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Abstract

The effects of cepharanthin (Ce), glycyrrhizin (G), verapamil (V), and G plus V on induced thermotolerance in NIH3T3 cells were studied. Cells were heated with or without the drug at 45 degrees C for 20 min (the first heating), incubated at 37 degrees C for 12h (the incubation period), and heated again at 45 degrees C for 0-210 min (the second heating). G and V were added throughout the experiment, while Ce was added throughout the experiment or during only the first or second heating, or the incubation period. The cells were harvested after the second heating to evaluate cell survival. In control experiments without any drug, thermotolerance developed and reached the highest peak in the cells incubated for 12h at 37 degrees C. However, thermotolerance in the control cells was suppressed by incubating them at 0 degree C, but developed by subsequent incubation at 37 degrees C. This suggests that the acquisition of thermotolerance by the cells required metabolic processes during the incubation at 37 degrees C. When each drug was present throughout the experiment, only Ce or the combined use of G and V was effective in reducing thermotolerance. Thermotolerance was also suppressed in the presence of Ce during the second heating. These results indicate that Ce reduces thermotolerance by enhancing thermosensitivity rather than by inhibiting the development of thermotolerance.

KEYWORDS: thermotolerance, hyperthermia, cepharanthin, glycyrrhizin, verapamil

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The effects of cepharanthin (Ce), glycyrrhizin (G), verapamil (V), and G plus V on induced thermotolerance in NIH3T3 cells were studied. Cells were heated with or without the drug at 45°C for 20 min (the first heating), incubated at 37°C for 12 h (the incubation period), and heated again at 45°C for 0-210 min (the second heating). G and V were added throughout the experiment, while Ce was added throughout the experiment or during only the first or second heating, or the incubation period. The cells were harvested after the second heating to evaluate cell surival. In control experi ments without any drug, thermotolerance developed and reached the highest peak in the cells incubated for 12 h at 37°C. However, thermotolerance in the control cells was suppressed by incubating them at 0°C, but developed by subsequent incubation at 37°C. This suggests that the acquisition of thermotolerance by the cells required metabolic processes during the incubation at 37°C. When each drug was present throughout the experiment, only Ce or the combined use of G and V was effective in reducing thermotolerance. Thermotolerance was also suppressed in the presence of Ce during the second heating. These results indicate that Ce reduces thermotolerance by enhancing thermosensitivity rather than by inhibiting the development of thermotolerance.

Key words : **thermotolerance, hyperthermia, cepharanthin, glycyrrhizin, verapamil**

Heat-induced thermotolerance raises serious problems in clinical hyperthermic treatment because it reduces the hyperthermic effects of subsequent heating. At present, thermotherapy is restricted to less than twice a week due to the development of thermotolerance. Therefore, it is important to discover effective substances to cope with acquisition of heat resistance by tumors in order to give move frequent thermotherpy.

A transient thermotolerant survival response in HeLa cells was first reported *in vitro* by Gerner *et al.* in 1976 (1). Since then it has been found that thermotolerance is also induced by ethanol, sodium arsenite and other drugs (2,3) and its development is inhibited by cycloheximide and D_2O (4-6). Several pieces of evidence suggest that cell membranes are one of the heat sensitive targets (7-9).

Cepharanthin (Ce), glycyrrhizin (G), and verapamil (V) have been reported to cause functional

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alterations in the transport of ions, fluidity or the activity of enzymes in cell membranes (10–14). Uda *et al.* observed that Ce inhibited thermotolerance in V-79 cells (15), but they did not clarify the mechanism of its inhibition.

In this study, we investigated the inhibitory effects of Ce, G and V, which have some common effects on cell membranes for the development of thermotolerance in NIH3T3 cells. The study was designed to elucidate the mechanisms

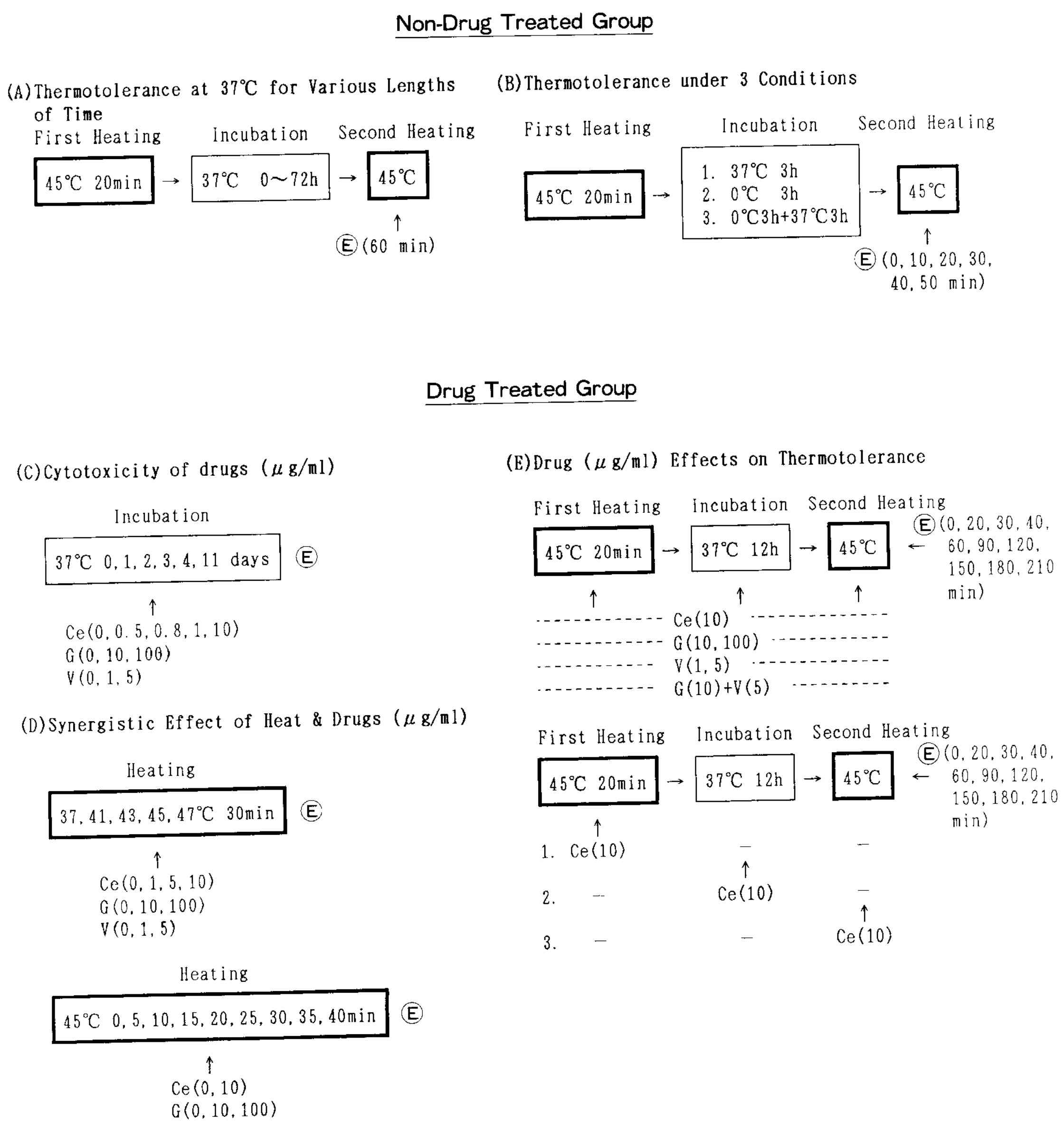

Fig. 1 Schema of the present experiments. Ce: cepharanthin, G: glycyrrhizin, V: verapamil. Ⓔ: evaluation of treatments for cell survival.

of inhibition of thermotolerance by Ce.

Materials and Methods

Cells and culture. NIH3T3 cells (a mouse fibroblast cell line) were maintained in Dulbecco's modified Eagle medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10 % bovine calf serum (Hyclone Laboratories, Inc., Utah, USA), 100 μg/ml streptomycin (Meiji Seika Kaisha, Ltd., Japan) and 100 units/ml penicillin (Meiji). Then 2×10^5 cells were seeded in 5 ml medium in a 25 cm^2 screw-topped polystyrene culture flask (Becton Dickinson and Company, New Jersey, USA) and cultured in a CO_2 incubator (Sanyo Electric Co., Ltd., Osaka, Japan) with 95 % air + 5 % CO_2 at 37°C. The screw-top of the flask was loosened during incubation of the culture. When the exponentially growing cells formed a monolayer in 24 h after seeding, experiments were started.

Heat treatment. Heat treatment was carried out by immersing the flask containing the cells in a temperature-regulated water bath (Taitec Co., Saitama, Japan) preset at 37–47°C. The temperature was maintained within an error of ± 0.05°C.

Drugs. Ce (Kaken Shoyaku Co., Ltd., Tokyo, Japan), G (Minophagen Pharmaceutical Co., Tokyo, Japan) and V (Eisai Co., Ltd., Tokyo, Japan) were diluted with distilled water and added to the medium to the final concentration indicated in each experiment.

Cell survival. Cell survival was determined by the colony forming ability of the cells. The cells, which were dispersed with trypsin (Difco Laboratories, Inc., Michigan, USA), were seeded in 5 ml medium in a 60 × 15 mm style tissue culture dish (Becton) at concentrations of 10^2, 10^3 and 10^4/dish, followed by incubation in a CO_2 incubator for 11 days. After incubation, viable cells were fixed with 10 % formaldehyde solution, and stained with 10 % Giemsa stain solution. Then cell survival was evaluated by the mean number of colonies containing more than 50 cells in three dishes under a phase contrast microscope.

Cell treatment. The medium was replaced before the treatment with 5 ml fresh medium prewarmed at 37°C. The control cells were heated for 20 min at 45°C (the first heating), incubated for 0-72h at 37°C and heated again for 60 min at 45°C (the second heating). They were harvested during the second heating to evaluate cell survival (Fig. 1A). The other control cells were tested by incubation for 3h at 37°C (Group 1–37) or 0°C (Group 2–0) after heating. The cells in Group 2–0 were further incubated for 3h at 37°C (Group 3–0/37). The cells in each group were heated again for 0–50 min at 45°C, and harvested at 10 min intervals to study thermotolerance (Fig. 1B). After each experiment, cell survival was evaluated as described above.

Drug cytotoxicity of Ce, G or V was tested by incubating cells at 37°C for 1–11 days. Then cell survival was evaluated (Fig. 1C).

In the presence of Ce, G or V, the synergistic effects of heat and drugs were tested by heating cells at 37–47°C for 30 min or at 45°C for 0–40 min. Then cell survival was evaluated (Fig. 1D).

The inhibitory effects of Ce, G, V or a combination of G and V on thermotolerance were examined (Fig. 1E). Cells were heated for 20 min at 45°C (the first heating), incubated for 12h at 37°C and heated again for 0–210 min at 45°C (the second heating). The cells were harvested during the second heating at 20–60 min intervals to evaluate cell survival. The effects of each drug were tested in the presence of Ce, G, V or GV in combination (5 ml each) throughout the experimental period. Ce was also tested during a specified period alone (the first or second heating, or the incubation period). At the conclusion of an experiment, each drug solution was eliminated and cells were rinsed twice with flesh medium. Then cell survivals were evaluated.

The evaluation of treatment. The cell survival rate was corrected by the seeding efficacy of the controls. The D_0 value, one of the criteria for evaluating cellular thermosensitivity, was adopted in this study. It indicates the treatment period (min) required to reduce the cell survival rate by $1-1/e$ in an exponentially regressing portion. The D_0 ratio, D_0 (control)/D_0 (with drugs), was used to express the drug effects on thermotolerance.

Results

Thermotolerance. First, the mechanism of development of thermotolerance was examined. In the absence of Ce, V or G, strong thermotolerance developed in the cells incubated for 0–72h at 37°C between the first and second heatings. The best cell survival was found in the cells incubated for 12h (Fig. 2A). The D_0 ratio at this time was 24.6, indicating the greatest thermotolerance. Thermotolerance developed in the

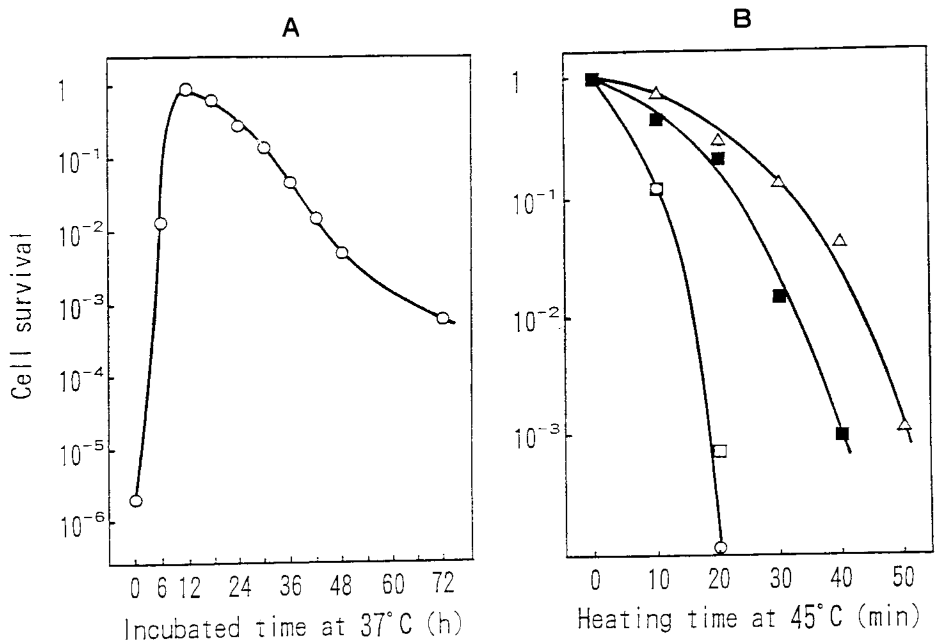

Fig. 2 (Above) Development of thermotolerance in non-drug treated cells. **A**. Thermotolerance was examined during the second heating after 0, 6, 12, 18, 24, 30, 36, 42, 48 and 72 h incubation at 37°C. The incubation was carried out between the first (20 min at 45°C) and second (60 min at 45°C) heatings. The horizontal axis indicates the period of incubation between the first and second heatings at 45°C. **B**. Thermotolerance was examined in three groups under three different conditions. (△——△), Group 1-37 ; (□——□), Group 2-0; (■——■), Group 3-0/37. Conditions of Groups 1-37, 2-0 and 3-0/37; See text. The horizontal axis indicates the heating time of the second heatings at 45°C. Cells were preheated for 20 min at 45°C. (○——○), The second heating without incubation (control).

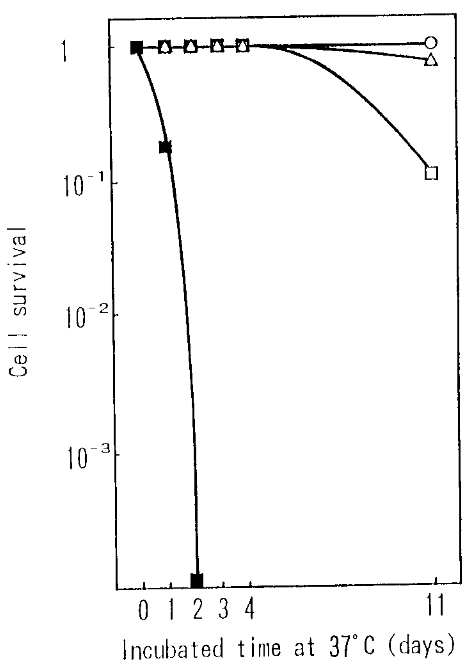

Fig. 3 (Left) Cytotoxicity by Ce. Ce. 0.5 μg/ml (○), 0.8 μg/ml (△), 1 μg/ml (□), 10 μg/ml (■). Ce: cepharanthin. The method of treatments is illustrated in Fig. 1C.

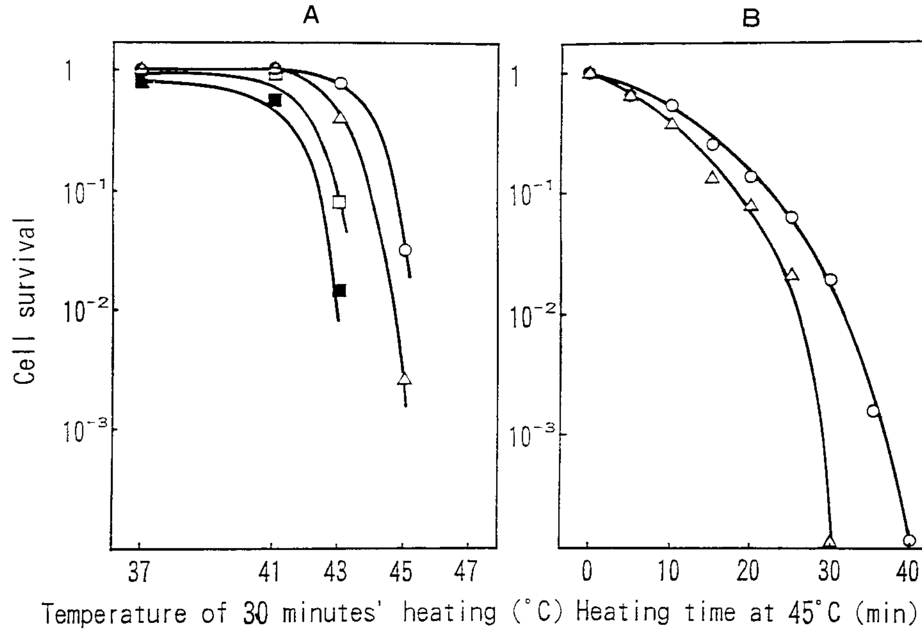

Fig. 4 Synergistic effects of Ce and heat on cell survivals.
A. Cells were heated for 30 min at various temperatures. Control (○), Ce-treated cells (1 μg/ml) (△), (5 μg/ml) (□), and (10 μg/ml) (■).
B. Cells were heated at 45°C for 10–40 min . Control (○), Ce-treated cells (10 μg/ml) (△). Ce: cepharanthin. The method of treatments is illustrated in Fig. 1D.

cells incubated for 3 h at 37°C between heatings (Group 1–37), but not in the cells incubated for 3 h at 0°C (Group 2–0) (Fig. 2B). Therefore, there were conditions at 0°C which inhibited cell metabolism and the development of thermotolerance. However, thermotolerance was induced in the cells incubated for 3 h at 0°C with subsequent 3 h incubation at 37°C (Group 3–0/37) (Fig. 2B). These results indicate that specific metabolic processes may be necessary for inducing thermotolerance during incubation.

Effects of drugs on cell survival. To examine cytotoxicity of Ce, G and V during incubation, each drug was added to the cells at the start of incubation. Cell survival was examined after incubation for 1, 2, 3, 4 or 11 days. During incubation, Ce became more cytotoxic as its concentrations and administration period increased (Fig. 3). Neither G or V was cytotoxic during incubation. To examine the synergistic effects of each drug during heating, each drug was added to the cells at the start of heating. Cell survival was examined after heating. When Ce was added during heating, its cytotoxicity increased with increases in the temperature and drug concentrations (Fig. 4A), and with prolongation of the heating time at 45°C (Fig. 4B). On the other hand, when G (10 μg/ml) or V (1 and 5 μg/ml) was added during heating, cytotoxicity was not induced even when the temperature was raised or the heating time at 45°C was extended. The only exception was a slightly increased cytotoxicity with G at a high concentration (100 μg/ml).

Drug effect on thermotolerance. The inhibitory effects of Ce, G, V or a combination of G and V on thermotolerance were examined. When Ce (10 μg/ml) was added throughout the experi-

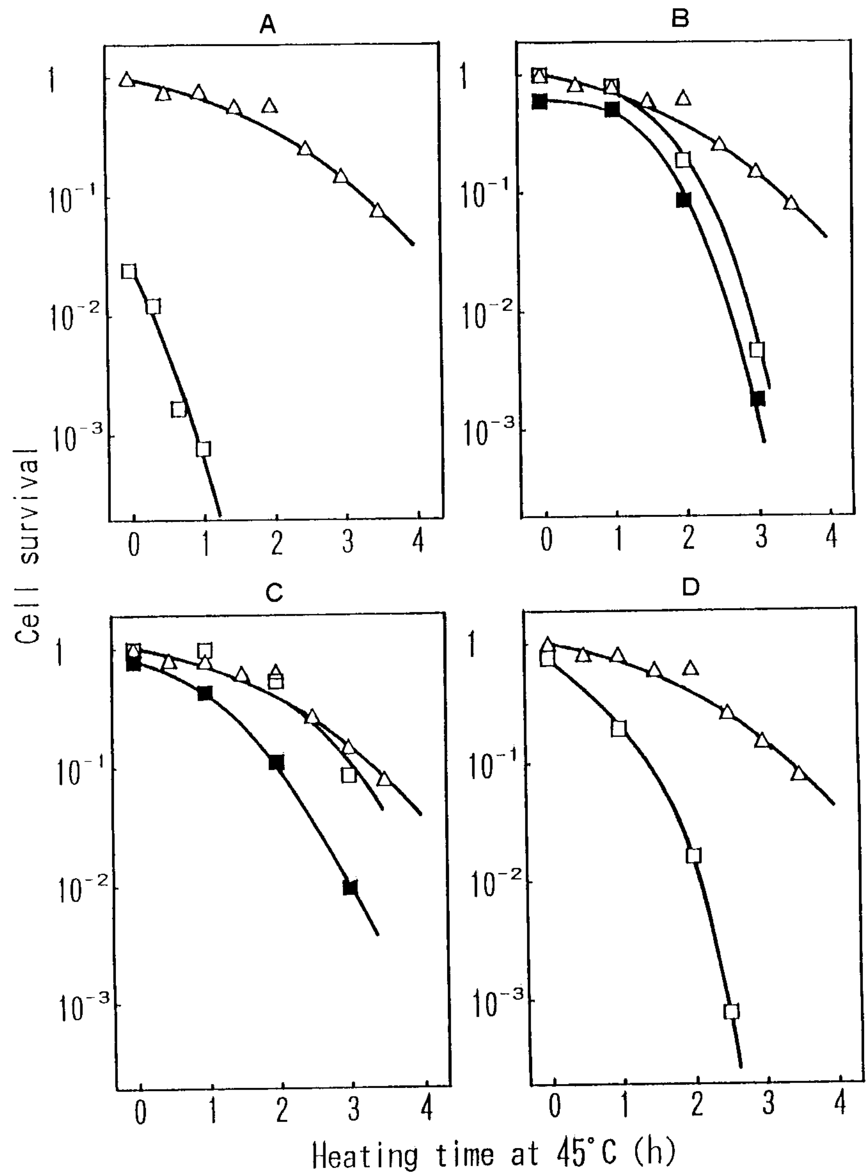

Fig. 5 Inhibition of thermotolerance by drugs added throughout experimental periods. The cells were incubated at 37°C for 12h between the first heating (20 min at 45°C) and the second heating (0–210 min at 45°C). The horizontal axis indicates the heating time of the second heating at 45°C. △——△, Heating twice without drugs (the first heating before incubation and the second heating after incubation) as a control. A, Ce 10 μg/ml (□); B, G 10 μg/ml (□), 100 μg/ml (■); C, V 1 μg/ml (□), 5 μg/ml (■); D, G (10 μg/ml) and V (5 μg/ml) in combination (□); Ce, G and V; See Fig. 1.

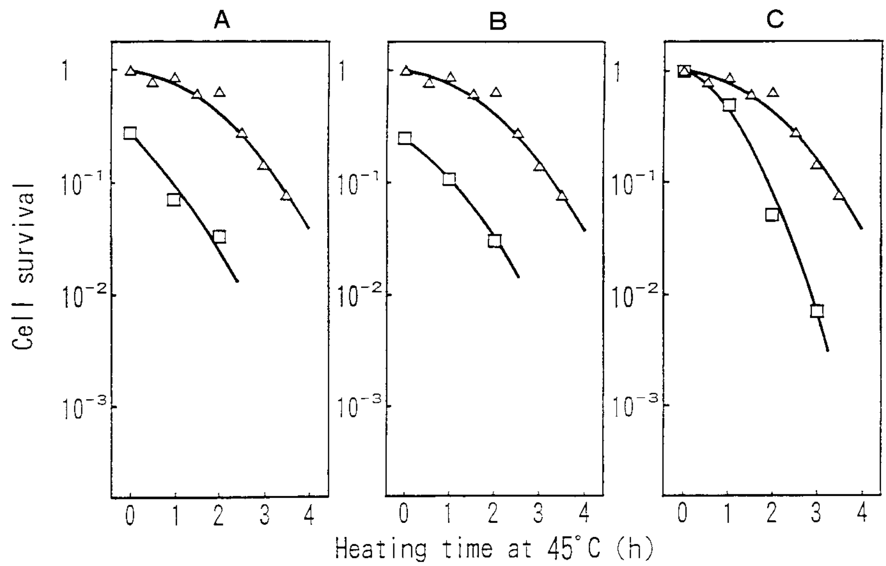

Fig. 6 Inhibition of thermotolerance by Ce added during a specified period (the first or second heating, or the incubation). Treatment procedures are the same as those described in Fig. 5 except for the period during which the drug was added. The horizontal axis indicates the heating time of the second heating at 45°C. (△——△), Heating twice without Ce with the incubation as a control. (□——□), treatment with Ce (10 μg/ml) during the first heating (A), the incubation period (B) or the second heating (C). Ce: cepharanthin.

Table 1 D_0 values showing the effects of heat treatment at 45°C with or without drugs.

Treatments	D_0 values (D_0 ratios) (min)
First heat treatment	
Without drugs	1.8
Ce	0.8 (2.3)
G	1.8 (1.0)
Second heat treatment	
12 h incubation after the first heat treatment	
Without drugs	44.3
Ce (A + B + C)	12.8 (3.5)
Ce (A)	40.5 (1.1)
Ce (B)	39.8 (1.1)
Ce (C)	22.5 (2.0)
G (A + B + C)	21.8 (2.0)
V (A + B + C)	24.0 (1.8)
G + V (A + B + C)	12.8 (3.5)

Ce; cepharnthin (10 μg/ml), G; glycyrrhizin (10 μg/ml), V; verapamil (5 μg/ml) A, B and C indicate the times when the drug was added: *i.e.*, A, during the first hating; B, during the post-heat incubation period; and C, during the second heating. D_0 values (min) and D_0 ratios: See text.

ment, thermotolerance induced during 12 h post heat incubation was suppressed (Fig. 5A). When G (10 μg/ml) or V (5 μg/ml) was added throughout the experiment, the thermotolerance induced during 12 h incubation was only slightly inhibited (Fig. 5B, C). G (10 μg/ml) and V (5 μg/ml) added together thoughout the experiment suppressed thermotolerance as strongly as Ce did (Fig. 5D). Ce did not inhibit induction and development of thermotolerance during the first heating and post heat incubation period, but inhibited it during the second heating (Fig. 6).

D_0 values and D_0 ratios obtained from the results in this experiment are summarized in Table 1.

Discussion

Although the heating targets of cells have not been completely clarified, changes in the structure and function of the cell membrane, inhibitions of DNA and protein syntheses, and chromosomal damage after heat treatment have been reported (7,16,17). Procain, ethanol and amphotericin B, which act on the membrane, are known to enhance themosensitivity (8,18–20). These facts suggest that the membrane is one of the heating targets.

In our study, Ce was shown to enhance thermosensitivity. Ce, a biscoclaurine alkaloid extracted and refined from *Stephania cepharantha* HAYATA, is known as an antihemolytic agent against hemolysis caused by snake venom due to its stabilizing effect on the cell membrane (21). Ce acts on the membrane, incorporates into the membrane (22) and decreases membrane fluidity as does cholesterol (11,12). On the other hand, an increase in membrane fluidity has been reported to enhance thermosensitivity (18,23–25). Therefore, the mechanisms of enhanced thermosensitivity by Ce need to be further studied to explain the role of membrane fluidity.

Not only heat, but also ethanol and sodium arsenite induce thermotolerance and heat shock proteins (2,3). Thermotolerance was modified by inhibition of the metabolism in the cells kept at 0 °C or treated with cycloheximide and D_2O during the post heat period (4–6,26). Therefore, thermotolerance may be the result of certain metabolic responses which occur in the stimulated cells during the post heat period. Our results also suggest that the cell metabolism plays an important role in the develpment of thermotolerance during the post heat incubation period, and that drugs-effectiveness in reducing thermotolerance may act on the cells during this period. Ce added during the first heat treatment or during the post heat incubation period did not inhibit induction or development of thermotolerance. However, when it was added during the second heat treatment or in mid-experiment, it strongly inhibited the survival of thermotolerant cells. Although the inhibiting effect of Ce on thermotolerance has been reported elsewhere (15), our results indicate that it does not inhibit induction or development of thermotolerance, but rather reduces the survival of thermotolerant cells by its thermoenhancing effect. Ce has been used clinically to treat snake or insect bites, leukopenia from irradiation, various allergic diseases and other diseases. When used clinically for hyperthermotherpy, Ce should be injected before the heat treatment so that a sufficient concentration may reach tumor cells during the treatment.

The combination of G and V markedly killed the thermotolerant cells, and showed the same D_0 ratio as that of Ce. Ce inhibits phospholipase A_2 (10) in a manner similar to G (14). Ce also prevents the release of Ca^{2+} into the extracellular medium (10), and the activity of membrane binding proteins such as P-glycoprotein (13). V is a calcium antagonist and changes the membrane permeability of anticancer drugs (27). It is believed to inhibit the mechanism of drug efflux by binding the receptor of P-glycoprotein (28). As mentioned above, the effect of the combination of G and V on the membrane was similar to that of Ce. This may explain why the D_0 ratio for the combination of G and V was the same as that for Ce.

We concluded from these results that Ce enhances the cell killing effect of heat treatment and reduces thermotolerance. These properties of Ce may be of some therapeutic value.

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References

1. Gerner EW, Boone R, Connor WG, Hicks JA and Boone ML: A transient thermotolerant survival response produced by single thermal doses in HeLa cells. Cancer Res (1976)

36, 1035–40.
2. Li GC and Werb Z: Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. Proc Natl Acad Sci USA (1982) **79**, 3218–3222.
3. Hahn GM and Li GC: Thermotolerance and heat shock proteins in mammalian cells. Radiat Res (1982) **92**, 452–457.
4. Henle KJ and Leeper DB: Modification of the heat response and thermotolerance by cycloheximide, hydroxyurea, and lucanthone in CHO cells. Radiat Res (1982) **90**, 339–347.
5 Fisher GA, Li GC and Hahn GM: Modification of the thermal response by D_2O. I: Cell survival and the temperature shift. Radiat Res (1982) **92**, 530–540.
6. Li GC, Fisher GA and Hahn GM: Modification of the thermal response by D_2O. II: Thermotolerance and the specific inhibition of development. Radiat Res (1982) **92**, 541–551.
7. Lepock JR: Involvement of membranes in cellular responses to hyperthermia. Radiat Res (1982) **92**, 433–438.
8. Yatvin MB: The influence of membrane lipid composition and procaine on hyperthermic death of cells. Int J Radiat Biol (1977) **32**, 513–521.
9. Harms-Ringdahl M, Anderstam B and Vaca C: Incorporation of [^{3}H] acetate into the membrane lipids of a murine tumour during the development of thermotolerance. Int J Radiat Biol (1989) **55**, 297–305.
10. Watanabe S: Inhibition of platelet aggregation by cepharanthine is accomplished during the early, membrane-related activation process. Acta Med Okayama (1984) **38**, 101–115.
11. Utsumi K, Miyahara M, Inoue M, Mori M, Sugiyama K and Sasaki J: Inhibition by cepharanthin of red blood cell potassium release induced by lead acetate and lysolecithin. Cell Struct Funct (1975) **1**, 133–136.
12. Utsumi K, Miyahara M, Sugiyama K and Sasaki J: Effect of biscoclaurin alkaloid on the cell membrane related to membrane fluidity. Acta Histochem Cytochem (1976) **9**, 59–68.
13. Shiraishi N, Akiyama S, Nakagawa M, Kobayashi M and Kuwano M: Effect of bisbenzylisoquinoline (biscoclaurine) alkaloids on multidrug resistance in KB human cancer cells. Cancer Res (1987) **47**, 2413–2416.
14. Okimasu E, Shiraishi N, Watanabe S, Morimoto Y and Utsumi K: Inhibitory effect of glycyrrhizin on the phospholipase A_2 activity. Igaku No Ayumi. (1982) **122**, 174–177 (in Japanese).
15. Uda M, Akagi K and Tanaka Y: Effect of cepharanthin and nicardipine on thermotolerance. Nippon Acta Radiol (1988) **48**, 1236–1242 (in Japanese).
16. Wong RSL and Dewey WC: Molecular studies on the hyperthermic inhibition of DNA synthesis in Chinese hamster ovary cells. Radiat Res (1982) **92**, 370–395.
17. Dewey WC, Westra A, Miller HH and Nagasawa H: Heatinduced lethality and chromosomal damage in synchronized Chinese hamster cells treated with 5-bromodeoxyuridine. Int J Radiat Biol (1971) **20**, 505–520.
18. Konings AWT: Development of thermotolerance in mouse fibroblast LM cells with modified membranes and after procaine treatment. Cancer Res (1985) **45**, 2016–2019.
19. Henle KJ: Interaction of mono- and polyhydroxy alcohols with hyperthermia in CHO cells. Radiat Res (1981) **88**, 392–402.
20. Hahn GM, Li GC and Shiu E: Interaction of amphotericin B and 43° hyperthermia. Cancer Res (1977) **37**, 761–764.
21. Hasegawa S and Takahashi K: Therapeutic application of cepharanthin for the snake venom injury. Saishin Igaku (1952) **7**, 627–631 (in Japanese).
22. Sato T, Kanaho Y and Fujii T: Relation of the characteristic action of biscoclaurine alkaloids on the erythrocyte membrane and their incorporation into the membrane. Cell Struct Funct (1980) **5**, 155–163.
23. Dennis WH and Yatvin MB: Correlation of hyperthermic senitivity and membrane microviscosity in *E. coli* K1060. Int J Radiat Biol (1981) **39**, 265–271.
24. Yatvin MB, Cree TC, Elson CE, Gipp JJ, Tegmo IM and Vorpahl JW: Probing the relationship of membrane "fluidity" to heat killing of cells. Radiat Res (1982) **89**, 644–646.
25. Konings AWT and Ruifrok ACC: Role of membrane lipids and membrane fluidity in thermosensitivity and thermotolerance of mammalian cells. Radiat Res (1985) **102**, 86–98.
26. Gerner EW and Schneider MJ: Induced thermal resistance in HeLa cells. Nature (1975) **256**, 500–502.
27. Tsuruo T, Iida H, Tsukagoshi S and Sakurai Y: Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res (1981) **41**, 1967–1972.
28. Qian X and Beck WT: Binding of an optically pure photoaffinity analogue of verapamil, LU-49888, to P-glycoprotein from multidrug-resistant human leukemic cell lines. Cancer Res (1990) **50**, 1132–1137.

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