to quartz flat cells for ESR measurements. To estimate the clonogenicity of the surviving FM3A cells immediately after sonication, colony-forming assay in soft agar was employed. For determination of single- and double-strand breaks of DNA in the cells, alkaline and neutral elution methods were employed[13,14].

When DMPO solutions were sonicated in the presence of O_2, an EPR spectrum consisting of a 1:2:2:1 quartet was observed [Figure 1(a)]. The equal nitrogen and hydrogen hyperfine coupling constants ($a_N = a_H = 1.49$ mT) are characteristic of the OH spin adduct of DMPO. When the DMPO solutions saturated with Ar were sonicated, DMPO-OH and DMPO-H lines, in which hyperfine coupling constant of nitrogen and hydrogen are $a_N = 1.66$ mT and $a_H^\beta = 2.25\,(2H)$ mT, were

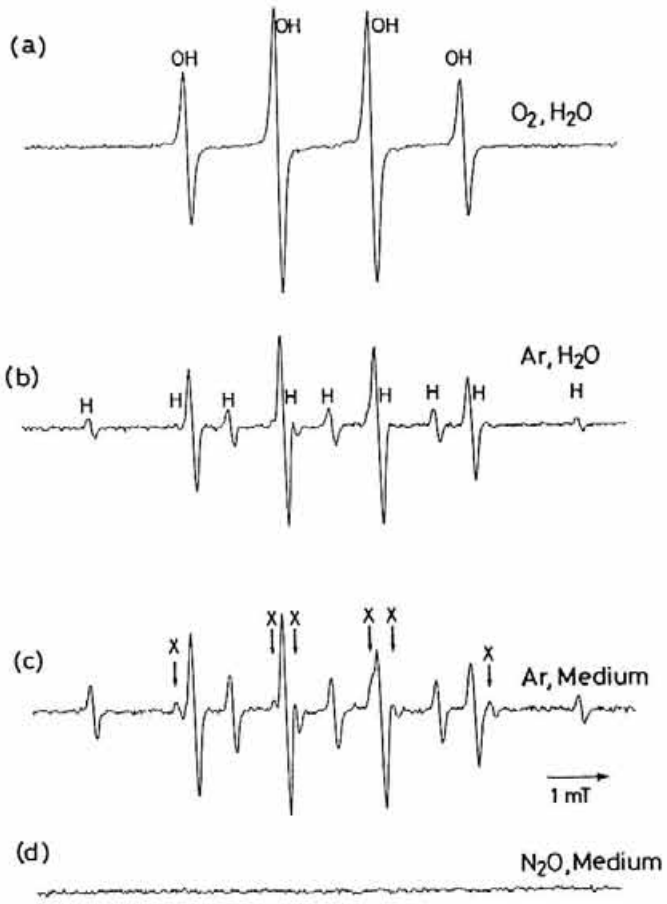

FIGURE 1 EPR spectra of spin trapped radicals obtained by sonolysis (10 min) of water or culture medium in the presence of 5 mM DMPO: (a) water saturated with O_2; (b) water saturated with Ar; (c) culture medium saturated with Ar; (d) culture medium saturated with N_2O.

observed [Figure 1(b)]. If the culture medium containing DMPO saturated with Ar was sonicated, additional lines, in which hyperfine coupling constants of nitrogen and hydrogen are $a_N = 1.55$ mT and $a_H^\beta = 1.88$ mT, on the EPR spectrum were obtained [Figure 1(c)]. These spin adducts seem to be formed mainly by the reaction of OH radicals and amino acids contained in the cultured medium. When the DMPO solution was saturated with N_2O, then sonicated, no spin adduct was observed, because triatomic N_2O gas creates low temperature cavitation bubbles due to the low γ values (Cp/Cv) [Figure 1(d)]. This condition did not produce high enough temperatures to create free radicals.

The EPR spctrum obtained after sonolysis of aqueous solutions of M_4PO, saturated with O_2 is displayed in Figure 2(a). The EPR spectrum consists of a primary triplet due to a nitrogen ($a_N = 1.53$ mT), with each line split into a doublet

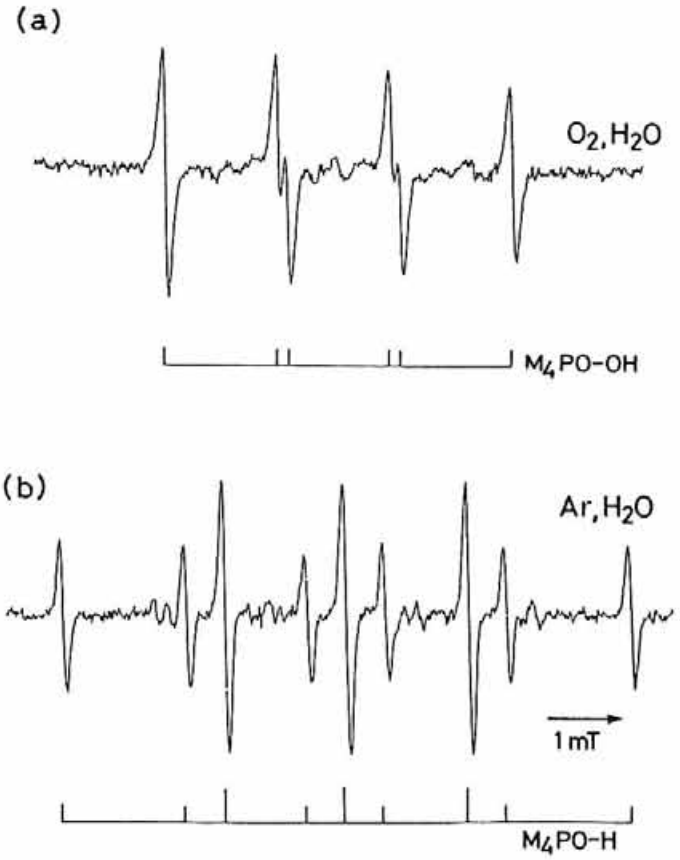

FIGURE 2 EPR spectra of spin trapped radicals obtained by sonolysis of water in the presence of M_4PO (5 mM) saturated with O_2 (a) and saturated with Ar (b).

by a secondary proton ($a_H^\beta = 1.69$ mT). These hyperfine coupling constants are in agreement with those reported previously for M_4PO-OH[15]. The EPR spectrum from sonicated Ar-saturated aqueous solution of M_4PO is shown in Figure 2(b). These lines were analyzed as a primary nitrogen triplet ($a_N = 1.65$ mT) further split by two secondary protons ($a_H^\beta = 2.17$ mT). This spectrum is consistent with the M_4PO spin adduct of the H atom[15]. When the DMPO-OH formation at different intensities of ultrasound was examined, the yields of DMPO-OH increased with ultrasonic intensity [Figure 3(a)]. However, above the intensity of 3.6 W/cm^2, the yields of DMPO-OH decreased. The dependence of cell killing of mouse mammary carcinoma FM3A cells on ultrasonic intensity was also examined. The cell survival decreased when increasing the ultrasonic intensity [Figure 3(b)]. However, the survival decreased above the intensity of 3.6 W/cm^2. This intensity dependence was a very good mirror image for that of DMPO-OH formation. The smaller yields of DMPO-OH formation and the higher survival rate seems to be due to the reduction of cavitation occurrence or the low efficiency of cavitation activity, because nucleation and growth of bubbles are inhibited by radiation pressure at high

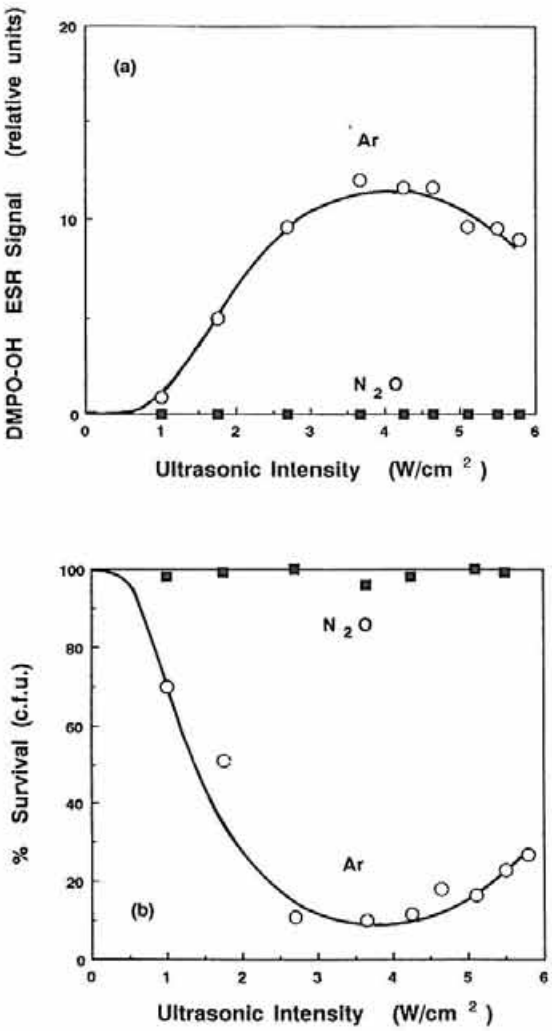

FIGURE 3 Effect of ultrasonic intensity on the relative yields of OH radicals (a) and on the cell survival of mouse mammary carcinoma FM3A cells (b). One unit of the Y-axis was calculated to be 10^{-7} M of DMPO-OH adduct by comparison with a standard nitroxide.

intensities. There was no decrease of cell survival when the cells were sonicated in the medium saturated with N_2O.

After sonication of the cells at the maximum intensity for DMPO-OH formation and cell killing, double-strand breaks and single-strand breaks were examined. Double-strand breaks were not observed when the cells were sonicated in either Ar or N_2O saturated condition. However, DNA single-strand breaks were observed when the cells were sonicated in the presence of Ar. In this condition, the yields of DNA single-strand breaks correspond to those induced by 1 Gy of X-irradiation (Figure 4). No single-strand breaks were observed in the cells sonicated in the medium in the presence of N_2O. To elucidate the mechanism of induction of

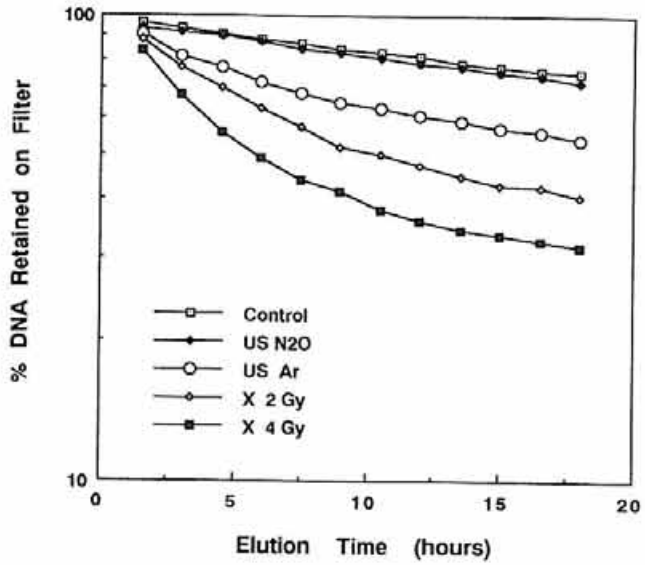

FIGURE 4 Alkaline elution profiles of mouse mammary carcinoma FM3A cells exposed to ultrasound for 2 min in the presence of N_2O or Ar. Positive controls were X-irradiated with 2 Gy or 4 Gy.

single-strand breaks, the effects of repair time and cysteamine on the formation of single-strand breaks were studied. If the cells were incubated after sonication for 30 min at 37°C, no repair was observed. Actual yields of single-strand breaks increased after incubation. In the presence of 50 mM cysteamine, which has been reported as effective concentration for the protection against thymine base damage induced by ultrasound[16], no prevention of single-strand break formation was observed. These results were different from those by free radical mechanism expected in X-irradiated cells. Therefore the formation of single-strand breaks in the cells due to mechanical effects or some other mechanism arising from cavitation is suggested. Recently, it was shown that ultrasonically induced single-strand breaks of DNA which were observed in cultured Chinese hamster ovary cells reside primarily in the nonviable fraction of cells[17]. Neither the production of OH radicals nor the mechanical effects of cavitation lead to the formation of double-strand breaks in the sonicated cells. In addition, the lack of free radical formation, cell killing, and DNA strand breaks were shown under conditions where collapse of high temperature cavitational bubbles does not occur for 1 MHz ultrasound.

These results indicate that the role of free radicals in aqueous solution induced by ultrasound is important for the formation of single-strand breaks of DNA *in vitro* when the time for sonication is long and/or the intensity is high. Besides this, the mechanism of ultrasound induced-strand breaks of DNA in cells and in aqueous solution is quite different.

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