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Mechanism of Cell Damage by Ultrasound in Combination with Hematoporphyrin

Shin-ichiro Umemura,[1,3] Nagahiko Yumita,[2] Ryuichiro Nishigaki[2] and Koshiro Umemura[2]

[1]*Central Research Laboratory, Hitachi, Ltd., 1-280 Higashi-Koigakubo, Kokubunji, Tokyo 185 and* [2]*School of Pharmaceutical Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274*

The mechanism of cell damage by ultrasound in combination with hematoporphyrin was studied. Mouse sarcoma 180 cell suspensions were exposed to ultrasound for up to 60 s in the presence and absence of hematoporphyrin, with and without active oxygen scavengers. The cell damage enhancement by hematoporphyrin was suppressed by adding histidine but not by mannitol. The enhancement was doubled in rate by substitution of deuterium oxide medium for normal water. Sonoluminescence was produced in a saline solution under similar acoustic conditions and observed to have spectral components that can excite hematoporphyrin molecules. These results suggest that cell damage enhancement is probably mediated via singlet oxygen generated by ultrasonically activated hematoporphyrin.

Key words: Ultrasound — Hematoporphyrin — Cell damage — Singlet oxygen — Active oxygen scavenger

Use of ultrasound irradiation for tumor treatment has been relatively well investigated with respect to thermal effects due to ultrasound absorption,[1] but few studies have been reported with respect to non-thermal effects such as chemical effects due to ultrasound cavitation. Furthermore, since the drugs used together with ultrasound in most of the past studies were anti-tumor drugs, it was difficult to identify the effects as being either synergistic or additive.[2,3]

Hematoporphyrin (Hp) is known to enhance the cell-killing effect of light irradiation[4] at a dose at which the chemical alone has no known biological effect. A hematoporphyrin derivative (HpD), which contains Hp dimers and accumulates well in tumors,[5] is used to treat tumors in combination with laser light irradiation.[6] This treatment is called photodynamic therapy. Recently, it was found that Hp also enhances the cell-damaging effect of ultrasound irradiation at a concentration at which the chemical alone causes no cell damage.[7] Implanted mouse tumors were also treated with ultrasound irradiation after administration of Hp and the tumor growth was completely inhibited, while ultrasound alone showed only a small inhibitory effect.[8] Since ultrasound penetrates much deeper into biological tissues than light, this newly found enhancement by Hp may be potentially useful for treating tumors located relatively deep in the body and can be called a 'sonodynamic' reaction.[9]

In the photodynamic reaction of Hp, several lines of evidence have suggested that singlet oxygen ($^1O_2$) generated by photochemically activated Hp is likely to be the most important cell-damaging mediator.[7] It seems natural to assume that the mechanism of the sonodynamic reaction of Hp is somewhat similar to that of its photodynamic reaction, but this needs to be confirmed. We have therefore investigated the mechanism by observing the sonodynamic cell damage in deuterium oxide medium or with active oxygen scavengers *in vitro*. Sonoluminescence of saline solutions under similar acoustic conditions was also examined.

MATERIALS AND METHODS

Chemicals Hematoporphyrin dihydrochloride, deuterium oxide (D_2O), histidine, mannitol, and superoxide dismutase (SOD) were purchased from Sigma Chemical (St. Louis, MO). All the other reagents were commercial products of analytical grade.

Tumor cells Sarcoma 180 cells were obtained and suspended in an air-saturated phosphate buffer solution (PBS) using the same procedure as in our previous paper.[7] To examine the effect of D_2O medium, isolated tumor cells were suspended in a PBS prepared by substituting D_2O for hydrogen oxide (H_2O).

Insonation apparatus for cell-damaging experiment An ultrasound transducer of a similar design to that in the previous paper,[7] with a larger diameter and the same resonant frequency (1.92 MHz), was used. The piezoelectric ceramic disk and the polymer acoustic matching layer had diameters of 40 mm and 42 mm, respectively. An insonation system of the same arrangement was used in the same way as in the previous paper.

Although the insonation experiments were performed in a standing wave situation, the output acoustic power from the transducer was calibrated in a propagation mode to avoid difficulty in acoustic power estimation. The output acoustic pressure was measured in degassed

[3] To whom correspondence should be addressed.

water 4 cm from the transducer surface using a 1-mm-diameter PVDF needle-type hydrophone (Medicoteknisk Institut, Denmark). Spatial average intensity was calculated by scanning the probe, ± 2 mm axially and laterally, to eliminate the effect of ripples in the field due to Fresnel diffraction. The measured intensity was approximately proportional to the square of the peak-to-peak driving signal voltage in the voltage range used in the insonation experiment. The driving signal was monitored by an oscilloscope during insonation. In the cell-damaging experiments, the transducer was driven at a voltage corresponding to a propagation mode intensity of 1.8 W/cm^2.

Insonation apparatus for sonoluminescence To produce and observe sonoluminescence, a transducer of a similar design, with a ceramic disk diameter of 12 mm and a resonant frequency of 1.93 MHz, was used with a quartz optical cell (Nihon Sekiei Glass T-3-UV-10, Tokyo) attached to it, as shown in Fig. 1. The optical cell was aligned in the optical path of a spectrophotofluorometer (Hitachi F-3000, Tokyo). An air-saturated saline solution (1 ml) with and without additives was contained in the cell, which has a horizontal cross-section of 1 cm^2, and was insonated with acoustic pressure in a similar range to that in the cell-damaging experiments. The peak pressure measured using the same needle-type hydrophone mentioned above in the center of the contained solution was 6×10^5 Pa. The measurement of the sonoluminescence spectrum was started 10 s after the insonation started.

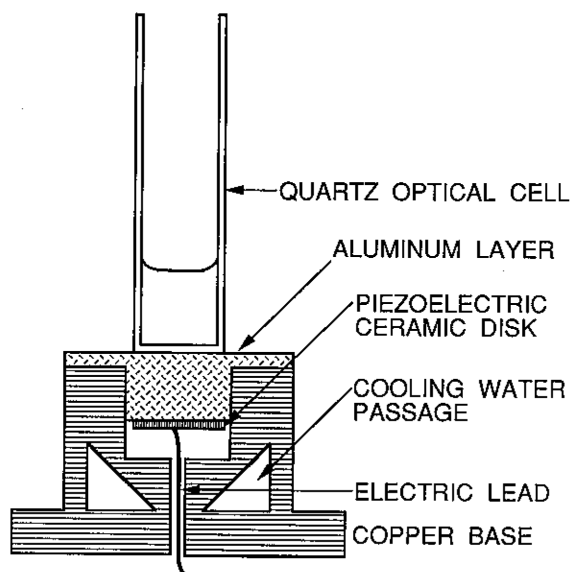

Fig. 1. Insonation apparatus for sonoluminescence.

Cell damage detection Cell damage induced by insonation in the presence and absence of hematoporphyrin in suspensions with and without active oxygen scavengers and in H_2O and D_2O medium was detected by staining of the cells with Trypan Blue dye as described in the previous paper.[7] The integrity of the cells was determined by counting the number of unstained cells on a hemocytometer glass plate using an optical microscope.

RESULTS

Effect of D_2O The unstained fractions of the isolated cells in the suspensions in D_2O medium with and without Hp (50 μg/ml) are plotted versus insonation time and compared to that in H_2O medium in Fig. 2. Note that the unstained fractions are plotted on a logarithmic scale. The ultrasound cell-damaging rate in the suspensions with Hp was about twice as high in the D_2O medium as in the H_2O medium, but in suspensions without Hp there was no significant difference.

Effect of active oxygen scavengers The effects on the ultrasound cell damage by adding histidine (10 mM), mannitol (100 mM), and SOD (100 μg/ml) to the cell suspensions with and without Hp (50 μg/ml) are shown in Fig. 3. Cell damage by ultrasound alone was partly

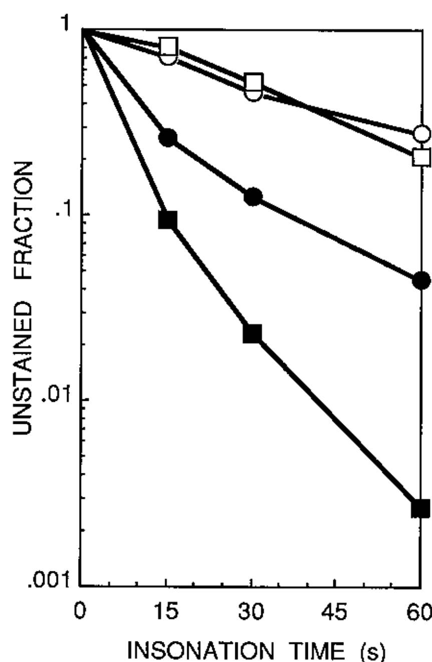

Fig. 2. Effect of D_2O on ultrasound cell damage. ○, without Hp in H_2O; ●, with Hp (50 μg/ml) in H_2O; □, without Hp in D_2O; ■, with Hp (50 μg/ml) in D_2O. Each point represents the mean unstained fraction of three to four experiments.

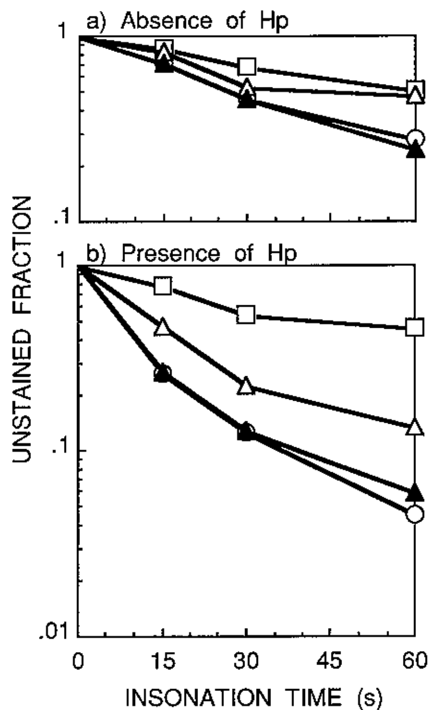

Fig. 3. Effect of active oxygen scavengers on ultrasound cell damage without Hp (a) and with Hp (50 μg/ml) (b). ○, no scavengers; □, histidine (10 m*M*); △, SOD (100 μg/ml); ▲, mannitol (100 m*M*). Each point represents the mean unstained fraction of four experiments.

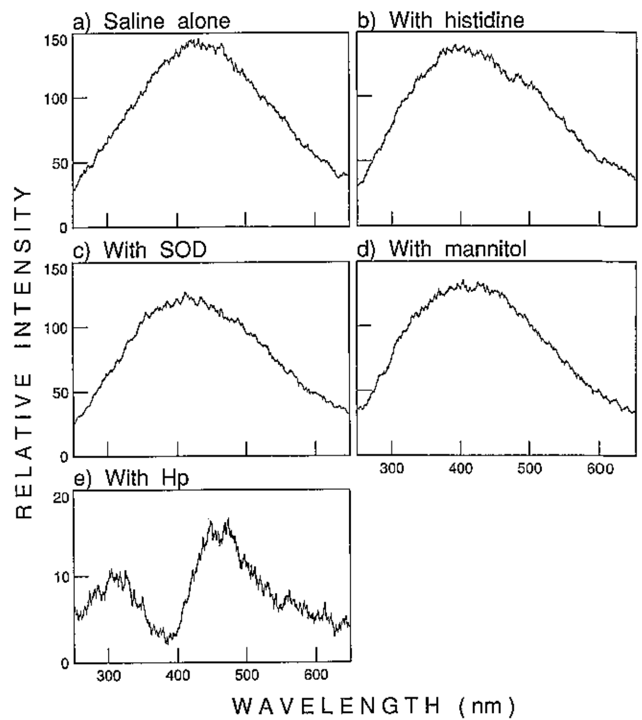

Fig. 4. Sonoluminescence spectra of air-saturated saline solutions; without additives (a), with histidine (10 m*M*) (b), with SOD (100 μg/ml) (c), with mannitol (100 m*M*) (d), and with Hp (10 μg/ml) (e).

Table I. Effects of D_2O Media and of Active Oxygen Scavengers on Ultrasound Cell Damage

Medium	Scavenger (conc.)	Hp conc. (μg/ml)	N[a]	Unstained fraction[b]
H_2O	(0)	0	4	0.279±0.044
		50	4	0.045±0.009
D_2O	(0)	0	3	0.210±0.029
		50	3	0.003±0.002[c]
H_2O	histidine (10 m*M*)	0	4	0.515±0.032[c]
		50	4	0.459±0.027[c]
	SOD (100 μg/ml)	0	4	0.477±0.055[c]
		50	4	0.134±0.027[c]
	mannitol (100 m*M*)	0	4	0.247±0.044
		50	4	0.059±0.014

a) Number of experiments.
b) Unstained fraction after insonation for 60 s.
c) Significantly different from the value of H_2O medium without active oxygen scavengers, based on Student's *t* test with $P \leq 0.05$ as the criterion of significance.

inhibited by histidine, but its enhancement by Hp was greatly inhibited. Mannitol showed no effect on either, while both were slightly inhibited by SOD. Note that there was no significant difference between the cell-damaging rates in the absence and presence of Hp when histidine was added to the suspensions.

The effects of D_2O and active oxygen scavengers on the unstained fraction are summarized in Table I.

Sonoluminescence Sonoluminescence spectra of air-saturated saline solutions with and without additives are shown in Fig. 4. The broad spectrum of saline alone has its peak at a wavelength of around 400–450 nm (Fig. 4, a). Sonoluminescence spectra of saline solutions with histidine, SOD, and mannitol of the same concentration as in the cell-damaging experiment are shown in Fig. 4, b, c, and d, respectively. No significant change was caused by these active oxygen scavengers in the shape or intensity of the spectrum. The main component of the spectrum of saline alone disappeared in that with Hp (10 μg/ml), but no fluorescence emission of Hp (around 630 nm) was observed (Fig. 4, e).

DISCUSSION

The half-life of singlet oxygen $^1\Delta_g$ in D_2O (~60 μs) is known to be much longer than in H_2O (~4 μs).[10,11] The remarkable increase in ultrasound cell-damaging rate with Hp due to the substitution of D_2O for the H_2O medium (Fig. 2) supports the suggested mechanism of sonodynamic cell damage with Hp being mediated by singlet oxygen.

Histidine is known to act as a scavenger of both singlet oxygen ($^1O_2$) and hydroxyl radical (OH^-), while mannitol is a scavenger of OH^- but not of $^1O_2$. A mannitol concentration of 50 m*M* has been reported to be effective to scavenge ultrasonically induced OH^-.[12] Thus, the protection by histidine from sonodynamic cell damage with Hp and the nonprotection by mannitol (Fig. 3) imply that $^1O_2$ is an important mediator in the sonodynamic reaction while OH^- does not mediate the reaction at all. Since SOD provided slight protection from the damage, superoxide seems also to play some role in the reaction, but not the most significant one. These results also support the suggested mechanism of sonodynamic cell damage with Hp being mediated mainly by singlet oxygen.

The mechanism of cell damage by ultrasound in the absence of Hp, on the other hand, seems to be much more complicated and might not involve a single dominant mediator. The result of the experiment in D_2O medium (Fig. 2) does not suggest that $^1O_2$ was playing an important role, and the results with active oxygen scavengers (Fig. 3) imply that only a part of the damage is likely to be mediated by $^1O_2$. Various mechanisms for cell damage induced by ultrasound (in the absence of sensitizing agents such as Hp) have been suggested by a number of researchers depending on the biological subjects and experimental set-ups.[13]

The above discussion may suggest that the presence of Hp in the suspensions particularly enhanced one mechanism of cell damage by ultrasound and thus made the roles of the other mechanisms less important. This may have allowed the mechanism of sonodynamic cell damage with Hp to be elucidated relatively easily compared to that of cell damage by ultrasound alone.

Sonoluminescence is known to be caused by ultrasonically induced cavitation. Several different hypotheses have been proposed for its origin, and the 'hot-spot' theory has been relatively successful among them in explaining most of the experimental facts.[14] Two possible mechanisms of light emission may be suggested to understand the observed sonoluminescence spectra in this context: direct emission from ultrasonically induced minute hot-spots, and recombination of active oxygen species generated by the hot-spots.

The observed sonoluminescence spectrum of saline solution looked similar to the broad spectrum of black-body radiation, and had a broad peak located around 400–450 nm, which can be estimated to correspond to a hot-spot temperature of 6000–7000°C (Fig. 4a). Since the sonoluminescence was not quenched at all by active oxygen scavengers (Fig. 4 b–d), it was likely to be emitted directly from the hot-spots rather than being due to the recombination of active oxygen species. These results can be assumed to be evidence that ultrasound irradiation can produce hot-spots having an energy level high enough to activate Hp molecules, since the sonoluminescence peak was located close to the absorption peak of Hp and its main spectral component was actually absorbed by Hp (Fig. 4, e).

Further investigation is needed to determine whether the hot spots of nonequilibrium high temperature directly activate the Hp molecules or whether the generated light photochemically activates them. Whichever the detailed mechanism of the activation is, Hp, used in combination with focused ultrasound irradiation, may be useful in furture for the 'sonodynamic' treatment of non-superficial tumors.

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