Abstract: Hyperthermia (HT) for cancer therapy combined with radiation and anticancer agents has been clinically used and has shown good results to a certain extent. However, clinical results by HT alone have not always been satisfactory. Although HT induces cancer cell death by apoptosis, the degree of apoptosis and its pathway varies in different cancer cell types. Therefore, attention has been focused on the search for substances to sensitize cancer cells to HT-treatment without affecting normal cells. Since it is known that HT can induce intracellular oxidative stress in cells and tissues because of increased production of reactive oxygen species (ROS) within cells, modification of intracellular oxidative stress could play a critical role in both sensitization and protection of apoptosis induced by HT.

In this review, some evidence on enhancement of HT-induced apoptosis by increase of intracellular oxidative stress, such as the use of temperature-dependent free radical generators, an intracellular hydrogen peroxide (H_2O_2) generator and an intracellular oxidative stress specific nitric oxide (NO') donor, will be shown and the mechanisms of the enhancement will be discussed.

Key words: apoptosis, heat sensitizer, hyperthermia, ROS
Introduction

The effectiveness of HT combined with radiotherapy in the treatment of various solid tumors has been demonstrated. Furthermore, recent clinical randomized trials for patients with brain tumors, recurrent or metastatic malignant melanoma, advanced breast carcinoma, locally advanced pelvic tumors, and malignant germ cell tumors have clearly indicated the advantages for patients treated with HT combined with radiotherapy or chemotherapy compared with radiotherapy alone. However, the uniform and precise delivery of heat to tumors still remains a challenge. In many circumstances the tumor cell killing has not been sufficient. Drugs that have been discussed to overcome this difficulty are heat sensitizers. An ideal heat sensitizer would be nontoxic at normal temperatures but could be cytotoxic at hyperthermic temperatures.

ROS is continuously generated in electron transfer reactions in aerobic cells. The generation of ROS is tightly controlled by the antioxidant systems of the cell, such as superoxide dismutase (SOD) and glutathione. However, abnormally high levels of ROS overcome the antioxidant system, and then oxidative stress will occur and subsequently damage and kill the cell.

There is mounting evidence that HT increases intracellular ROS such as superoxide (O$_2^-$) and lipid peroxide (LPO) to induce cellular oxidation events. It has been reported that the generation of intracellular O$_2^-$ by HT is due to elevated activation of xanthine oxidase (XO) and/or mitochondria respiratory reaction chain. Intracellular SOD converts O$_2^-$ to H$_2$O$_2$, a well-known apoptotic agent, and reaction of these ROS with low molecular weight iron complexes can induce LPO.

The increases of intracellular ROS by HT are considered to be the major mechanism for HT-induced cell injury and the principle of hyperthermic treatment in cancer therapy. In liver tissue exposed to hyperthermic perfusion, several markers of oxidative injury have been determined, including the oxidation and depletion of reduced glutathione and the appearance of products of lipid peroxidation. A particular goal to enhance HT-induced apoptosis is to modulate the intracellular generation of ROS.

Previously we reviewed the chemical sensitization of apoptosis induced by HT, and here we will discuss the enhancement of HT-induced apoptosis via the modulation of intracellular ROS.

Effects of temperature dependent free radical generators on heat-induced apoptosis.

An ideal heat sensitizer would be a tumor cell specific agent that exerts its cytotoxicity locally in the heated tumor volume. A potential candidate for heat activated production of toxic species is a water soluble azo compound, 2,2'-azobis- (2-amidinopropane dihydrochloride) (AAPH). This agent has been used as a free radical generating compound by heat- or light- induced activation. Upon activation by heat or light, AAPH decomposes to yield two alkyl radicals per molecule. These initial radicals could attack critical target molecules, or in the presence of oxygen, initiate lipid peroxidation and thus exert cell and tissue damage. Although the agent has been used as a heat sensitizer to increase the thermosensitivity of Chinese hamster V79 cells at 42.0, 43.0 and 45.5°C estimated by the colonogenic assay, the detailed mechanisms responsible for the enhanced lethal effect, especially on inducing apoptosis, are not fully understood. Recently, we found that HT increased the intracellular calcium ion concentration ([Ca$^{2+}$]i) arising from increased expression of type 1 inositol-1,4,5-trisphosphate receptor.
(IP3R1) and LPO in human myelomonocytic lymphoma U937 cells, which are sensitive to apoptotic stimuli, while an additional increase in [Ca\(^{2+}\)]\(_i\) due to further increased LPO and the activation of a mitochondria-caspase-dependent pathway plays a major role in thermosensitization by AAPH. However, the mechanism how AAPH enhances apoptosis induced by HT in resistant cells against apoptotic stimuli remains to be elucidated. We have examined whether the combination of AAPH and HT enhances apoptosis or not in human uterine cervical adenocarcinoma HeLa cells and squamous cell carcinoma CaSki cells. When combined with nontoxic AAPH (50 mM), significant enhancement of apoptosis induced by HT at 44°C for 60 min was observed, where the initial rate of free radical formation was about twice as high as that at 37°C. Augmentation of the growth delay, LPO and an increase in [Ca\(^{2+}\)]\(_i\) were also observed after the combined treatment. A water-soluble vitamin E, Trolox, blocked the increase in [Ca\(^{2+}\)]\(_i\) and an intracellular Ca\(^{2+}\) chelator, bis-(O-aminophenoxy)-ethane-N, N, N', N'-tetraacetate acid-acetoxymethyl (BAPTA-AM), prevented the DNA fragmentation induced by the combination. However, no significant change in mitochondrial

![Fig. 1a. A schematic presentation of the mechanism of enhancement of HT-induced apoptosis by AAPH in a human lymphoma cell line. HT combined with AAPH which generates alkyl radicals (A) and alkyl peroxy radicals (AOO), increased intracellular oxidative stress and intracellular calcium ion concentration to enhance apoptosis. In addition, the combination enhanced the activation of the mitochondria-caspase pathway in apoptosis.](image)

![Fig. 1b. A schematic presentation of the mechanism of enhancement of HT-induced apoptosis by AAPH in a human cancer cell line. Relatively long term HT combined with AAPH which generates alkyl radicals (A) and alkyl peroxy radicals (AOO), increased intracellular oxidative stress and intracellular calcium ion concentration to enhance apoptosis with membrane damage (secondary necrosis). In addition, the combination enhanced the activation of the mitochondria-caspase pathway in apoptosis. In this case, typical features of apoptosis, lowering of mitochondrial membrane potential and release of cytochrome c from mitochondria were not observed.](image)
membrane potential and expression of Bax and Bcl-2 was observed. A slight increase in Fas expression was observed only in CaSki cells after the combined treatment. These results indicate that HT and AAPH induce enhanced apoptosis and subsequent cell killing via an increase in LPO and the increase in \([\text{Ca}^{2+}]_i\), but not the activation of a mitochondria-mediated apoptosis pathway in these cell lines (25) (see Fig. 1).

Recently, we attempted to enhance apoptosis by increasing the incorporation of the agent through combination with low intensity therapeutic ultrasound with AAPH. Based on the idea of transient pore formation on cell membranes as a result of ultrasonic exposure, we hypothesized that if cells are sonicated in the presence of AAPH, the generated pores would allow the agent to enter the cells more easily, thereby improving the effectiveness of cell killing. Significant enhancement of apoptosis at 25 and 37°C was observed when cells were treated with the combination of AAPH and ultrasound even at 25 and 37°C (26). Another attempt to utilize more lipophilic free radical generator, 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN) combined with HT in U937 cells showed that AMVN is about two orders of magnitudes more potent than the water soluble radical generator, AAPH, on the DNA fragmentation induced by the combination (27).

Since it is known that cancer cells are generally resistant to physical and chemical stress-induced apoptosis, free radical generators appear to be a useful thermosensitizer for hyperthermic cancer therapy.

Enhancement of HT-induced apoptosis by intracellular \(\text{H}_2\text{O}_2\) generator 6-formylpterin

Pterins, the 2-amino-4-hydroxy-pteridine derivatives, occur in conjugated or unconjugated form in a wide range of biological systems. Six-formylpterin (6-FP), a metabolite of folic acid, contains a CHO-residue in the 6-substituted conjugated pterin and the agent was found to be more abundant in some cancer cells (28). Among the pterins, 6-FP has some unique properties, especially transfer of electrons from NAD (P) H to oxygen, and this process leads to intracellular \(\text{H}_2\text{O}_2\) generation in living cells (29). Since 6-FP enhances radiation-induced apoptosis, it is a good agent to produce \(\text{H}_2\text{O}_2\) intracellularly, potentially yielding an effect on the cells different from that induced by extracellular \(\text{H}_2\text{O}_2\). Based on the implied low toxicity of 6-FP and its ability to continuously generate intracellular \(\text{H}_2\text{O}_2\), we hypothesized that 6-FP could be an enhancer of apoptosis at nontoxic concentrations in combination with ROS associated physical stimuli such as ionizing radiation and HT. When radiation-induced apoptosis and its possible enhancement in the presence of 6-FP were examined in U937 cells, a significant enhancement of radiation-induced apoptosis was observed (28).

In addition, the enhancement of heat-induced apoptosis by 6-FP, an intracellular generator of \(\text{H}_2\text{O}_2\), was examined in U937 cells. The cells were treated with either 6-FP alone at a nontoxic concentration of 300 \(\mu\text{M}\) (37°C), heat shock (44°C, 20min) alone, or a combination of the two, and then incubated at 37°C for 6 h. Assessments of apoptosis, mitochondrial membrane potential, and caspase-3 activation were performed by flow cytometry. Moreover, caspase-8 activation, and changes in the \([\text{Ca}^{2+}]_i\) were examined. Bax, Bel-2, Bel-X\(_1\), Bid, cytochrome c, and PKC\(\delta\) were determined by Western blotting. The enhancement of heat-induced apoptosis evaluated by morphological observation and DNA fragmentation assay were promoted by the addition of 6-FP. Mitochondrial membrane potential was decreased and the activation of caspase-3 and -8 was enhanced in the cells treated with the combination.
A decreased-expression of Bid was noted, although no significant changes in Bax, Bcl-2, and Bcl-XL expressions were observed after the combined treatment. Furthermore, both the release of cytochrome c from mitochondria to cytosol and the translocation of PKC δ from cytosol to mitochondria, which were induced by heat shock, were enhanced by the addition of 6-FP. The number of cells with a higher \([\text{Ca}^{2+}]_i\) was also increased by the addition of 6-FP. These findings suggest that the increase in \([\text{Ca}^{2+}]_i\), the activation of the mitochondria-caspase dependent pathway, and the translocation of PKC δ to mitochondria play principal roles in the enhancement of heat-induced apoptosis by 6-FP 30) (see Fig. 2).

Intracellular ROS modification by an intracellular generator of \(\text{H}_2\text{O}_2\) appears to be useful for enhancement of radiation- and HT-induced apoptosis in cancer cells.

Enhancement of HT-induced apoptosis by release of NO\(^{\bullet}\) from PBN under hyperthermic condition

A spin trap \(\alpha\)-phenyl-tert-butyl nitrone (PBN), is known as a potent protector against oxidative stress \textit{in vitro} and \textit{in vivo}. Recently, it has been shown that, under oxidative conditions, PBN can be decomposed with hydroxyl radicals by the Fenton reaction, generating \(\text{NO}^{\bullet}\) 31) 32).

Based on the phenomenon of NO\(^{\bullet}\) generation from PBN under oxidative stress, we hypothesized that PBN could be an enhancer of heat-induced apoptosis at nontoxic concentrations because HT causes intracellular oxidative stress. When U937 cells were treated with the combination of PBN and HT, a significant enhancement of heat-induced apoptosis was observed. Decrease in mitochondrial trans-membrane potential, cleavage of Bid, release of cytochrome c from mitochondria to cytosol, and activation of caspase-8 and -3 were significantly induced by the combined treatment. \([\text{Ca}^{2+}]_i\) was also increased by the combined treatment. The enhancement of apoptosis was partly inhibited by an intracellular \(\text{Ca}^{2+}\) chelator, BAPTA-AM. Increase in Fas externalization, and decrease in the levels of both Hsp70 and phosphorylated HSF1 were observed in the combined treatment. Heat-induced generation of intracellular \(\text{O}_2^{\bullet-}\) was decreased by the addition of PBN, while nitrite concentration in the medium was increased only by the combined treatment. Furthermore, a NO\(^{\bullet}\) scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide) and a putative peroxynitrite...
ONOO⁻ scavenger (uric acid), but not an inducible NO synthase inhibitor (N⁰-nitro-L-arginine methyl ester) significantly inhibited the enhancements of apoptosis, Fas externalization, and the increase in [Ca²⁺]i by the combined treatment. Imaging using diaminofluorescein-2-diacetate, an intracellular fluorescence dye for NO, stained intracellularly only after the combined treatment. These results suggest that, 1) NG is released from PBN by HT, and subsequently reacts with O₂⁻ to form ONOO⁻, 2) NO and ONOO⁻ are involved in the enhancement of apoptosis through the Fas-mitochondria-caspase pathway and [Ca²⁺]i dependent pathways, and 3) decrease in the level of Hsp70 and phosphorylated HSF1 contributes to the enhancement of apoptosis (see Fig. 3).

Since it was reported that NO is able to induce apoptosis, oxidative stress specific NO donors such as PBN derivatives also could be a candidate for a heat sensitizer to generate NO intracellularly.

**Conclusion**

Although physical development, such as precise heating of tumors and noninvasive thermal therapy, has not been sufficient in treating tumors and has not reached the level of practical use for cancer therapy, the clinical application of HT has a strong biological basis. The utilization of the combination of HT and drugs also needs further attention, because most pharmacological research in drugs has been made at 37°C and only limited data are available on the interaction between cells and drugs and on cellular signal transduction under hyperthermic conditions. Here, we have shown that somehow a different signal transduction in apoptosis is induced by the different combinations of heat and drugs, which produce alkyl and alkyl peroxy radicals, H₂O₂, or NO. Elucidation of the molecular mechanism responsible for the HT-induced apoptosis and its enhancement may provide a rational basis for the further effective use of HT and heat sensitizers in cancer therapy.

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References

細胞内酸化ストレスの修飾による温熱誘発
アポトーシスの増強

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要 旨：がん治療としてのハイパーサーミアは生物学的な合理性に欠け、放射線あるいは抗癌剤との併用によりある種の癌では良好な臨床治療効果が得られている。しかし、ハイパーサーミア単独治療では、十分な癌治療効果が期待できない症例が多い。ハイパーサーミアによる細胞死の一つにアポトーシスがあるが、その程度はがん細胞の種類により異なり、その分子機構は必ずしも明らかになっていない。ハイパーサーミアによるアポトーシスの分子機構の解明およびがん細胞のアポトーシスを選択的に増強する治療法の開発が重要となる。ハイパーサーミアは、細胞内で活性酸素を生成し、その結果、細胞と組織に酸化ストレスを与える。そこで、ハイパーサーミアが誘導する細胞内酸化ストレスの修飾によるアポトーシスの増強を目指して、一連の研究を行った。

この総説では、温度依存性のフリーラジカル発生剤、細胞内過酸化水素誘導剤および細胞内酸化ストレスにより一酸化窒素を発生する薬剤を用いたハイパーサーミアによるアポトーシスの増強効果を示し、その分子機構について考察した。